

# **GENE EXPRESSION PROFILES OF COLUMBIA RIVER WHITE STURGEON EXPOSED TO TWO INDUSTRIAL EFFLUENTS**



Photo courtesy of: Streamside Adventures at [www.fishonbc.com/sturgeon\\_gallery.html](http://www.fishonbc.com/sturgeon_gallery.html)

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Prepared for:

Upper Columbia White Sturgeon Recovery Initiative  
Sturgeon Contaminants Working Group (SCWG)

## **Foreword**

This document was created in fulfillment of James Edwards' contract. It was heavily edited by both Joy Bruno and Rachel Skirrow of the Pacific Environmental Science Centre, with valuable experimental design information contributed by Heather Osachoff. The study was limited from the outset by RNA pooling immediately following extraction. RNA pooling reduced the sample size from the six animals to one animal, which limited the statistical power of the study. Data was analyzed across time and across concentration, but the treatment of data at each time point is unclear in this report. Graphical data is presented for each gene (10 genes total) at each concentration of effluent tested; however, it is unclear what timepoint this data represents. Furthermore, the expression data obtained from day 7 and day 17 50% Teck Cominco exposure was presented as an average of the two distinct timepoints. This manipulation of the data was incorrect.

The sturgeon exposure experimental design included animal collection every five days for fifty days. The molecular analysis was conducted on animals collected every ten days. Ideally, this portion of the study should be repeated to include tissues collected at each five day time point. Furthermore, future analysis should be conducted on individual animals (not animal pools) to allow rigorous statistical analysis.

## Abstract:

White sturgeon, *Acipenser transmontanus*, are an endangered species in the Columbia River system and natural recruitment for the aging population is virtually non-existent. The numerous studies conducted on the effects of contaminants on white sturgeon have shown that these fish can be extremely sensitive to environmental contaminants. The toxic chemical exposure response involves a change in cellular function that is often linked to a change in gene expression. This change in gene expression precedes overt toxic effects and can serve as a marker for exposure. However, little is known of the sturgeon's molecular level response to contaminant exposure. Consequently, a juvenile white sturgeon effluent bioassay was conducted in the summer of 2002 to assess gene expression using Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) technology. The sturgeon were exposed to two industrial effluents found in the lower Columbia River, pulp mill and smelter, for a period of 50 days at concentrations of 1%, 50% (one effluent only) and 100%. The expression of 10 genes was analyzed using RNA extraction and RT-qPCR. These genes include: structural gene -  $\alpha$  actin (ACTA1), housekeeping gene -  $\beta$  actin (ACTB), housekeeping/structural genes - L22 ribosomal protein (RPL22) and 18S ribosomal RNA (18S), signal transducer/receptor gene - aryl hydrocarbon receptor (AHR), stress-responsive gene- heat shock protein 70 (HSPA), receptor/endocrine gene- mineralocorticoid receptor (MR), metabolic/enzymatic gene- phosphoenolpyruvate carboxykinase (PCK2), regulatory gene - secreted protein, acidic, cysteine-rich (SPARC), and endocrine/structural gene - vitellogenin (VTG). ACTA1, ACTB, RPL22 and 18S were evaluated as possible normalizer genes. AHR, HSPA, PCK2, SPARC and VTG are known to respond to contaminants and MR has an endocrine/receptor function. Although not analyzed for significance, the sturgeon exposed to the 100% Teck Comino effluent showed a marked increase in gene expression when compared to control. This response illustrates an overload to the metabolic system which resulted in complete mortality of all test organisms within 5 days of exposure. Sturgeon exposed to the 50% effluent showed suppression of SPARC and  $\alpha$  actin, genes that code for proteins vital in cell growth and repair. This downregulation culminated in complete mortality. RT-qPCR results showed a significant increase in the expression of the AHR and MR genes in animals exposed to 100% pulp mill effluent compared to controls. AHR expression is associated with exposure to halogenated aromatic hydrocarbons and in this case possibly from dioxin exposure. It is not understood why the MR genes are being expressed but any disruption in the normal biochemical processes is of concern. No significant differences in gene expression were observed in fish exposed to 1% pulp mill or 1% metal smelter effluent. Therefore, AHR and MR may serve as possible biomarkers of exposure. Recommendations for subsequent studies include: testing additional genes such as growth regulatory hormones; further investigation of the metal smelter discharge; increasing sample size for time point analysis; and detailed investigations of a single time point.

## *Acknowledgments*

The study was conducted under the direction of Graham C. van Aggelen and Joy B. Bruno in support of the Upper Columbia River White Sturgeon Recovery Initiative (UCRWSRI) Contaminants Working Group Sub Committee.

The work presented was conducted at Environment Canada's Pacific and Yukon (P&Y) Regional Environmental Toxicology Laboratory, located at the Pacific Environmental Science Centre (PESC) in North Vancouver, British Columbia and at the University of Victoria (UVIC), in Dr. Caren C. Helbing's Laboratory in the Department of Biochemistry and Microbiology. Heather Osachoff (PESC) designed the sturgeon QPCR assay. Heather Osachoff (PESC) and Dr. Nik Veldheon (UVIC) provided guidance and training to James Edwards. We thank Dr. Helbing for her RT-qPCR expertise and for reviewing the document. We also thank the members of the Sturgeon Contaminant Working group for reviewing the document.

The members of the study would like to especially acknowledge and thank the following agencies and individuals for their help and support for making this study possible: William Green, Director of Canadian Columbia River Inter-tribal Fisheries Commission; Chris Beers, Columbia River white sturgeon stewardship; Ktunaxa Kinbasket Tribal Council and Sue Ireland, Kootenai Tribal Fish and Wildlife Program.

## **Introduction:**

White sturgeon, *Acipenser transmontanus*, is an endangered species in the Columbia River system. This long living, late maturing and bottom feeding species exists in a river system that is highly impacted by dams, industry, agriculture, and municipal development. Of the approximate 1400 individuals remaining, it is estimated that less than one percent are below the age of 40, and natural recruitment is virtually non-existent. These factors have led the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) to list white sturgeon as “endangered” in Canada.

While the White Sturgeon Recovery Initiative (WSRI), an international partnership of interested stakeholders, including regulatory agencies, industry, universities and First Nations, has listed habitat loss as the major cause of recruitment failure, the possible impact and significance of pollutants cannot be dismissed. Numerous studies have been conducted on the effects of contaminants on white sturgeon. For example, tissue residues from Fraser River white sturgeon have levels of mercury that exceed human consumption criteria and levels of PCBs, dioxins and furans that exceed human health guidelines (Sutherland et al., 1997). Studies that have focused on the traditional toxicological endpoints such as mortality (Szenasy et al.), and hatch rate (Kruse and Scarnecchia, 2002) have shown that white sturgeon can be extremely sensitive to environmental contaminants (Wood et al., 1998). In addition to known acute toxicological effects, contaminant exposure may cause numerous non-lethal impacts.

Little is known of the molecular level responses of white sturgeon to contaminant exposure. Toxicogenomics, an emerging field of toxicology, is providing the tools to identify and quantify the non-lethal effects. Toxicogenomics is defined as the application of genomic technologies to define large scale changes in gene expression resulting from contaminant exposure (Selkirk and Tenent, 2003). Significant differences in gene expression have been detected between European flounder, *Pleuronectes flesus*, captured from polluted and unpolluted estuaries in the United Kingdom (Williams et al., 2003). While microarray technology can identify genes with altered expression levels, real time quantitative PCR (RT-qPCR) is required to more accurately determine the extent and direction of the change. RT-qPCR uses the polymerase chain reaction and fluorescent DNA dye incorporation to quantify the amount of DNA in a sample. The main problem in using RT-qPCR to analyze gene transcription levels stems from two factors: the precise nature of RT-qPCR analysis and the difficulty in achieving reproducible RNA extraction from tissues (Radonic et al., 2003). Both RT-qPCR and microarrays require the use of normalizer genes. The ideal normalizer gene, also called a housekeeping gene or control gene, is invariantly expressed between control and treated samples and can be used to calculate normalization factors that correct for the differences in tissues and sample handling (Ambion Inc., Tech Notes 8(5)).

An early lifestage white sturgeon bioassay was conducted in the summer of 2002 to assess gene expression after exposure to specific industrial effluents. Final discharge effluent was collected from the two dominant industries in the Lower Columbia River, Celgar Pulp Company Limited (Celgar) in Castlegar and the Teck Cominco Metals

Limited (Teck Cominco) smelter in Trail. Ten sturgeon genes were analyzed using RT-qPCR. These genes were chosen either for their suspected interaction with contaminants or to provide a candidate normalization gene. The ten genes are:

| <b><u>Gene (Gene Code)</u></b>                 | <b><u>Description</u></b>  |
|--|--|
| $\alpha$ Actin<br>(ACTA1)                      | Codes for a structural muscle protein and was evaluated as possible normalization gene. Actin monomers assemble to form microfilaments, characteristic of striated muscle.   |
| $\beta$ Actin<br>(ACTB)                        | Codes for a structural protein and was evaluated as possible normalization gene. $\beta$ Actin has been used and evaluated as a control gene in some applications (Lupberger et al., 2001). It is found in nonmuscle cells, roles include: organelle transport, regulation of ion transport, and receptor-mediated responses of the cell to external signals.  |
| Aryl hydrocarbon<br>Receptor (AHR)             | Codes for a transcription factor that activates the expression of drug metabolizing genes that mediate the biochemical and toxic effects of halogenated aromatic hydrocarbons. (GeneCard – AHR; Hahn, 2002).   |
| Heat Shock Protein<br>70 (HSPA)                | Codes for a major stress-inducible heat shock protein that plays an important role in protecting the cell against major stresses and in repairing stress induced damage (GeneCard – HSP70; InvivoGen – HSP70). Its expression has shown to be induced by heavy metal stress (Wu et al., 1986; Yui et al., 1994).   |
| Mineralocorticoid<br>Receptor (MR)             | Codes for a member of the steroid/thyroid super family of receptors. The mineralocorticoid effects of cortisol are linked to the osmoregulatory processes required for adaptation to seawater and restoring salt and water homeostasis.  |
| L22 ribosomal<br>Protein (RPL22)               | Codes for a ribosomal protein that has been used in some organisms as a control gene. (Primerano et al., 2002)   |
| Phosphoenolpyruvate<br>Carboxykinase<br>(PCK2) | Codes for an enzyme that plays a central role in glucose regulation (Scott et al., 1998). PCK2 gene expression is affected by environmental contaminants, including heavy metals, organic pollutants ( $\beta$ -naphthoflavone), and dioxins (TCDD) (Fana and Rosman, 1994; Stahl et al., 1993). These contaminants impair the liver gluconeogenic capacity, which may adversely affect the animal's capacity to cope with stress. |

(Continued)

**Gene (Gene Code)**    **Description**

Secreted protein,  
Acidic, cysteine-rich  
(SPARC)    Codes for a glycoprotein responsible for regulating cell growth and is heavily expressed in tissues undergoing morphogenesis, remodelling and wound repair (GeneCard – SPARC). SPARC is sensitive to estrogens; therefore, estrogen mimetic compounds could potentially affect the transcription of this gene.

18S ribosomal RNA  
(18S)    Constitutively expressed at high levels. It is one of the most common control genes used. (Powell and Howel, Eurogentec; Ambion Tech Notes 8(5); Carson et al., 2002; Ma et al., 2002).

Vitellogenin (VTG)    Codes for egg protein. Vitellogenesis is the process through which maturing oocytes in the ovary accumulate yolk. Vitellogenesis is normally restricted to maturing females; however, immature females and males can produce VTG following exposure to estrogen and their mimics. Any factor causing an impairment of the vitellogenic cycle can dramatically reduce the reproductive success (number of eggs, hatching rate and viability of embryos) of an individual, which in turn has the potential to affect the entire population of the species. (Holbech et al., 2002; Goodbred et al., 1996; Cyr et al., 2002)

**Materials and Methods:**

*Exposure and sampling*

The white sturgeon early life stage toxicity test was conducted at Environment Canada's Pacific Environmental Sciences Centre (PESC) in North Vancouver, BC. For full details on exposure refer to Sturgeon Report (J. B. Bruno, March 2004). In summary, eleven to fourteen day old sturgeon from Hill Creek Hatchery were exposed for 50 days to either effluent from Teck Cominco or Celgar. The laboratory well water (pH 7.3, dissolved oxygen 9.2 mg/L, hardness 100 mg/L, temperature 14°C) served as control/dilution water. Seven replicates for each solution were set up: three replicates were used to harvest sturgeon every five days during the study, and four replicates were used for mortality, and behavioural and physiological observations. The concentrations of effluent used in testing were 100%, 50% (Teck Cominco only), 1%, and 0% (Control). Each replicate contained approximately 65 developing sturgeon. For the full strength Teck Cominco maintenance-period effluent, the LT<sub>50</sub> was 4 days (96 hours), while for the half-strength effluent, the LT<sub>50</sub> was 7 days. All sturgeon exposed to the 100% effluent were dead after 5 days of exposure while all sturgeon in the 50% effluent died within 17 days. Toxicity effects were not evident after exposure to any of the tested concentrations (100%, 1%) of the Celgar effluent. The LT<sub>50</sub> for sturgeon mortality (%) was not determinable as less than 50% of the fry died in any of the treatments.

Fish for genomic analysis were euthanized using tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd., Vancouver, BC) according to PESC Standard Operating Procedures (LC50FF15). The entire hatchling or alevin was preserved and stored in RNA-Later™ (Ambion Inc., Austin, Texas, USA) and archived at -80°C. Fifteen animals were stored in 1.5 ml of RNA-Later. Due to the acute toxicity of the Teck Cominco effluent, sturgeon exposed to the 100% Teck Cominco maintenance-period effluent were collected at day 2 only and those exposed to the 50% Teck Cominco effluent only up to day 17. Matching control animals were collected at each timepoint.

### *RNA Extraction*

RNA was extracted from 6 pooled animals collected at day 2 (Teck Cominco control and 100% only), 7 (Teck Cominco 50% only), 10, 17 (Teck Cominco 50% only), 20, 30, 40, and 50. Total RNA was extracted and purified using an RNeasy Lipid Tissue extraction kit as described by the manufacturer (animals pooled) (Qiagen Inc., Mississauga, Ontario, Can.). Extracted RNA was suspended in RNase-free water and stored at -60 °C. An Ultraspec 3000 Spectrophotometer nucleic acid scan was used to determine the concentration of the total RNA extracted and to calculate the A260 / A280 ratio (Absorbance at 260 nm versus Absorbance at 280 nm) to assess purity. RNA quality was further assessed using agarose gel electrophoresis, with ethidium bromide stain. The appearance of whole 28S and 18S rRNA bands indicated that the RNA was intact and not degraded.

### *Preparation of cDNA*

cDNA for each pooled time point / exposure level was made as described by the manufacturer using 1 µg of total RNA with the cDNA synthesis kit reagents Superscript™ II RNase H Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, California, USA) and RNaseOUT™ Ribonuclease Inhibitor (Invitrogen Life Technologies, Carlsbad, California, USA).

### *Real time – quantitative PCR*

RT-qPCR analysis was carried out on a Stratagene MX4000 Real-time Instrument (Stratagene, La Jolla, California, USA). Each RT-qPCR reaction contained 2 µL 20 fold diluted sturgeon cDNA, 1.5 µL 10X qPCR Buffer (100 mM Tris-Cl pH 8.3, 500mM KCl, 30 mM mgCl<sub>2</sub>, 0.1% Tween-20, 8% glycerol, 2.5 X SYBR Green 1 (4000 X dilution of SYBR Green 1 Stock (10,000x)), 0.3 µL dNTPs (40 mM total; 10 mM each dATP, dGTP, dCTP, dTTP) (Fisher Scientific, Nepean, Ontario, Canada), 0.25 µL ROX reference dye (diluted 200 fold; Stratagene, La Jolla, California, USA ), 9.75 µL RNase-free water, 0.2 µL (1 Unit) Platinum® Taq (Invitrogen Life Technologies, Carlsbad, California, USA) and 0.5 µL each of the specific gene primers (20 µM working stock primer solution ) (synthesized by Alpha DNA, Montreal, Quebec, Canada). Each sample was run in quadruplicate for precision control. The data was normalized using the reference dye as a volume standard. The thermocycle used a 9 minute 95 °C enzyme activation step followed by 40 cycles of 95 °C for 15s, 58 °C or 60 °C for 30s, 72 °C for

45s (Table 1). The 60 °C annealing temperature was used for the following gene primer pairs: 18S, RPL22, MR and AHR. The other primer pairs were run with 58 °C as the annealing temperature. Negative controls that lacked Taq polymerase or cDNA were included to ensure the specificity of cDNA amplification. Standard curves were obtained for each gene using known quantities of plasmids with the relevant gene sequence inserted to determine the linear relationship between the log of the copy number and the cycle threshold ( $C_T$ ) of the RT-qPCR.

Table 1. Oligonucleotide primers and gene targets for QPCR analysis.

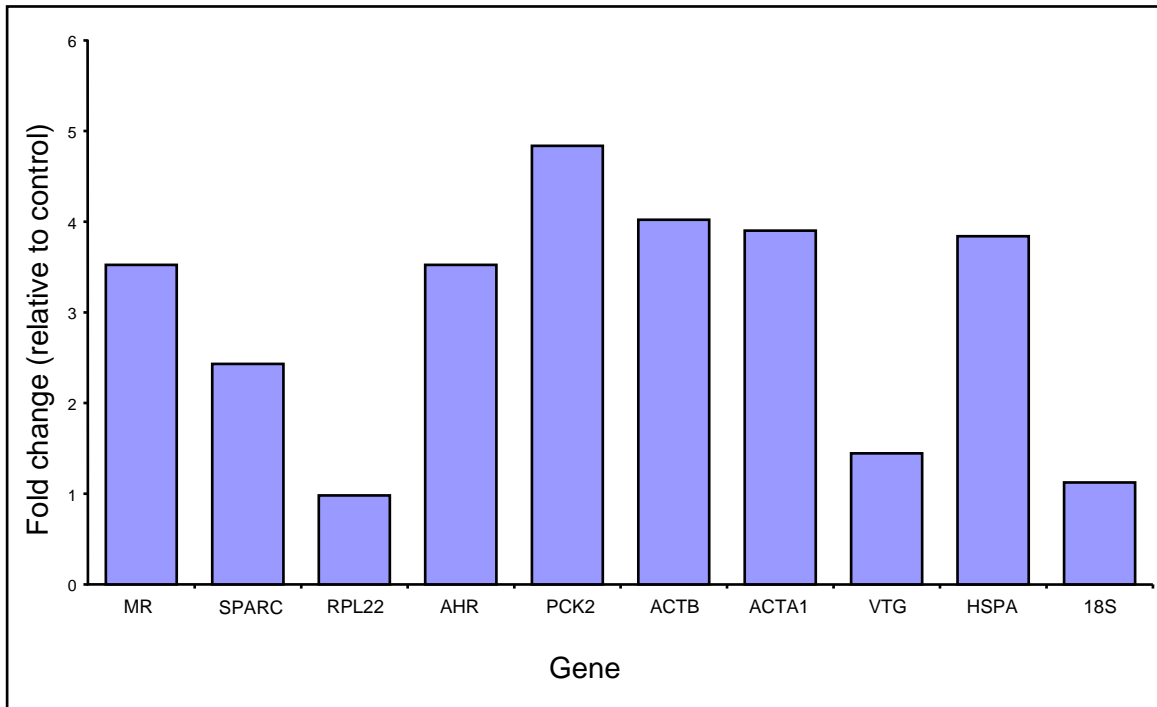
| Gene                       | Size | Annealing (°C) | Primer direction <sup>a</sup> | Primer Sequence       | GenBank Accession # |
|----------------------------|------|----------------|-------------------------------|-----------------------|---------------------|
| 18S rRNA                   | 307  | 60             | S                             | GGTCCGAAGCGTTTACTTTG  | AF188379            |
|                            |      |                | AS                            | AGTCGGCATCGTTTATGGTC  |                     |
| $\alpha$ -actin            | 345  | 58             | S                             | GTGGGTATGGGTCAGAAAGA  | AY880258            |
|                            |      |                | AS                            | AACACCGTCACCAGAGTCAA  |                     |
| aryl hydrocarbon receptor  | 414  | 60             | S                             | CTTCACTTCGCCCTCAATCC  | AY880254            |
|                            |      |                | AS                            | GTGTAACCCAGCACCCACCTT |                     |
| $\beta$ -actin             | 216  | 58             | S                             | TTGCCATCCAGGCTGTGCT   | AY878120            |
|                            |      |                | AS                            | TCTCGGCTGTGGTGGTGAA   |                     |
| heat shock protein 70      | 175  | 58             | S                             | GACCTGTTCCGTGGTACTCT  | AY880255            |
|                            |      |                | AS                            | CTGGGTTGATGCTCTTGTT   |                     |
| L22-like protein           | 217  | 60             | S                             | CAGGAGCGGATCAAGGTAAA  | AY880260            |
|                            |      |                | AS                            | GGAAGTAGCGCAGTTCGTAG  |                     |
| mineralocorticoid receptor | 194  | 60             | S                             | AGCAGAAATCCAGCAGAC    | AY880259            |
|                            |      |                | AS                            | GAGCACGGTTCCCAAAGA    |                     |
| PEPCK                      | 280  | 58             | S                             | TGATGACCTTGCCTTTGTG   | AY880257            |
|                            |      |                | AS                            | GTGACCTCCTGGAAGAACC   |                     |
| SPARC                      | 257  | 58             | S                             | GTCGCACTGGTCAAGAAG    | AY880253            |
|                            |      |                | AS                            | GCGAGAAGCAGAACTCAGG   |                     |
| vitellogenin               | 173  | 58             | S                             | ACCTTTCCTGGTTCCTTC    | U00455              |
|                            |      |                | AS                            | GAGACAGTCCTTGCCTTG    |                     |

<sup>a</sup> S = Sense; AS = Antisense

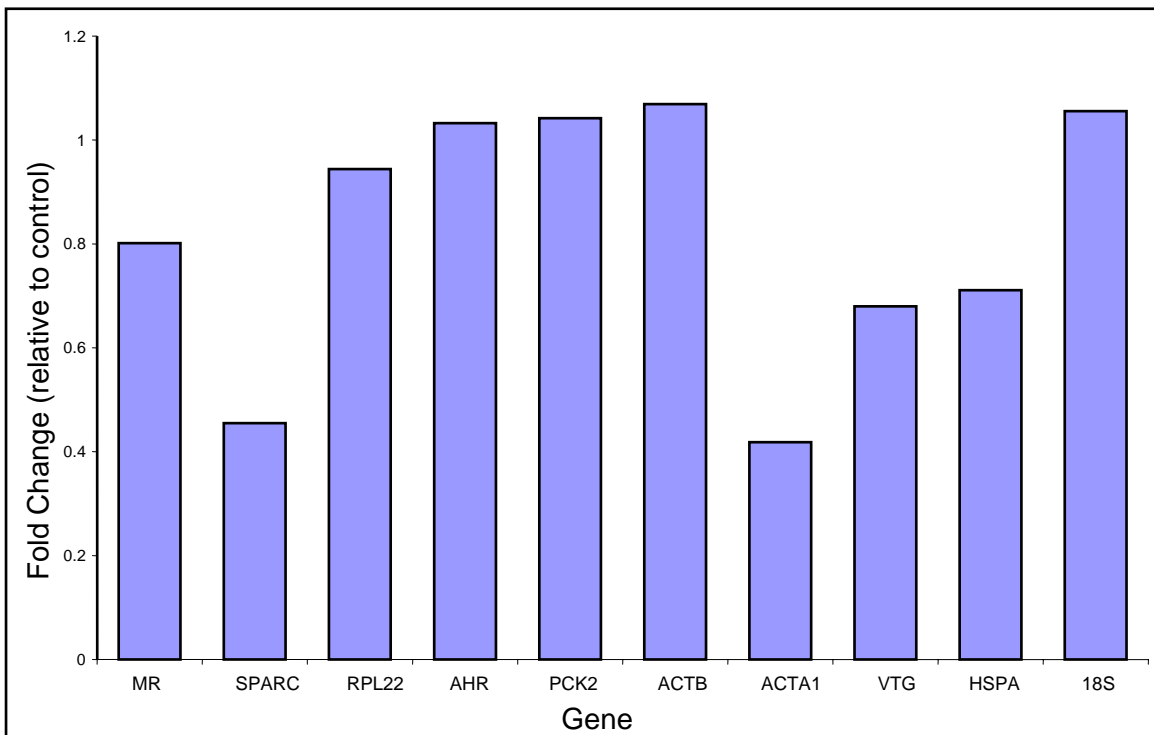
Quadruplicate data was averaged with outliers excluded using the rejection quotient test ( $\alpha=0.05$ ). Averaged  $C_{TS}$  were used to calculate copy numbers which were then normalized to the 18S ribosomal RNA gene. RPL22,  $\alpha$ - and  $\beta$ -actin were not deemed suitable normalizers as discussed in the results section.

**Results:**

The genotoxic response of White Sturgeon was assessed after exposure to two types of industrial effluent found in the Columbia River system, pulp and paper effluent from Celgar Pulp Company Limited in Castlegar and metal smelter effluent from Teck Cominco Metals Limited’s operations in Trail. A novel approach was utilized by analyzing the response of ten genes directly using real time quantitative PCR. Due to the acute lethal toxicity of Teck Cominco’s Trail effluent, organisms exposed to 100% effluent (Figure 1) and 50% effluent (Figure 2) were only collected up to day 2 and 17, respectively. Gene expression was analyzed using RT-qPCR but the data could not be statistically analyzed due to animal pooling at the outset of the experiment. Figure 1 shows that PCK2 and ACTB experienced the largest increases in gene expression after a 2 day exposure to 100% Teck Cominco effluent (>4-fold). ACTA1, HSPA, AHR and MR were all expressed greater than three-fold compared to the control, while SPARC expression was greater than two-fold. By contrast, VTG showed only slight upregulation after exposure. The large fold expression increases of ACTB and ACTA1 confirms that these genes are not appropriate normalizer genes for this application. RPL22 and 18S were expressed at near-control levels and therefore constitute good normalizer genes.



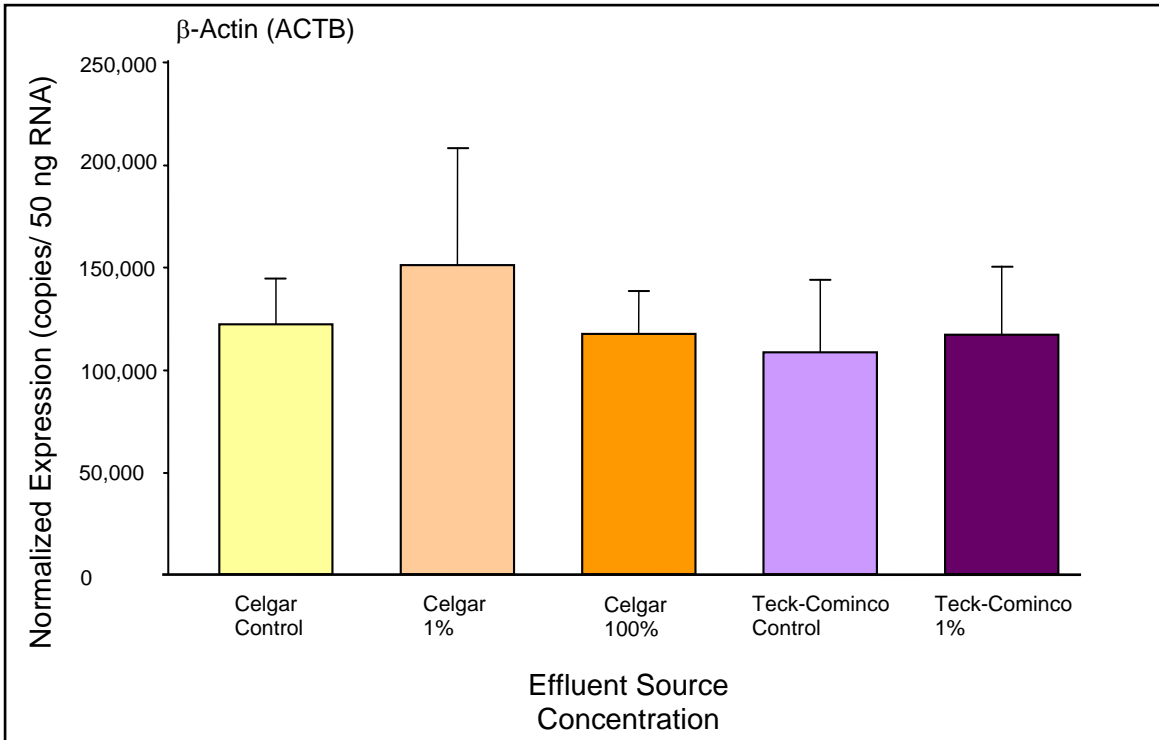
**Figure 1.** Gene expression of Teck Cominco test group exposed to 100% effluent for 2 days. Expression is depicted as fold change versus control. Each gene assayed represents a minimum of 6 pooled individuals. Biological replicates were not assayed; therefore, statistical analysis was impossible.



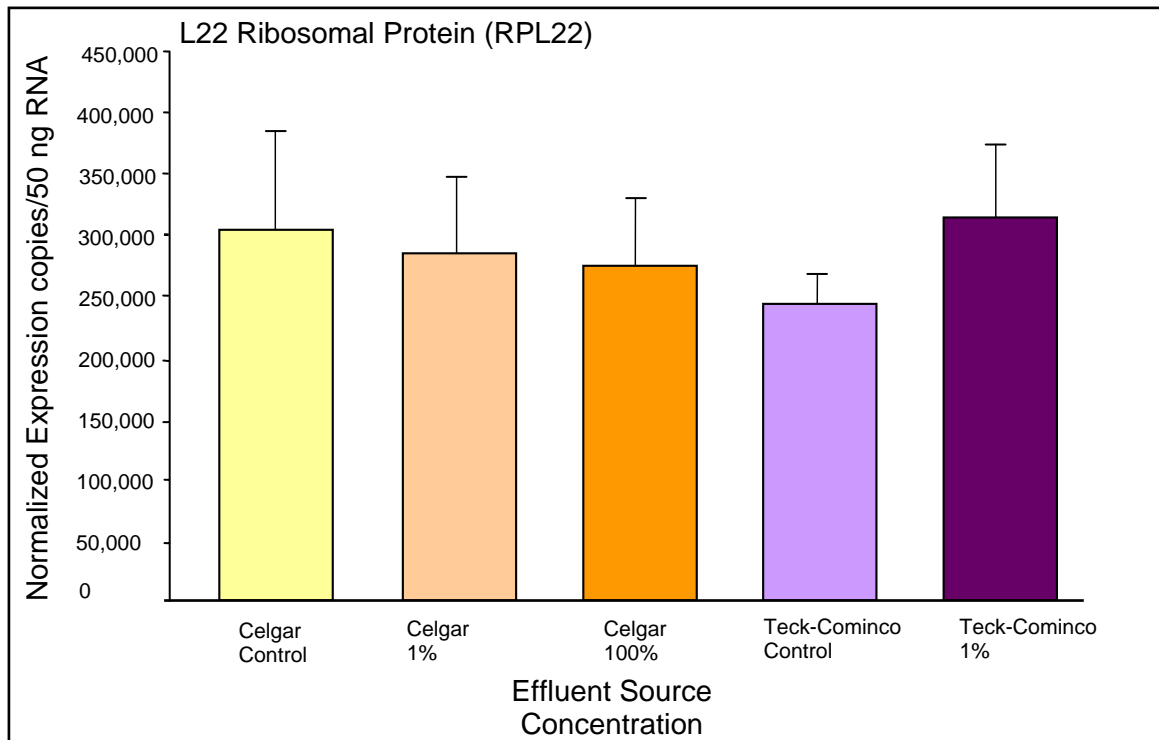
**Figure 2.** Gene expression of Teck Cominco test group exposed to 50% effluent for 7 and 17 days (averaged). Expression is depicted in fold change versus control. Each gene assayed represents a minimum of 6 individuals; however there was insufficient data to perform a statistical comparison.

Figure 2 depicts the gene expression for the sturgeon exposed to the 50% Teck Cominco effluent. It is unfortunate that the data was averaged since the study was designed such that we could compare the expression of the genes over the time course of the experiment. That being said we can see that ACTA1 is not a good normalizer since it is not being expressed equally in the exposed versus control organisms while expression of RPL22, ACTB and 18S remains constant. Exposure to the effluent decreases the transcription of SPARC and ACTA1 (down-regulated).

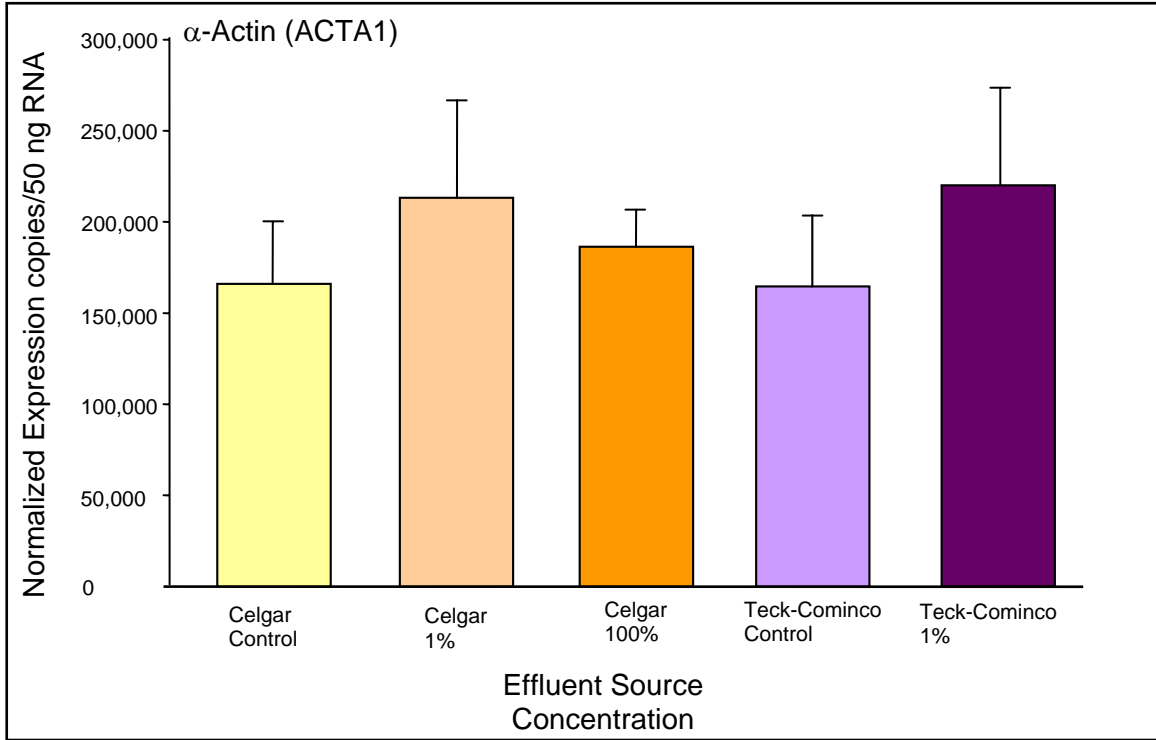
Since three points (control, 1% and 100%) were not adequate for statistical analysis the sturgeon exposed to the Teck Cominco control and 1% effluent were analyzed with those exposed to the Celgar effluent. As stated earlier, a suitable normalizer gene is critical in gene expression analysis using RT-qPCR. Since this was the first study conducted using this species and this method, four candidate normalizer genes were evaluated (ACTA1, ACTB, RPL22 and 18S).  $\beta$ -actin (ACTB) was eliminated as a candidate normalizer gene due to significant differences between the control and the 100% Celgar test group (Wilcoxon signed rank test,  $\alpha=0.05$ ) (data not shown). Both RPL22 and  $\alpha$ -actin also had higher variations between control versus treated (data not shown). Only 18S was determined to be suitable as a normalization gene due to consistent expression between control and treated. Figures 3-5 describe  $\beta$ -actin, RPL22 and  $\alpha$ -actin steady state transcript levels following 18S normalization, respectively. Figure 6 describes 18S steady state transcript levels.



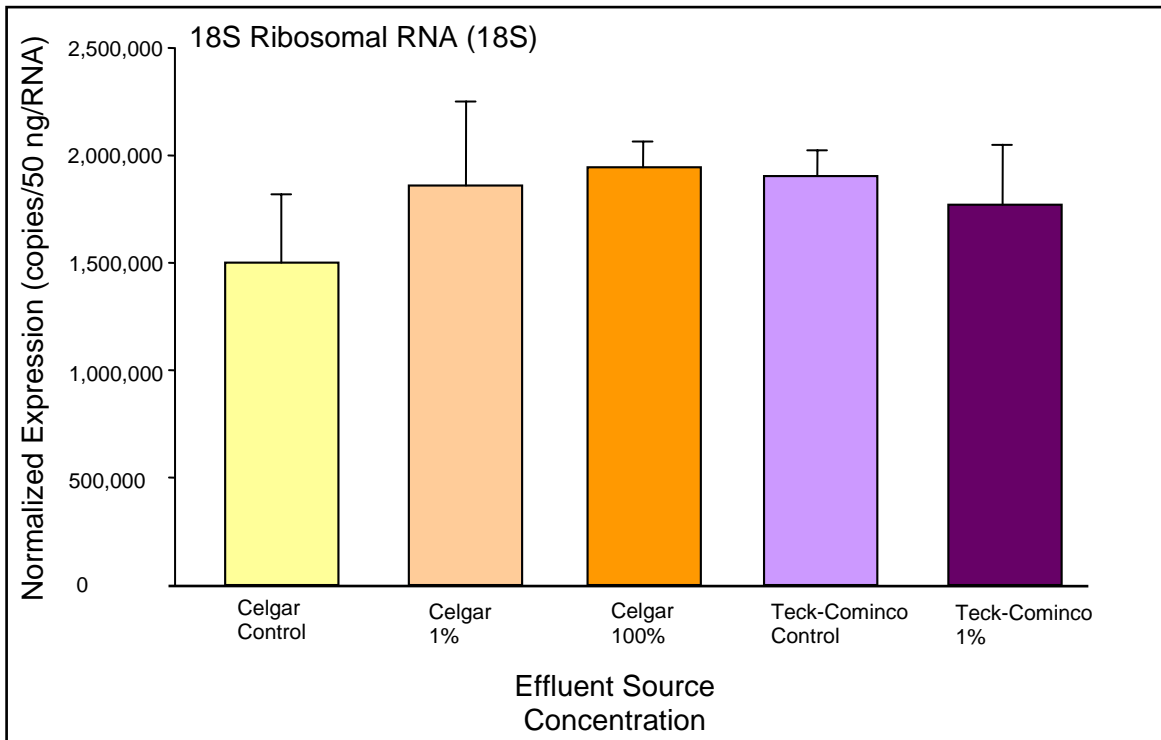
**Figure 3.** Gene expression (copy number) of the  $\beta$ -actin gene normalized to the 18S rRNA gene. Error bars represent standard error of the mean.



**Figure 4.** Gene expression (copy number) of the RPL22 Ribosomal protein gene normalized to the 18S rRNA gene. Error bars represent standard error of the mean.

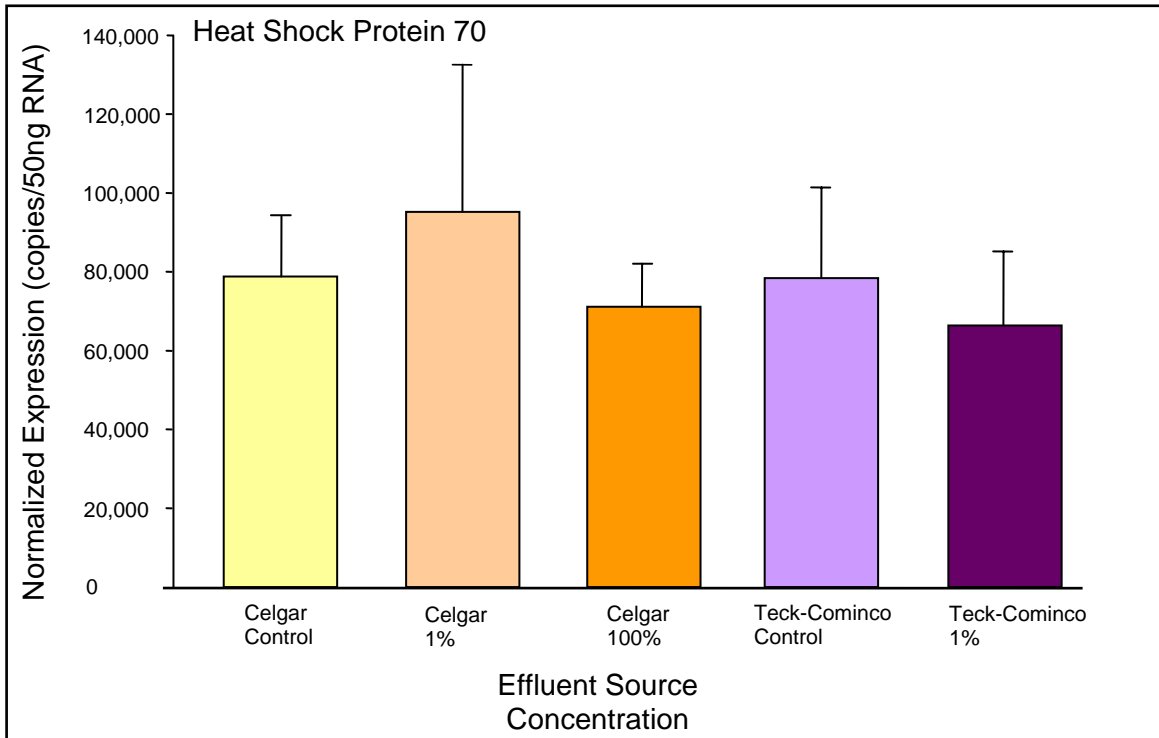


**Figure 5.** Gene expression (copy number) of  $\alpha$ -actin gene normalized to the 18S rRNA gene. Error bars represent standard error of the mean.

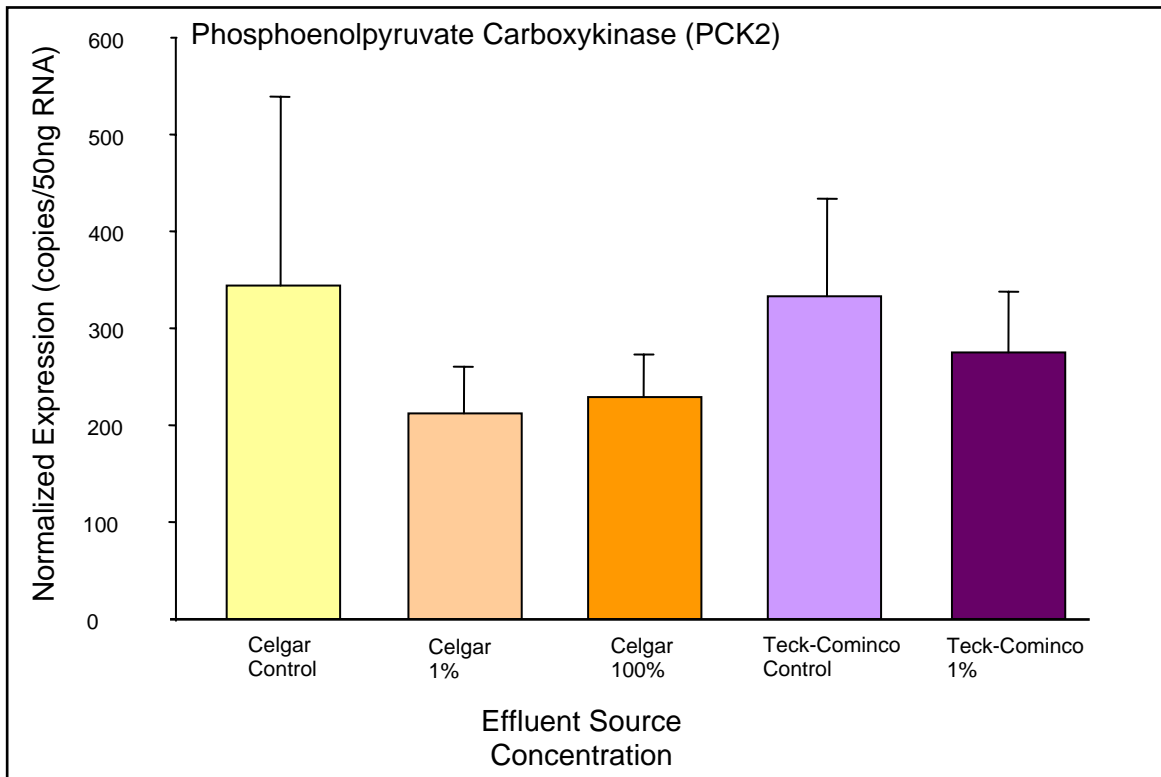


**Figure 6.** Gene expression (copy number) of the 18S rRNA gene. Error bars represent standard error of the mean.

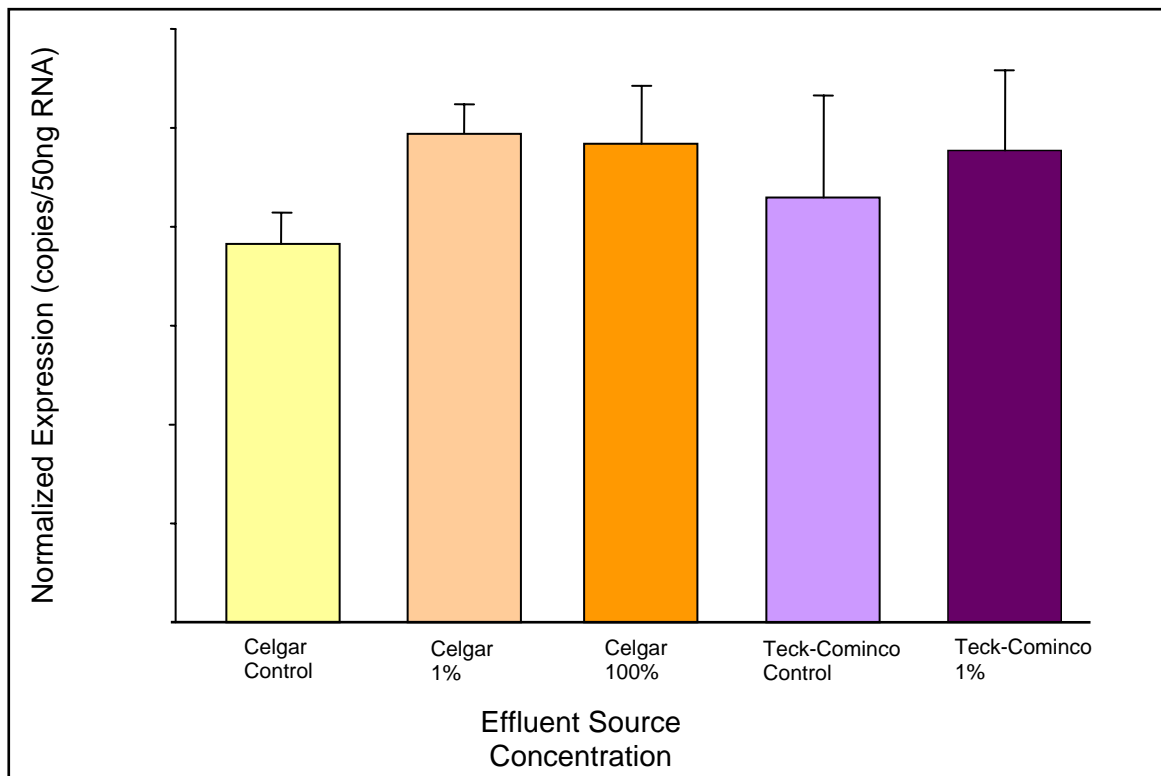
The RT-qPCR  $C_T$  data was averaged and normalized to the 18S rRNA gene. The normalized data was not uniformly normal (normal probability plot,  $\alpha=0.05$ ); therefore, non parametric methods of analysis were employed. The Wilcoxon signed rank test for matched pairs was used with  $\alpha = 0.05$  to compare control versus treated (Wackerly et al., 2002). Gene expression data for HSPA (Figure 7), PCK2 (Figure 8), SPARC (Figure 9) and VTG (Figure 10) did not show statistically significant differences from the control. The mineralocorticoid receptor, MR, showed a significant increase from control for the 100% Celgar test group (Figure 11). The aryl hydrocarbon receptor, AHR, showed a significant increase from control for the 100% Celgar test group (Figure 12).



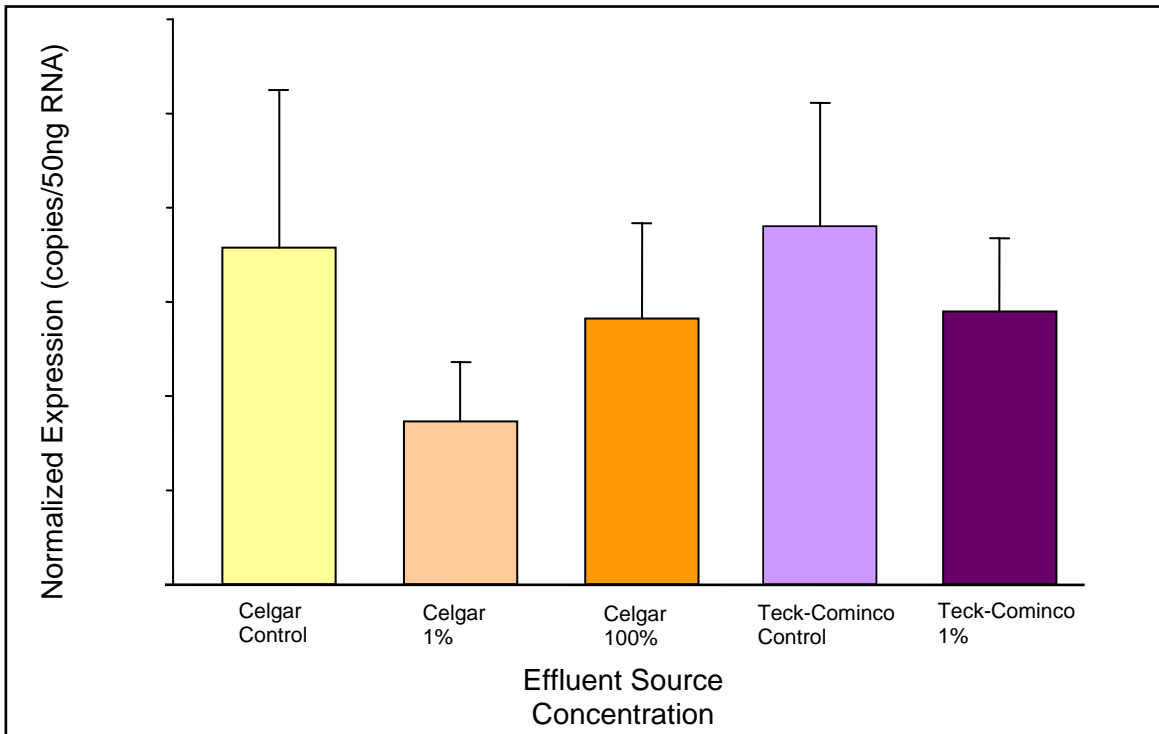
**Figure 7.** Gene expression (copy number) of the heat shock protein 70 gene, normalized to the 18S rRNA gene. Error bars represent standard error of the mean.



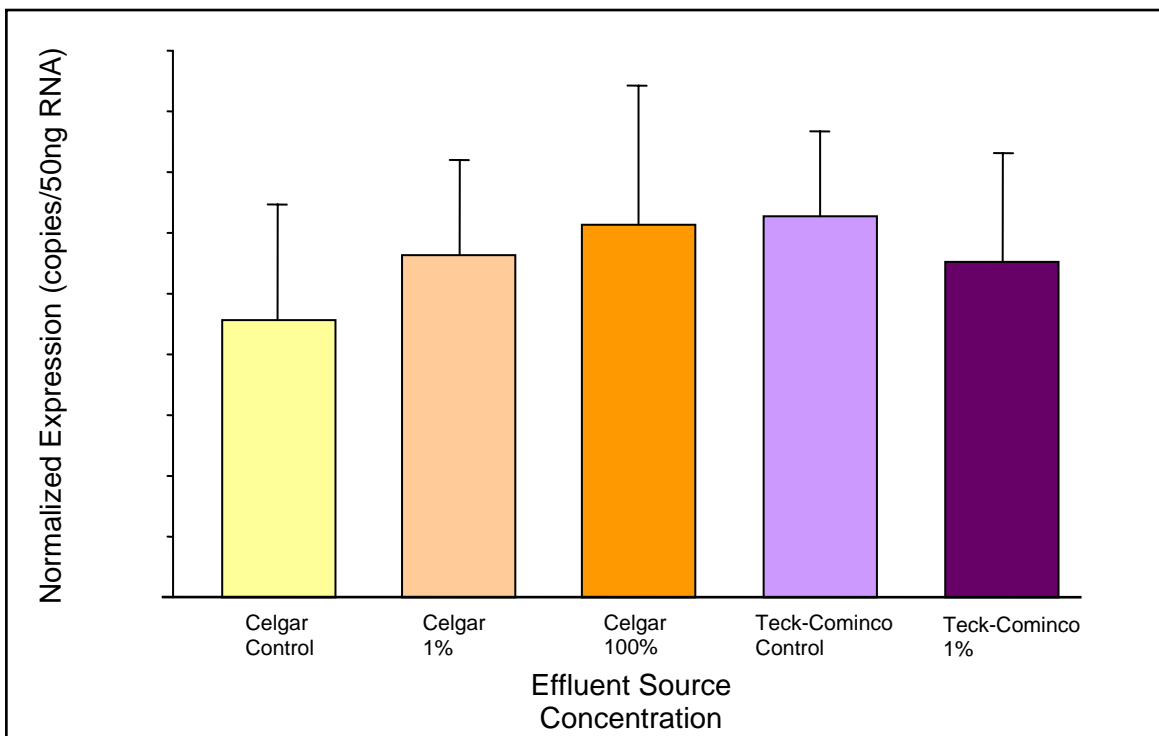
**Figure 8.** Gene expression (copy number) of PCK2, normalized to 18S rRNA. Error bars represent standard error of the mean.



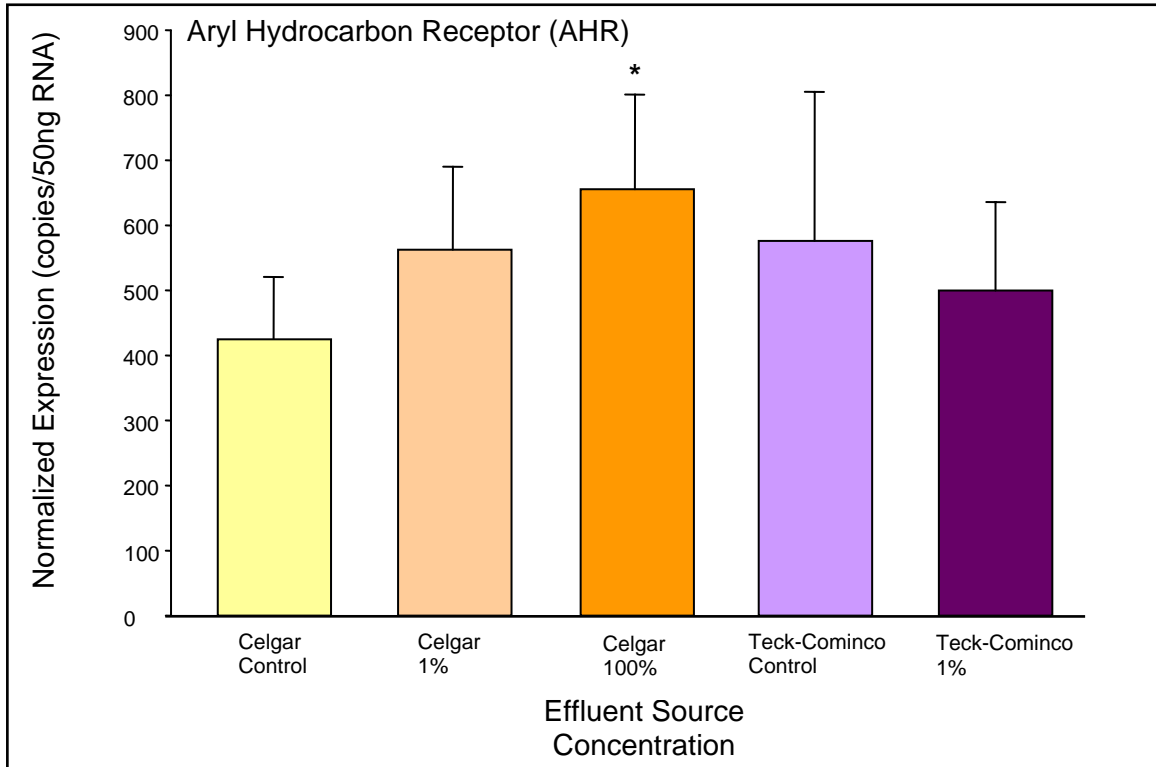
**Figure 9.** Gene expression (copy number) of the SPARC gene, normalized to the 18S rRNA gene. Error bars represent standard error of the mean.



**Figure 10.** Gene expression (copy number) of the Vitellogenin gene, normalized to 18S rRNA. Error bars represent standard error of the mean.



**Figure 11.** Gene expression (copy number) of the minerolocorticoid gene(MR), normalized to the 18S rRNA gene. The asterisk represents significant change versus control at 95% confidence (Wilcoxon signed rank test,  $\alpha=0.05$ ). Error bars represent standard error of the mean.



**Figure 12.** Gene expression (copy number) of the aryl hydrocarbon receptor normalized to the 18S rRNA gene. The asterisk represents significant change versus control at 95% confidence (Wilcoxon signed rank test  $\alpha=0.05$ ). Error bars represent standard error of the mean.

### Discussion:

Analysis of the Teck Cominco 100% test group at day 2 and the Teck Cominco 50% test group at day 7 & 17 yielded the most interesting data. Fish exposed to 100% effluent for two days showed a marked increase in the transcription level of almost all genes when compared to control, illustrating a shock response to the contaminants. PCK2 showed the largest increase in gene expression (>four-fold). PCK2 codes for an enzyme central to amino acid metabolism and gluconeogenesis and its expression has been linked to contaminant exposure from heavy metals, organics and dioxin. ACTB and ACTA1 both showed a greater than three-fold increase in expression. Both genes code for cell growth and repair proteins. As well, HSPA (heavy metals and a general stress response), AHR (halogenated aromatic hydrocarbon exposure), and MR (endocrine/receptor function) were all upregulated by more than three-fold. These fish were undergoing massive metabolic changes and it is not surprising that they all perished soon after exposure to the 100% Teck Cominco effluent. This massive upregulation of gene expression contrasts dramatically with the downregulation evident in the Teck Cominco 50% test group. Exposure to this effluent had the greatest effect on SPARC and  $\alpha$  actin expression. Both SPARC and  $\alpha$  actin code for proteins essential in cell growth and repair and both were suppressed two-fold.

Metal analysis of the 100% effluent showed the following levels: zinc 128-273 ug/L (average 166); lead 12.5-54 ug/L (average 21.6); copper 1-6 ug/L (average 2.5); and cadmium 2.28-3.35 ug/L (average 2.55). The zinc, lead and cadmium values are above the Canadian Environmental Quality Guidelines for water: aquatic life freshwater (zinc 30 ug/L, lead 1-7 ug/L, copper 2-4 ug/L and cadmium 0.2-1.8 ug/L). The fish exposed to 50% Teck Cominco effluent were also observed to be visually smaller and thinner than their counterparts in the control and Celgar test groups. These results suggest that the metal contamination present in Teck Cominco's maintenance-period effluent is affecting growth regulation through an unknown mechanism. It would be beneficial to test this by analyzing growth hormone levels in subsequent experiments and metallothionein gene expression (genetic indicator of metal exposure).

The sturgeon exposed to the 100% Celgar showed significant increases in the steady state levels of the mineralocorticoid receptor and the aryl hydrocarbon receptor transcripts. It is not understood why the mineralocorticoid receptor is being differentially expressed but the aryl hydrocarbon receptor transcripts are associated with halogenated aromatic hydrocarbon exposure and could indicate a response to low levels of dioxin in the effluent. Unfortunately, dioxin was not assessed in this effluent. The chemistry results for the 100% Celgar included: low levels of resin and fatty acids and PAHs (polycyclic aromatic hydrocarbons); organohalides (AOX) at 0.87-2.0 mg/L; low levels of total metals except for weeks 6 and 7 when the zinc levels rose from an average range of 7.1-33 µg/L to 176 and 141 ug/L, respectively; detection of 5 out of 18 sterols above the detection limits. The top three sterols were:  $\beta$  sitosterol ranging from 0.15-1.04 ug/L, stigmasterol from 0.06-0.49 ug/L, and campesterol 0.03-0.24 ug/L.

Although it was not unexpected for the 100% Celgar test group to show gene expression effects, it was surprising to see no effect for the 1% test group. Also, given that the 100% and 50% Teck Cominco test groups were acutely toxic, the need for additional test concentrations is evident (e.g. 1%, 3%, 10%, 30% and 100%). As previously mentioned, this toxicogenomics approach is novel. The experimental design was modified from the standard toxicology early lifestage bioassay and the results of this exposure suggest that improvements must be made in experimental design to better accommodate toxicogenomics.

The goal of this study was to describe gene transcript steady state levels at multiple time points and concentrations. Consequently, animals were exposed to control (0%), 1% and 100% effluent for 50 days with 10 discrete collection time points (every 5). Unfortunately, the data was not analyzed according to plan due to animal pooling at the outset of the experiment which reduced the sample size per treatment and timepoint to one.

RT-qPCR is extremely laborious and only suitable analysis of a small number of genes; therefore, when analysis of a large number of genes is desired cDNA microarray should be considered. This study was limited to 10 genes; therefore, changes in many other genes were not captured. For this study, genes in the following classes were analyzed: apoptotic (0), binding/transport (0), embryonic (0), endocrine (2), enzymatic (1), housekeeping (3), immune (0), metabolic (1), oncogenic (0), receptor (2), regulatory (1),

signal transducer (1), stress-responsive (1), structural (4), transport (0). Recommendations for gene additions include:

- at least one representative from each gene class;
- more estrogen-responsive genes. Although vitellogenin steady state transcript levels were measured, the vitelline envelope proteins (VEPs) were neglected. Transcripts for these genes have been suggested as earlier, and potentially more sensitive, indicators of estrogen exposure. Aspartate protease should also be included since it is a highly estrogenic sensitive hepatic gene;
- more genes responsive to organic toxicants, such as cytochrome P450 (CYP 1A3) and carbonyl reductase;
- genes responsive to metals; although more than one year was spent trying to obtain metallothionein, there is still a need to include this metal-responsive gene;
- genes responsible for key functions such as prostaglandin D synthase. This gene is involved in paracrine control of numerous metabolic pathways and immune response and is known to be altered by short-term exposure to heavy metals.

Using the tools of molecular biology to assess environmental impacts is a relatively new field. These results clearly show that undiluted industrial effluents can affect the white sturgeon gene expression profile in a laboratory setting. However, it cannot yet be determined if these effects are deleterious or if they will occur at environmentally relevant levels. Even if more genes are incorporated into the genomics method, the procedure retains a fundamental limitation: gene transcript levels do not always correlate with functional protein levels. Regardless, exposure-induced changes in gene expression are cause for concern, especially if those changes occur in a group of related genes (e.g. estrogen responsive genes) and if a valid correlation between mRNA and functional protein can be established. It remains necessary to develop better means of analyzing gene expression data, including: developing additional tools for statistical analysis; and increasing data sets correlating mRNA transcript levels (genomics) with protein levels (proteomics).

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