# TOXICOLOGICAL EVALUATION
OF EMERGING CHEMICALS IN
MUNICIPAL WASTEWATER EFFLUENTS
WITHIN THE
GEORGIA BASIN
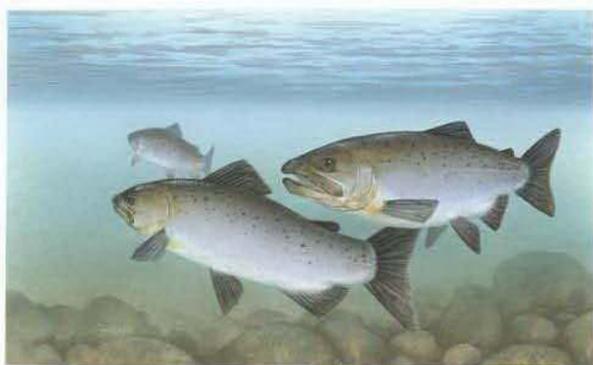
Chinook

Rainbow trout

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EXECUTIVE SUMMARY

In British Columbia, more than 0.58 trillion litres of municipal wastewater effluent (MWWE) are released annually into the Georgia Basin. While sewage treatment plants attempt to remove the majority of nutrients, pathogens and chemicals from the effluent prior to its release into the environment, not all substances are removed or neutralized. Of particular concern to scientists and the public are the exposure effects from emerging chemicals of concern, or ECCs.

ECCs have novel toxicity, are accumulating in the environment, are being detected in living organisms, and may have the potential to cause adverse effects to public health and/or the environment. ECCs are a diverse group that includes: bis-phenol A, phthlates, nonylphenols, brominated flame retardants, nanoparticles, industrial chemical additives, synthetic musks and other personal care products. Some ECCs are endocrine disruptors. Endocrine disruptors affect normal hormone function at the parts per billion or parts per trillion levels. Documented MWW effects on aquatic life (fish) include: endocrine disruption, altered immune function, growth reduction, feminization and mortality. Vitellogenic alterations are widely documented. Expression of vitellogenic genes in sexually immature fish is indicative of estrogen exposure.

Functional genomics tools (cDNA microarrays, QPCR, QuantiGene Plex) were used to determine how salmonids (rainbow trout (*Oncorhynchus mykis*) and chinook (*Oncorhynchus tshawytscha*)) respond at a molecular level to municipal wastewater effluent (MWWE).

In particular, the objectives were:
to determine if emerging chemicals of concern affect fish by assessing if:
- o chemistry data are predictive of biological effect,
- o sewage exposure causes measureable changes in gene expression within the liver of exposed salmonids,
- o gene expression changes are permanent,
- o the lowest sewage concentration (environmentally-relevant) casues significant changes in gene expression,
- o gene expression changes are dose responsive,
- o gender influences the molecular response to sewage exposure.

and to create toxicogenomic capacity and expertise.

MWWE from Greater Vancouver Regional District (GVRD or Metro)'s Annacis Island, and Capital Regional District(CRD)'s Clover Point and Macaulay Point were examined. Annacis (Annacis Island) MWWE was chosen for the study since it represented the most processed effluent, enhanced secondary. The plan was to study the most processed and least processed effluents which empty into the Georgia Basin, with the expectation that there would be marked differences in their effects on fish. It was also anticipated that the lowest concentrations of the secondary sewage would not have any molecular level

effects and a threshold value would be found where only effluent concentrations above this level would have an effect. The MWWE from the Victoria area, Macaulay Point and Clover Point, represented the least treated effluent, preliminary, and this effluent discharges into the marine environment. Annacis discharges into freshwater in the Vancouver area. The effluents were collected over a four year period with chemical analysis (metals, sterols, acidic drugs, musks and general parameters) of the sewage conducted through out the study. Salmonids were exposed to an exposure regime consisting of two 4 day back to back effluent bioassays and then an 8 day recovery in control water. The concentrations ranged from NOEC (no observed effect concentration) to environmentally relevant receiving water. Through out the study at specific time intervals (Days 1, 4, 8 and 16) fish were sacrificed and tissues (liver, brain, muscle flap) collected for gene expression analysis. Gene expression experiments compared control (clean water) and municipal wastewater effluent (MWWE) exposed salmonids.

Chemistry data was somewhat predictive of biological effect and depended on which chemicals were being analyzed. Metals, non-steriodal anti-inflammatories and fibrates were not predictive of biological response whereas known estrogens do have a predicted biological response. As shown in Figures A and B, secondarily treated sewage (A Fresh 2005/2006) is more homogenous than preliminary treated sewage (B C Marine), thus resulting in a more predictable molecular response in exposed fish.
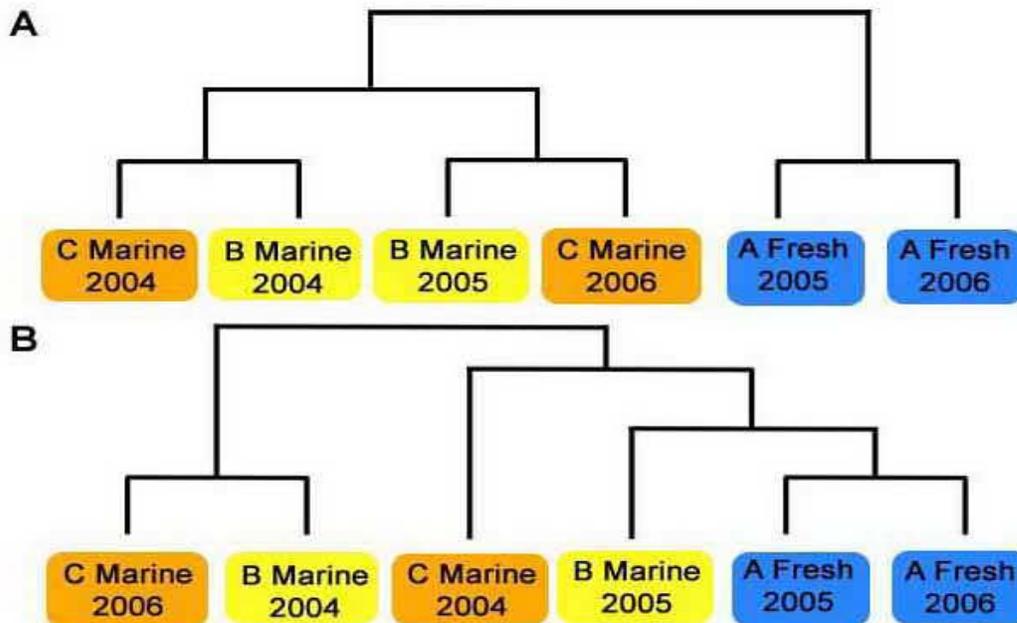
Figures A and B. Euclidean distance tree of (A) chemistry data and (B) gene expression profiles.

Sewage exposure did cause measureable changes in gene expression with the liver of exposed salmonids. Between all six exposures, 98% of the genes responded at some time point and concentration; however, most of these genes were not consistently altered in

every exposure. Gene expression changes were seen within one day of exposure and were sometimes maintained even after eight days of depuration. Figure C. highlights the classes of genes on the array and the impact of the sewage exposure on the expression of the genes within the classes. The top gene classes affected by sewage exposure were: metabolism, endocrine and binding/transport. Synthesis or removal of gene transcripts has an inherent metabolic cost which could result in resources from other functions such as growth or ion regulation being diverted to perform this function, which could ultimately impair the organism's ability to function and/or increase its chance of predation.
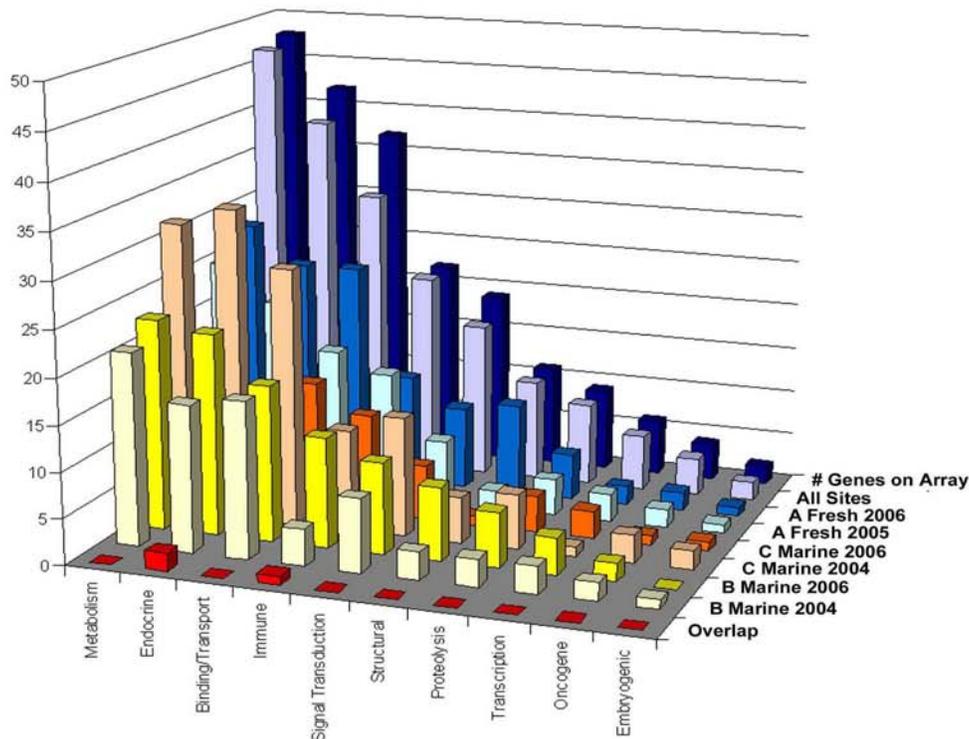
Figure C. Gene Class Distribution on the Array and Impact of Sewage Exposure on the Expression of Genes within those Classes.

Most gene expression changes were not permanent. Between 64 to 98 genes recovered after the 8 days of depuration that followed the 8 day sewage exposure. The array is a designer or focused array with 207 genes so this represents a large portion of genes recovering. As depicted in Figure D., 61% to 74% of the genes recovered, 1% to 17% (1-25) did not recover while 15% to 34% (22-38) were newly altered only after 16 days of sewage/control exposure. However, it must be remembered that although most genes did recover there is still a metabolic cost to synthesis or removal of gene expression transcripts. This diversion of resources might have negative consequences on other functions, including growth or ion regulation. At a minimum, the observed gene expression changes are early warning signs with potential physiological consequences.

	B Marine 2004	B Marine 2005	C Marine 2006	A Fresh 2005	A Fresh 2006
Recovered	61%	71%	74%	69%	68%
Did not recover	5%	8%	1%	12%	17%
Altered	34%	21%	25%	19%	15%
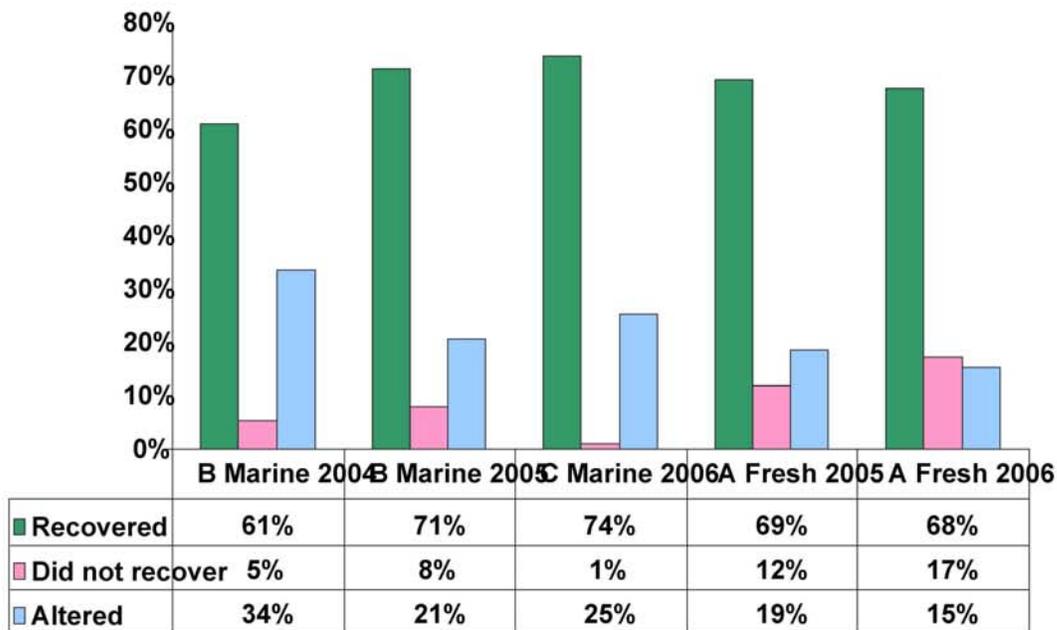
Figure D. Gene Transcripts at Day 16: genes that recovered to control levels, genes that did not recover, and those that were altered on Day 16 only.

The lowest sewage concentrations did cause significant changes in gene expression. The genes responding to low levels of sewage were distinct from the genes responding to the higher sewage concentration. As shown in Figure E, the low doses had a greater affect on genes in the immune and signal transduction classes while the higher concentrations had a greater affect on genes in the proteolysis and endocrine classes.
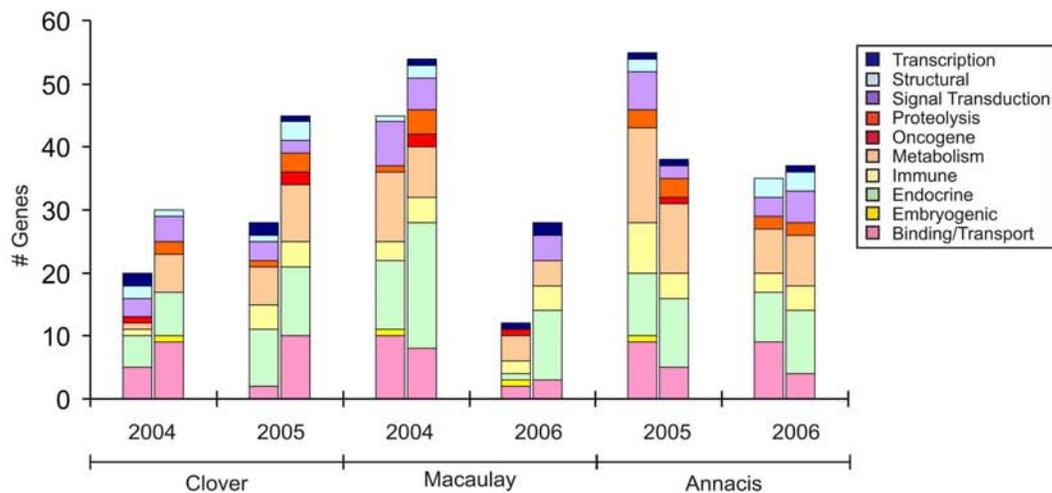
Figure D. Class Distribution for genes responding to low and high MWWE concentrations.

Only some of the gene expression changes were dose-responsive. As depicted in Figure E., of the 207 genes on the array, only 2-31 were dose responsive. The percentages that were directly proportional to the sewage concentration ranged from 35 to 75%. While the percentage that were inversely proportional to he sewage concentration ranged from a low of 25% to a high of 65%.

Site	Year	# Genes responding dose-responsively	% Directly Proportional to sewage concentration	% Inversely Proportional to sewage concentration
B Marine	2004	5	40%	60%
	2005	15	67%	33%
C Marine	2004	20	75%	25%
	2006	2	50%	50%
A Fresh	2005	31	35%	65%
	2006	14	71%	29%

Figure E. Genes Responding to Sewage Exposure in a Dose-Responsive Manner.

The sewage did differentially affect gene expression in males as compared to females but the difference was rarely statistically significant. To evaluate this factor, QPCR data analysis was conducted on one of the effluents. QPCR is much more focused than microarrays and typically only examines a handful of genes intensely. Due its laborious nature, only one effluent was studied to this extent. As shown in Figure F.,

	%MWWE	Day 1		Day 4		Day 8	
		F	M	F	M	F	M
VTG	0.7	1.2	3.6	2.2	3.0	1.8	1.8
	2	4.6	2.5	7.5	7.5	3.1	4.5
	5	1.6	2.1	3.7	4.0	2.3	6.9
VEPA	0.05	1.8	2.1	2.5	4.5	2.5	2.4
	0.7	2.0	2.1	6.8	15.6	1.8	3.0
	2	3.2	2.6	23.9	16.6	2.5	5.0
	5	2.4	4.9	10.8	18.1	4.3	8.0
VEPB	0.05	1.2	2.1	1.3	2.0	2.1	1.3
	0.7	1.5	2.2	2.8	4.9	1.7	1.5
	2	1.5	2.5	6.3	4.8	1.8	2.5
	5	1.0	2.6	3.3	4.1	3.7	3.3
ESR1	0.05	1.5	2.2	1.2	1.2	2.0	1.3
	0.7	1.6	3.1	2.3	2.0	2.0	1.4
	2	2.3	3.1	2.5	1.7	1.6	1.6
	5	1.8	4.0	2.1	1.5	1.0	1.1

highlighted the xenoestrogenic changes in gene expression. Xenoestrogens can alter the expression of genes associated with adult fish reproductive processes, particularly those relating to egg production (female-specific): vitellogenin and the vitelline envelope proteins.

An in-depth analysis of the Chinook exposed to CRD Clover sewage revealed that there were very few significant differences in the expression of female-specific genes between the sexes. Gene expression patterns were examined for trends across time and sewage concentrations. Trends due to fish exposure to CRD Clover effluent included U-shaped and linear dose response curves. All CRD Clover effluent concentrations examined caused gene expression alterations, including the very low environmentally relevant concentrations.
Some of these changes were confirmed by real time PCR. (Possibly omit this information. Report might not include this type of analysis.)

In addition to the data from the toxicogenomics analysis, the report describes the development of: (1) high-throughput, optimized microarray hybridization protocols, (2) microarray data analysis methods, (3) an absolute quantification Quantitative Polymerase Chain Reaction (QPCR) assay for estrogenic genes, and (4) QPCR data analysis methods.

The gene expression experiments demonstrated the applicability of the different techniques in the study of the effects of toxicants.

Keywords: toxicogenomics, microarray; QuantiGene plex, QPCR, gene expression; Chinook, salmon, liver;, xenoestrogens, endocrine; sewage, MWWE; Georgia Basin Action Plan, Environment Canada

ABBREVIATIONS AND SYMBOLS

bp	base pair
°C	degree(s) Celsius
CAEAL	Canadian Association for Environmental Analytical Laboratories Inc.
CCME	Canadian Council of Ministers of the Environment
CRD	Capital Regional District
Cy3	Cyanine 3 fluorescent dye
Cy5	Cyanine 5 fluorescent dye
DNA	Deoxyribonucleic acid
E2	17β-ethinylestradiol (natural estrogen)
ECOC	Emerging Contaminants of Concern
EDC	Endocrine Disrupting Compound
EE2	17α-ethinylestradiol (synthetic estrogen)
g	gram(s)
g	gravitational
GVRD	Greater Vancouver Regional District

h	hour
L	litre
LC50	median lethal concentration
LOEC	lowest-observed-effect-concentration
LWMP	Liquid Waste Management Plan
M	Molar
mg	milligram(s)
min	minute
ml	milliliter
MM	Master Mix
MOU	memorandum of understanding
mRNA	messenger RNA
ng	nanogram
nmole	nanomole
NOEC	no-observed-effect-concentration
OECD	Organization for Economic Cooperation and Development
pmol	picomole
^{o}/oo, ppt	parts per thousand
PESC	Pacific Environmental Science Centre
PPCP	pharmaceutical and personal care products
SETAC	Society of Environmental Toxicology and Chemistry (North America)
QA/QC	Quality Assurance/Quality Control
QPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
s	second
SD	standard deviation
SETAC	Society of Environmental Toxicology and Chemistry (North America)
μ	micro
μg	microgram
μl	microlitre
μm	micrometer
V	volts
WQG	water quality guideline
WWT	wastewater treatment
WWTP	wastewater treatment plant

TERMINOLOGY

Note: all definitions are given in the context of this report, and might not be appropriate in another context.

Acclimation means to become physiologically adapted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions (Environment Canada, 1990).

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period of exposure to a test material, usually $\leq$ 4 days for fish (Environment Canada, 1990).

Bioavailability is used to describe the fraction of a dose of a compound that reaches the systemic circulation; when a compound is administered intravenously, its bioavailability is 100%. When a compound is administered via other routes (such as ingestion or through the gills), its bioavailability decreases due to incomplete absorption and first-pass metabolism (www.wikipedia.org).

Biotransformations are processes by which a chemical or other foreign substance is modified by a living organism. They can be divided into two phases: phase 1 and phase 2. In phase 1 transformations, a reactive polar group is added onto the target substance. In phase 2 transformations, an additional compound is added to the polar group from the phase 1 reaction to facilitate excretion (see Phase 1 and Phase 2 Transformations).

Chronic toxicity is a property of a substance that has toxic effects on a living organism, when that organism is exposed to the substance continuously or repeatedly. Compared with acute toxicity (www.wikipedia.org).

Complementary Deoxyribonucleic Acid (cDNA) is a single-stranded DNA molecule that is complementary to messenger ribonucleic acid (mRNA). The DNA is synthesized *in vitro* from isolated RNA, using a reverse transcriptase enzyme and may be utilized in hybridization studies, including microarray experiments.

Compliance means in accordance with governmental permitting or regulatory requirements (Environment Canada, 1990).

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution
Ct is the cycle at which the amplification curve crosses the threshold, see PCR procedures.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test condtions (e.g., quality of the control/dilution water, health or handling of test organisms) (Environment Canada, 1990).

Control/dilution water is the water used for diluting the test material, or for the control test, or both (Environment Canada, 1990).

Cyanine 3 fluorescent dye (Cy3) is a reactive water soluble fluorescent dye of the cyanine family (www.wikipedia.org). Cy3 is excited maximally at 550 nm and emits maximally at 570nm, in the red part of the spectrum It is usually synthesized with

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Environment Canada Environnement Canada   GVRD and CRD MWWE Study

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reactive groups on either one or both of the nitrogen side chains so that it can be chemically linked to either nucleic acids or protein molecules. Labelling is done for visualization and quantification purposes. See Fluorophores.

Cyanine 5 fluorescent dye (Cy5) is a reactive water soluble fluorescent dye of the cyanine family (www.wikipedia.org). Cy5 is excited maximally at 649 nm and emits maximally at 670nm, in the far-red part of the spectrum. It is usually synthesized with reactive groups on either one or both of the nitrogen side chains so that it can be chemically linked to either nucleic acids or protein molecules. Labelling is done for visualization and quantification purposes. See Fluorophores.

Deleterious means causing harm or damage (www.wikipedia.org). The deposit of deleterious substances into water frequented by fish (Canadian fisheries waters) is prohibited under subsection 36(3) of the Federal *Fisheries Act*. Environment Canada has the legislative responsibility for the administration and enforcement of this portion of the law, which prohibits unauthorized pollution.

Deleterious substance Under section 34 of the Federal *Fisheries Act* a deleterious substance is defined as: (a) any substance . . . [or] (b) any water that contains a substance in such quantity or concentration, or that has been so treated, processed or changed, by heat or other means, from a natural state that it would, if added to any water, would degrade or alter or form part of a process of degradation or alteration of the quality of that water so that it is rendered or is likely to be rendered deleterious to fish or fish habitat or to the use by man of fish that frequent that water.

Deoxyribonucleic Acid (DNA) is a nucleic acid found in the nucleus of the cell consisting of a polymer of nucleotides and shaped like a double helix. DNA carries the genetic information of the cell and is capable of self-replication and synthesis of RNA.

Dilution capacity is the capability of a body of water to dilute materials; the larger the body of water, the larger the capacity to dilute the materials.

Dilution water is the water used to dilute a test material in order to prepare different concentrations of the material for use in toxicity testing (Environment Canada, 1990).

Ecosystem or an ecological system is an assemblage of organisms (plant, animal and other living organisms—also referred to as a biotic community or biocoenosis) living together with their environment (or biotope), functioning as a loose unit. That is, a dynamic and complex whole, interacting as an "ecological unit".

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment (Environment Canada, 1990).

Electrophoresis is a method of separating large molecules (such as DNA fragments or proteins) from a mixture of similar molecules, based on the rate of movement of each molecule in a colloidal suspension while in an electric field. A DNA microarray is created by spotting specific pieces of DNA onto a glass slide. This DNA is often

generated by PCR and its purity must be assessed by gel electrophoresis and spectrophotometry before it can be spotted onto a glass slide. PCR can generate multiple products (i.e. non-specific amplification of DNA) resulting in heterogeneous DNA being deposited onto the microarray, which can lead to non-specific hybridization.

Emerging Chemical Contaminants of Concern (ECC) are new toxic substances, including pharmaceuticals and nanomaterials, which occur in industrial and municipal effluents. These contaminants may disrupt endocrine systems, cause morphological anomalies or reduce the reproductive rate of aquatic organisms.

End point means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (LC50, etc.) (Environment Canada, 1990).

Endocrine Disrupting Compounds (EDC) are compounds that alter the function of the endocrine system, thereby harming an individual, its offspring, or populations. Endocrine systems, also referred to as hormone systems, are found in all mammals, birds, fish, and many other types of living organisms. They include: glands, hormones, and receptors. The glands release the hormones, chemical messengers that circulate throughout the body. Hormones bind to their compatible receptors, like a key (hormone) in a lock (receptor), and once bound, the receptor carries out the hormone's instructions by either altering the cell's existing proteins or turning on genes that will build new proteins. Both actions create reactions throughout the body. Any compound that interferes with the endocrine system is labelled an EDC. EDCs are associated with adverse developmental and reproductive effects.

Fold Change is the ratio of gene expression in a treatment to gene expression in a control. A value of 1 indicates equal gene expression in both treatment and control; a value of 2 indicates a two fold increase in gene expression in the treatment as compared to the control. A value of 0.5 indicates a two fold decrease in gene expression in the treatment as compared to the control.

Fluorophores are substances that fluoresce. Fluorescence is the molecular absorption of light energy at one wavelength and its nearly instantaneous re-emission at another wavelength. Microarrays use fluorescent DNA dyes (Cy3, Cy5) that carry an N-hydroxysuccinimidyl ester group. The ester group reacts with aminoallyl-modified nucleotides which are incorporated into cDNA during reverse transcription from the target RNA.

Fork length is the length of a fish, measured from the tip of the nose to the fork of the tail (Environment Canada, 1990).

Gene Transcript is the portion of the gene is transcribed from DNA to mRNA. The cDNA generated from an mRNA template is also referred to as a "gene transcript".

Guidelines are statements on policy or procedure to determine a course of action (www.answers.com). Canadian Council of Ministers of the Environment (CCME) sets

environmental quality guidelines as numerical concentrations or narrative statements recommended to support and maintain a designated use of water.

Hybridization is the pairing (tight physical bonding) of two complementary single strands of DNA to give a double-stranded molecule. It is the process by which the gene transcripts bound to the microarray pair with the gene transcripts extracted from the organism of interest.

Inorganic chemicals are chemicals that are **not** organic chemicals. Inorganic chemicals are traditionally derived from mineral sources of non-biological origin but also include all metal-containing compounds, even those found in living systems (www.wikipedia.org).

Lethal means causing death by direct action. Death of fish is defined as the cessation of all visible signs of movement or other activity (Environment Canada, 1990).

Lowest-Observed-Effect Concentration (LOEC) is the lowest tested concentration of a material that has an observable effect on the organism that is statistically different from the control.

Median Lethal Concentration (LC50) is the concentration of substance in effluent that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities after a fixed period of exposure.

Messenger ribonucleic acid (mRNA) is a single-stranded RNA molecule that is transcribed from a gene and acts as the template for protein synthesis.

Microarray refers to a piece of glass, plastic, or silicon onto which gene transcripts have been chemically attached. Microarrays (arrays, biochips, DNA microarrays) can be utilized to study how large numbers of genes interact with each other and how a cell's regulatory networks control vast batteries of genes. The microarrays serve as hybridization targets for the cDNA derived from isolated tissue. RNA extracted from the tissue is reverse transcribed, with simultaneous incorporation of a label, into cDNA. Labelled cDNA is hybridized onto the microarray slide and the incorporated label provides a signal where cDNA from tissue binds to the cDNA previously spotted on the slide. This technique allows the analysis of multiple genes to develop gene expression profiles.

No-Observed-Effect Concentration (NOEC) is the next lowest concentration below the LOEC, among those concentrations tested.

Organic chemicals are chemicals composed of carbon and hydrogen, which may contain any number of other elements, such as nitrogen, oxygen, halogens, and more rarely phosphorus or sulphur (www.wikipedia.org).

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions

on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions (Environment Canada, 1990).

Photoperiod is the duration of illumination and darkness within a 24-h day (Environment Canada, 1990).

Polymerase Chain Reaction (PCR) is a method used to make multiple copies of DNA from a DNA template using a thermostable DNA polymerase enzyme, a primer pair, deoxynucleotides, buffer and divalent cations. PCR includes three temperature-controlled steps (denaturation, annealing, and elongation) which are repeated 20-40 times to achieve exponential amplification of the target DNA. The template DNA is first **denatured** by heating the reaction to 94-96°C, thereby disrupting the hydrogen bonding between complementary bases of the DNA strand and generating single-stranded DNA. The reaction is then cooled to 50-65°C, which allows the short primer pair (~18-22 nt) to **anneal** to complementary bases within the single-stranded DNA template. The DNA polymerase then binds the primer-template hybrid and begins DNA synthesis. The temperature of the reaction is then increased to 72°C, which promotes **elongation** of the new DNA strand. The DNA polymerase enzyme elongates the new strand by adding dNTPs that are complementary to the template in 5' to 3' direction. PCR and its registered trademarks are the property of F. Hoffmann-La Roche & Co. AG, Basel, Switzerland.

Phase 1 Transformations comprise every possible stage in the enzymatic modification of a xenobiotic by oxidation, reduction, hydrolysis, hydroxylation, dehydrochlorination and related reactions.

Phase 2 Transformations comprise all reactions concerned with modification of a xenobiotic by conjugation. The general mechanism is to add a polar compound to the functional group to facilitate excretion.

Preliminary Treatment is a screening process where grit, and larger solids and objects are removed from the effluent. It is the most basic level of treatment (www.ec.gc.ca).

Primary Treatment is a physical process that uses a series of separators to remove solids from the water stream. No controlled biological treatment takes place. Primary clarification removes a fraction of the solids and organics from the water stream, producing primary sludge and scum outputs. (www.ec.gc.ca).

Proteomics is the study of the full set of proteins encoded by the genome with the aim of measuring the changes in protein expression patterns in different environments and conditions.

QuantiGene Plex Assays (QGP) combine two technologies-xMAP® (multi-analyte profiling beads) and bDNA (branched DNA signal amplification technology) to allow the simultaneous measurement of multiple mRNA targets directly from tissue homogenates without the need for RNA purification or reverse transcription.

Quantitative Polymerase Chain Reaction (QPCR) refers to the use of PCR to quantify the amount of a DNA sequence within a sample by monitoring the increase in fluorescence emitted by a fluorophore incorporated into double-stranded DNA during PCR amplification.

Receiving water is surface water (e.g., in a stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just upstream from the discharge point) (Environment Canada, 1990).

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms, and to assist in establishing validity of the toxicity data obtained for a test material. In most cases, a toxicity test with a reference toxicant is performed to assess (a) the sensitivity of the organisms at the time the test material is evaluated, and (b) the precision of results obtained by the laboratory over a period of time which includes several or many tests of that reference toxicant.

Ribonucleic acid (RNA) is a single-strand nucleic acid made up of nucleotides. It is an intermediary between DNA and protein and therefore essential for the transmission of genetic information.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of seawater. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter and is reported in parts per thousand (‰) (Environment Canada, 1990).

Secondary Treatment uses biological methods (bacteria) to consume the organic waste.

Spectrophotometer is an instrument that measures the fraction of the light that is absorbed by a solution when a beam of light is applied. The concentration and purity of a compound can be determined from its absorbance value. Before DNA is deposited on the microarrays, the product concentration and purity is verified using spectrophotometry (A260/280 absorbance measurement) and gel electrophoresis.

Static describes toxicity tests in which test solutions are not renewed during the test (Environment Canada, 1990).

Sterols are a group of natural steroid alcohols derived from plants or animals. They contain a multi-ring (steroid) structure and a hydroxyl group (-OH).

Stock solution is a concentrated aqueous solution of the material to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions (Environment Canada, 1990).

Sublethal means detrimental to the fish, but below the level which directly causes death within the test period (Environment Canada, 1990).

Toxicity is the inherent potential or capacity of a material to cause adverse effects on fish (Environment Canada, 1990).

Toxicity test is a determination of the effect of a material on a group of selected organisms under defined conditions. An aquatic toxicity test usually measures the proportion of organisms affected by their exposure to specific concentrations of chemical, effluent, elutriate, leachate or receiving water (Environment Canada, 1990).

Toxicogenomics is a branch of toxicology that investigates the molecular response of organisms exposed to toxins. Often the earliest detectable cellular event initiated in response to a potential toxin is alteration in mRNA levels; hence, the interest in examining these responses and developing techniques to measure differentially expressed genes.

Wastewater is a general term which includes effluents, leachates, and elutriates (Environment Canada, 1990).

Xenobiotics are compounds that are foreign to the biological system. Xenobiotics may include such chemicals as pesticides.

Xenoestrogens are estrogens foreign to an organism; they are man-made chemicals that have EDC effects.

OBJECTIVES

Using salient indicator species and novel biological approaches determine presence and impact of "emerging chemicals" in MWWE within the Georgia Basin. Specifically, the study objectives were:

- To expose salient indicator species at critical life stages to environmentally relevant concentrations of liquid effluents and evaluate the molecular level impacts through toxicogenomic (gene transcript expression) methodologies.

- To further expand the in-house toxicogenomic capabilities by improving current microarray methodologies and developing a QPCR methodology.

- To evaluate the magnitude of gene transcript expression response to receiving water concentrations by including supra-environmental effluent concentrations in the exposure regime. Standardized 96 hour lethal concentration (96 hr LC50) bioassays will be used to establish the top range of the concentrations for these exposures.

- To conduct a broad chemical characterization of liquid effluents from Municipal Wastewater Treatment Plants (MWTP) in the Georgia Basin, specifically those of Greater Vancouver Regional District (GVRD) and Capital Regional District (CRD).

- To develop the in-house capability to measure key chemical components and "emerging chemicals of concern" found in Municipal Wastewater Effluent (MWWE) including pharmaceuticals and personal care products (PPCPs), and antibiotics suspected of being responsible for endocrine disruption.

INTRODUCTION

The following report outlines and discusses a five-year project (2003-2007) conducted at the Environment Canada (EC) Pacific Environmental Science Centre's (PESC) Environmental Toxicology Section. Organism exposures to the effluent were performed from 2004 to 2006, followed by RNA extraction, application to the microarray (2005-2007), subsequent analyses and report writing.

Fish laboratory exposures were conducted using British Columbian municipal Wastewater effluent (MWWE), otherwise known as sewage. The sewage exposures were conducted at PESC (an EC Laboratory in North Vancouver, BC, Canada) and involved a team of EC staff members and volunteers.

The main purpose of this project was to evaluate the effects of environmentally relevant concentrations of sewage on liver gene expression in juvenile salmonids (rainbow trout and Chinook). The gene expression analysis was conducted to address five major concerns:

(1) Does chemistry data predict biological effect?

(2) Does sewage exposure cause measurable changes in gene expression within the liver of exposed salmonids? If so, are those changes in gene expression permanent?

(3) What concentration of sewage is sufficient to elicit changes in gene expression within the liver?

(4) Are gene expression changes dose-responsive?

(5) Does gender influence gene response?

Microarrays, QPCR and QGP Assays were used to evaluate the toxicogenomic effects of sewage on salmonids. The genetic sex of each fish was determined in Macaulay 2004 and 2006 and in Clover 2004, 2005 and 2006. The genetic sex data from Clover 2005 contributed to data analysis and interpretation. Due to the relatively short length of the experiments, it was not possible to determine the phenotypic sex of the fish.

Whole organisms were exposed to sublethal levels of the suspected pollutant, municipal wastewater, and the effects on the liver were evaluated. The objective was to assess the molecular differences between fish exposed to sewage and fish exposed to clean laboratory water, with the aim of predicting the effects of sewage exposure on wild fish. One of the problems with this approach is that in addition to the target effluent, wild fish may be exposed to other contaminants during their lifetime; therefore, the laboratory test may represent a 'best case scenario' for the fish.

Given that very low levels of pollutants might disrupt endocrine processes at levels well below those which cause mortality, it is evident that simple survival tests are no longer sufficient to protect the fish (Kime, 2001). Current standardized fish testing methods typically use mortality as an endpoint for evaluation of effluent toxicity. Pollutants such as municipal wastewater can affect lifetime fecundity (ability to reproduce), a parameter not easily measured using current standardized test methods. This study, therefore, used toxicogenomic methods to determine if molecular markers could be used to predict eventual reproductive disruption in fish exposed to sewage.

ENVIRONMENT CANADA MANDATE

EC's mandate is to preserve and enhance the quality of the natural environment including water, air and soil quality; conserve Canada's renewable resources; conserve and protect Canada's water resources; carry out meteorology; enforce the rules made by the Canada - United States International Joint Commission relating to boundary waters; and coordinate environmental policies and programs for the federal government. In terms of wastewater, EC's vision is to ensure that, across the country, the release of wastewater effluents does not pose unacceptable risks to human and ecosystem health and fishery resources. While all levels of government share the responsibility for managing the collection, treatment and release of wastewater effluent, the Government of Canada is responsible for managing the risks posed by substances listed under the *Canadian Environmental Protection Act, 1999* (CEPA 1999) and for protecting fish and fish habitat from harm caused by deleterious substances under the *Fisheries Act.*

FUNDING

Funding for the project was provided by Georgia Basin Action Plan (GBAP) and Strategic Technology Applications of Genomics in the Environment (STAGE).

Georgia Basin Action Plan

The Georgia Basin Action Plan (GBAP) is a multi-partnered initiative working to improve environmental sustainability in the Georgia Basin. The vision of GBAP is for "healthy, productive and sustainable ecosystems and communities in the Georgia Basin". GBAP began as the Georgia Basin Ecosystem Initiative, a five-year program which commenced in 1998. This program was renewed as GBAP in 2003. The GBAP partnership has undertaken many projects ranging from research studies, planning initiatives, monitoring, outreach and education. These projects can be grouped under the following four key areas: Supporting Sustainable Communities, Achieving Clean Air, Conserving and Protecting Habitat and Species, and Achieving Clean Water. This study was in part funded through the Achieving Clean Water area whose mission is to improve the aquatic ecosystem health through research and inventories of key toxic substances leading to the development of best management practices.

Strategic Technology Applications of Genomics in the Environment

Environment Canada developed the Strategic Technology Applications of Genomics in the Environment (STAGE) program in 2000. STAGE is administered by the Environmental Biotechnology Applications of the Technology Strategies Directorate

(formerly Environmental Technology Advancement Directorate). The broad objectives of STAGE have evolved from (i) curiosity driven research to (ii) enhancing EC's capacity and understanding of the applications of genomics-based technologies to address research problems in the environmental sciences, and (iii) to exploring the potential for responsible application of genomics-based tools to improve decision making and fulfill departmental priorities.

MUNICIPAL WASTEWATER

Municipal wastewater consists of the liquid waste collected from a community's sanitary sewage and storm water. Sanitary sewage derives from households, commercial establishments, institutions and industries while storm water originates from rain or melting snow draining off rooftops, lawns, roads and other urban surfaces. In Canada, there are more than 3,500 municipal wastewater facilities which annually release more than 3 trillion litres of effluent into surface waters, with treatment processes ranging from no treatment or screening to state-of-the-art technologies (CCME 2007). According to the Municipal Water Use Database (MUD), 77.74% of the Canadian municipal population is on secondary and/or tertiary treatment (EC 2000). Furthermore, it is estimated that 19% of all Canadians are served by primary treatment and 38% are served by secondary treatment. Most coastal communities have either no treatment or primary, while inland communities have predominately secondary or tertiary treatment (CCME 2006). Sewage treatment plants attempt to remove the majority of nutrients, pathogens and chemicals before releasing the treated sewage into the environment. However, even with the best treatment, not all substances, compounds or biological organisms are removed and there are documented effects on aquatic life. The effects on fish include: altered immune function, growth reduction, endocrine disruption and mortality.

Municipal wastewater effluents are complex mixtures of approximately 200 identified chemicals plus many unknowns (EC 2001). Effluents typically contain human and other organic waste, nutrients, pathogens, microorganisms, grit, debris, suspended solids, household and industrial chemicals not removed by the treatment process. They are also the leading source of biochemical oxygen demand (BOD), total suspended solids (TSS), nutrients (including ammonia), organic chemicals, metals and endocrine disrupting chemicals (EDCs).

Aluminum and iron are the most abundant metals present as salts and are often used in the sewage treatment process. Other metals, including cadmium, copper, lead, zinc, manganese, molybdenum and nickel, may be present at lower levels (CCME 2006a, EC 2001). Mercury, which is a metal of considerable environmental concern, may be present as well, although in trace quantities.

Organic chemicals, such as polychlorinated biphenyls (PCBs), dioxins and furans, tetrachloroethylene and trichloroethylene, tend to be present at even lower levels than the metals. However, some of these contaminants can be toxic at low levels and can persist in the environment for very long periods of time. MWWE surpasses direct industrial

discharges as the dominant source of waterborne PCBs and mercury entering lakes Superior and Ontario, according to estimates for 1991 and 1992 (EC 2001).

Nations around the world have recognized that sewage discharge poses enormous potential risk to both human and environment health due to the presence of pathogens, nutrients and chemical compounds. Pollutants do not act in isolation and fish exposed to wastewater are actually exposed to a cocktail of chemicals. Each chemical may act individually or they may have a synergistic effect such that the total effect is not simply the sum of the individual effects. These contaminants diminish water quality and are a major environmental concern as they can kill aquatic life and may persist to bioaccumulate in the food-web and eventually be consumed by humans.

TREATMENT PROCESSES

Conventional sewage treatment consists of three basic levels: primary, secondary and tertiary. Each treatment, from primary to tertiary, increases the quality of the end-of-pipe effluent. One step above raw sewage is screened sewage. Screening removes grit and solid material before sewage receives further treatment or is released into the environment (National Sewage Report Card). Screening makes sewage less offensive to the eye but no less dangerous to the environment or to human health (Nat'l Sewage Report Card). Screening does not significantly reduce the level of suspended solids, BOD, toxic pollutants or microorganisms and pathogens (Sewage Report Card). Effluent from the Capital Regional District (Macaulay and Clover Points) undergoes screening before release into the marine environment. MWWE from the Greater Vancouver Regional District's Annacis Island treatment plant undergoes secondary treatment prior to discharge into the freshwater environment (Table 1, Figure 1).

TABLE 1 MMWE Treatment Used in the Study

	Sewage Treatment Plant		
	Annacis Island	*Clover Point*	*Macaulay Point*
Treatment	Fixed film Secondary	Preliminary Screened	Preliminary Screened
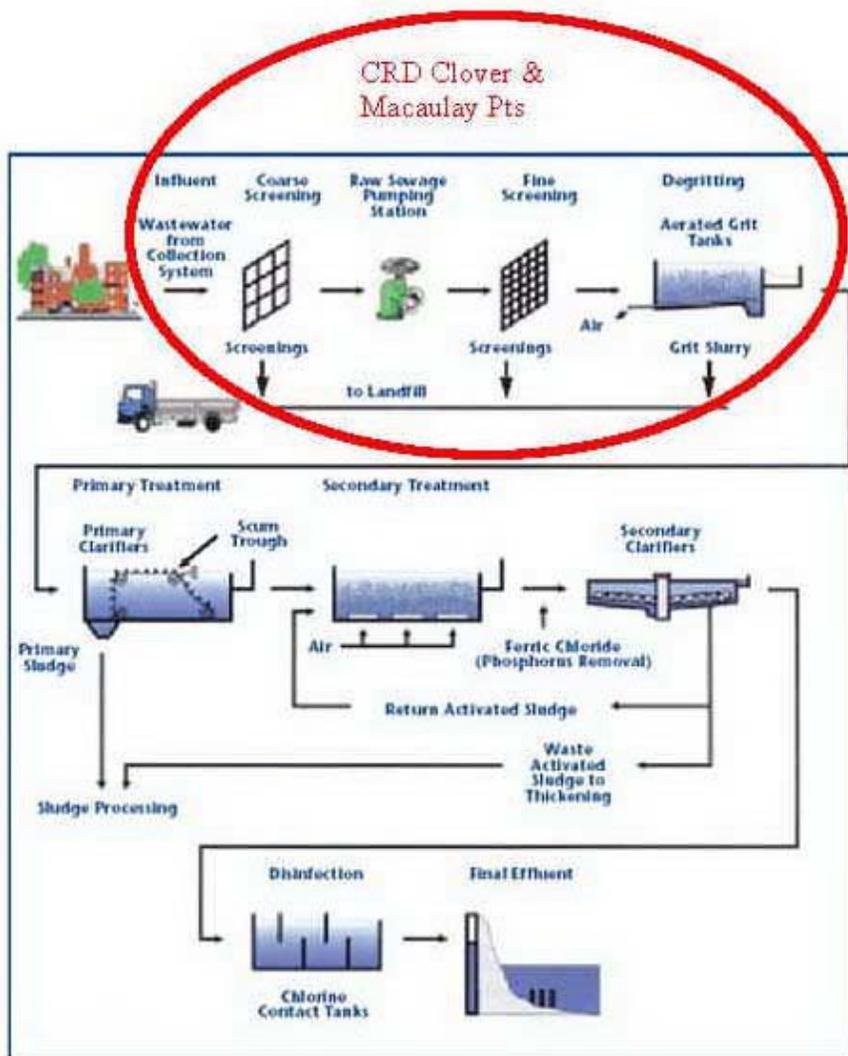
FIGURE 1 MWWE Treatment Processes (modified from image: www.ec.gc.ca/soer-ree/English/SOER/MWWE1.cfm)

Preliminary Treatment

Preliminary treatment is the most basic level of treatment. It is a physical process whereby the effluent is screened or pulverized/ground to remove grit, larger solids and objects before it is discharged into the receiving environment. This treatment process removes coarse solids such as sticks, rags and other debris from the incoming wastewater. The purpose of preliminary treatment is to protect downstream treatment components such as pumps and reduce maintenance or operational problems. Preliminary treatment is a common first step to all wastewater facilities.

Primary Treatment

Primary treatment follows preliminary treatment and includes the use of primary devices that reduce the effluent flow allowing solids to settle due to gravity. Sedimentation tanks commonly detain effluent flows for 2 to 6 hours, to allow the solids to settle, facilitating

removal for separate treatment. With standalone primary treatment, primary effluents can be treated by chemical disinfection prior to release. Primary treatment can also be enhanced using chemicals in which inorganic or organic flocculants are introduced into the wastewater to help clarify and improve the effluent quality over primary treatment alone.

Secondary Treatment

Secondary treatment follows the preliminary and primary treatments. Secondarily-treated effluent is subjected to biological processes (bacteria and other microorganisms) to remove biodegradable organic matter and suspended solids. Following this biological treatment, the effluent is settled to remove suspended solids The Annacis Island facility has enhanced secondary treatment, which includes treatment with fixed film plants and trickling filters Fixed film plants rely on the development of biological growth through direct contact with air. They are also referred to as "attached growth" or "supported growth" plants. The most common fixed film configurations are Trickling Filters (TFs) and Rotating Biological Contactors (RBCs) (Figure 2). For TFs, the secondary aerobic stage comprises a system of fixed nozzles or rotating distribution arms to evenly distribute the raw influent over a tower or filter bed of artificial or natural biomass support media (e.g. plastic or stone).

Tertiary Treatment

Tertiary treatment removes suspended, colloidal, and dissolved constituents remaining after conventional secondary treatment. In Canada this term can refer to physical processes that further remove suspended solids, such as sand filtration.

Advanced or Quaternary Treatment

Advanced or quaternary treatment processes include: reverse osmosis, membrane filtration and activated carbon technologies. This level of treatment is required for enhanced source water protection or for water reuse applications. This level of treatment is currently used in the Orange County Groundwater Replenishment System, California, USA (Royte, 2008).
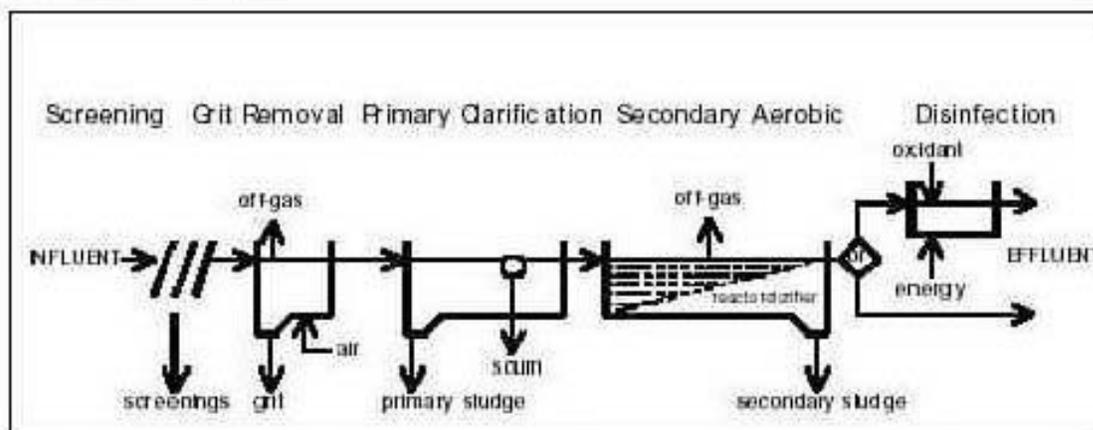

FIGURE 2 Secondary Treatment Fixed Film (Trickling Filter, Rotating Biological Contactor) (image: http://www.ec.gc.ca/pdb/npri/documents/ToolBox/docs/sect_4_4_6_e.cfm).

FISH

The salmonids, rainbow trout (*Oncorhynchus mykiss*) and Chinook (*Oncorhynchus tshawytscha*), were used in this study. Salmonids belong to the infraclass Teleostei or Telestomi, a large group of fishes with bony skeletons, including most common fish (Farlex, free dictionary). Rainbow trout (Annacis 2005) and Chinook (Annacis 2006) were used with the freshwater discharging effluent (Annacis). Chinook were used in the 2006 Annacis exposure since, unlike rainbow trout, their sex could be identified and therefore could potentially offer more information. Salt water acclimated Chinook were used with the marine effluent discharges (Clover, Macaulay).

Rainbow trout are native to western North America, mostly west of the Rocky Mountains, but can now be found in the waters of all Canadian provinces due to intentional or unintentional releases (EPS RM9). They thrive in most cool, freshwater bodies and are one of the most widespread of the salmonids. In Canada and elsewhere, rainbow trout are widely reared in hatcheries for sports fishing stocks and are among the most common species used in commercial aquaculture (RM9). The rainbow trout is also the world's standard cool-water fish for freshwater pollution studies and aquatic toxicity research (RM9). It is also the test species used to establish federal (e.g. USEPA, Environment Canada) regulatory limits (effluents, chemicals). Rainbow trout usually spawn yearly, starting at age three or four and typically live to be six or seven years old.

Freshwater-reared Chinook were used for one of the Annacis effluent collections (freshwater discharge) while saltwater-acclimated Chinook were used for the exposures to marine water discharging effluents. Chinook are the largest of the Pacific salmon species and have recreational fishery importance. They spawn once in their lifetime anywhere from two to seven years of age. Both Chinook and rainbow trout species are highly valued in sport fishing and are consumed as food thereby having implications in human health (Genomic Approaches, 2007).

Fish are the main species used to evaluate the potential harm of wastewater as their risk is greatest given that their natural habitat is the final wastewater depository. Many wastewater compounds are deposited onto the sediment where they are then absorbed by bottom-dwelling aquatic organisms which are in turn consumed by large carnivorous fish and marine mammals and subsequently resulting in bioaccumulation (Kime, 2001). Besides being suitable laboratory test animals, fish may also be used as an early-warning indicator of potential long-term health effects on humans (Kime, 2001). While the complexity of the endocrine system may differ between fish and mammals, the basic functionality is conserved; therefore, chemicals which affect fish have a very high probability of having similar effects in humans (Kime, 2001).

While it is recognized that small rapidly maturing fish, such as medaka and fathead minnow, are currently being used in many studies, they may not provide a suitable model for those species of greatest environmental relevance to North America. A warm water fish, which takes only a few months to progress from hatching to spawning, may differ greatly in its endocrine control from a large marine fish, such as Pacific salmon, which matures only after several years and has a single spawning migration followed by death

(Kime, 2001). Environmental toxicants can bioaccumulate over several years in long-lived carnivorous species; therefore, they may be exposed to low levels of pollution during a prolonged adulthood. The impact of this bioaccumulation cannot be properly assessed by laboratory assays, which are typically brief.

Liver

The liver was chosen as the tissue of interest since, among other functions, it is the major organ of detoxification and the chief site of bioaccumulation for heavy metals and organic pollutants. The liver is also the primary site of steroid hormone catabolism (deactivation) and synthesis of the egg protein, vitellogenin. For these reasons, the liver has been the focus of many toxicological studies.

Some toxicants are not acutely lethal but they can harm fish populations by accumulating and causing physical harm. This harm may shorten a fish's lifespan and ultimately result in decreased population through premature mortality and reduced spawning years (Kime, 2001). Pollutants can also affect the reproductive system and decrease the fertility of otherwise apparently healthy fish. Larval survival and development and the fertility of surviving larvae may be also affected (Kime, 2001).

Many internally produced (endogenous) and environmental (exogenous) toxicants are fat-soluble and have a high affinity for fat tissues, such as the lipid-rich liver. The liver detoxifies harmful substances through a complex series of enzymatic reactions which convert fat soluble toxins into water soluble substances that can be excreted. The enzymatic processes usually occur in two steps, each referred to as phase 1 and phase 2 detoxification. Phase 1 either directly neutralizes a toxin or modifies the toxic chemical to form activated intermediates which are then neutralized by one or more of the phase 2 enzyme systems. The hepatic enzymes used for detoxifying xenobiotics are closely related to the enzymes used for synthesis and deactivation of natural chemicals in the body. For example, the cytochrome p450 family of hemoproteins plays a major role in deactivating steroid hormones. If these enzymes are preferentially used to metabolize pollutants, hormones may be less rapidly deactivated, resulting in higher plasma concentrations (Kime, 2001). Therefore, some of the detoxification enzymes and proteins are used as bioindicators of environmental contamination exposure (Kime, 2001). Common indicators, such as the metallothioneins (MTs) and the P450 enzymes, play a natural role within the fish in maintaining homeostatic equilibrium.

Heavy metals have been shown to accumulate in the body, particularly the liver, and the food chain. The metal-binding family of proteins, MTs, are synthesized in the liver and are known to bind physiological (Zn, Cu, Se . . .) and xenobiotic (Cd, Hg, Ag. . .) heavy metals. MTs maintain constant cellular or tissue levels of essential metals and their physiological levels are directly proportional to the heavy metal content of the target tissue.

Persistent organic pollutants (POPs), including industrial chemicals such as polychlorinated biphenyls (PCBs) and pesticides, also accumulate in the liver. The cytochrome p450s are synthesized in response to POP exposure and function to catalyze a monooxygenase reaction in which an atom of oxygen is inserted into an organic

substrate to facilitate excretion (Kime, 2001). The measurement of cytochrome p450 levels can therefore be indicative of POP exposure.

The liver is also responsible for the estrogen-induced synthesis of the egg yolk protein, vitellogenin (Kime, 2001, Figure 5). The liver is therefore a useful tissue in evaluating a fish's exposure to environmental estrogens (Kime, 2001).

Salmonids Endocrine System including Vitellogenesis

The endocrine system along with the nervous and immune systems are the primary means of communication between cells and organs in multicellular animals (Giulio, R.T. et al, 2008). The regulation of many physiological functions involves the integration of the endocrine and nervous systems (Giulio, R.T. et al, 2008). The endocrine system regulates activities such as body fluid homeostasis, stress management and reproduction and fertility, which are necessary for propagation of the species (Kime, 2001). It comprises of glands such as the hypothalamus, pituitary, thyroid and reproductive organs (testes and ovaries) and is particularly sensitive to very low levels of pollutants (Kime, 2001). The endocrine system secretes hormones into circulation where they bind to specific receptors in target tissues and elicit intracellular responses. Many hormones function together in pathways or groups, for example: steroid biosynthesis, growth, HPT (hypothalamus-pituitary-thyroid) axis, the HPI (hypothalamus-pituitary-interrenal) axis, and the HPG (hypothalamus-pituitary-gonadal) axis.

The HPT axis regulates metabolism, ultimately affecting fish activity and growth (Kime, 2001). It also affects larval development, behavior and reproduction through interactions between growth, gonadal and thyroid hormones (Kime 2001). It is also involved in the parr-smolt transformation, spawning migration and osmoregulation (Kime, 2001). Thyroid activity is regulated in a similar manner to that of the other hypothalamus-pituitary axes. Metabolic or neural stimuli act on the hypothalamus which secretes thyrotropin-releasing hormone (TRH) which in turn acts on the pituitary to release the thyroid–stimulating hormone (TSH) causing the thyroid to secrete the hormones thyroxine (T_4) and triiodothyronine (T_3). The 5'-monodeiodinase enzyme, present in both the liver and thyroid, converts T_4 to the active hormone T_3. Like many enzymatic reactions, this conversion could be affected by xenobiotics which act on hepatic enzymes. The HPI axis coordinates a number of physiological processes. In the brain, the hypothalamic central nervous system discharges corticotropin-releasing hormone (CRH) which stimulates the function of corticotrophs in the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn causes the interrenal gland to synthesize and secrete corticosteroids (Figure 3). The interrenal gland in fishes is homologous to the adrenal cortex of mammals; however, it is not distinct, but is rather a scattering of small clumps. The interrenal gland is the principal source of teleost corticosteroid hormone which, in fish, functions as both glucocorticoid and mineralocorticoid hormones, regulating intermediary metabolism and electrolyte balance respectively (Giulio, R.T. et al, 2008).
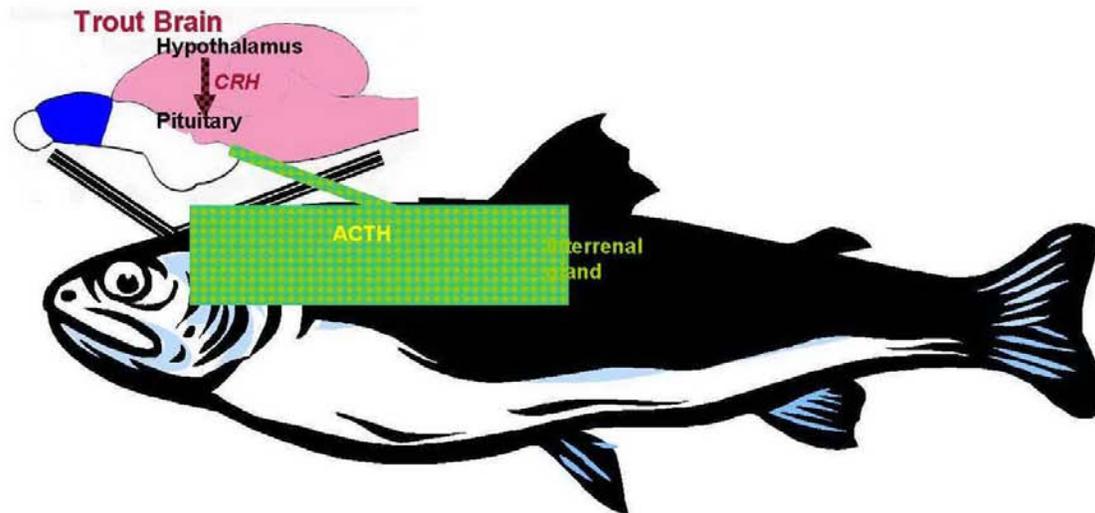
FIGURE 3 Teleost HPI axis

Rapid increases in corticosteroid hormones, along with increases in catecholamine secretion, occur in response to a wide range of stressors (Giulio, R.T. et al, 2008).
These hormones regulate a wide variety of adaptive responses that may be critical for short term survival; however, prolonged elevations of cortisol can negatively influence growth and impair immune and reproductive functions.

Normal sexual development requires both the HPI and HPG axis, also referred to as the reproductive axis. These two axes are regulated in a similar manner. The HPG axis is depicted in Figure 4. The HPG axis regulates sex steroid levels by first secreting gonadotropin releasing hormone (GnRH) from the hypothalamus to stimulate the gonadotrophs in the pituitary gland to release gonadotropins. The gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) are then released from the pituitary to stimulate secretion of the sex steroids from the gonads. Both the HPI and HPG axes synthesize steroid hormones. All steroid hormones are synthesized from the same precursor, cholesterol. The gonads (testes and ovaries) synthesize and secrete the important reproductive hormones, androgen and estradiol.

Hypothalamus-pituitary-gonadal (HPG) Axis
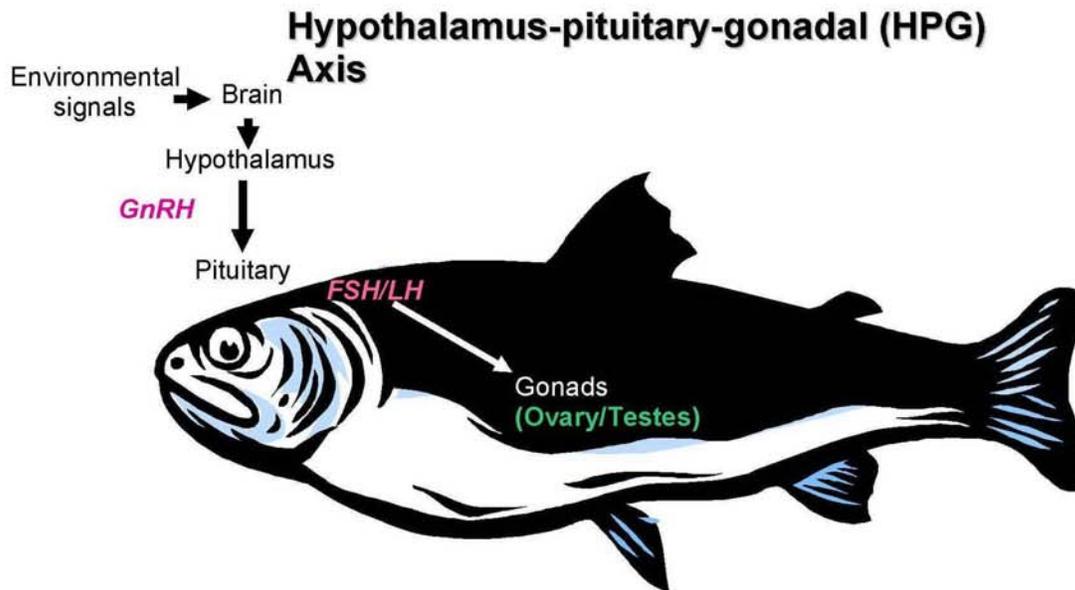
FIGURE 4 Teleost HPG axis

Reproductive activity in most fishes is seasonal, with both external environmental stimuli and endogenous circannual rhythms influencing reproductive cycles in teleosts (Giulio, R.T. et al, 2008). In females, 17β-estradiol regulates the hepatic production of vitellogenin, the egg yolk precursor protein. Estradiol, a sex hormone, is the major
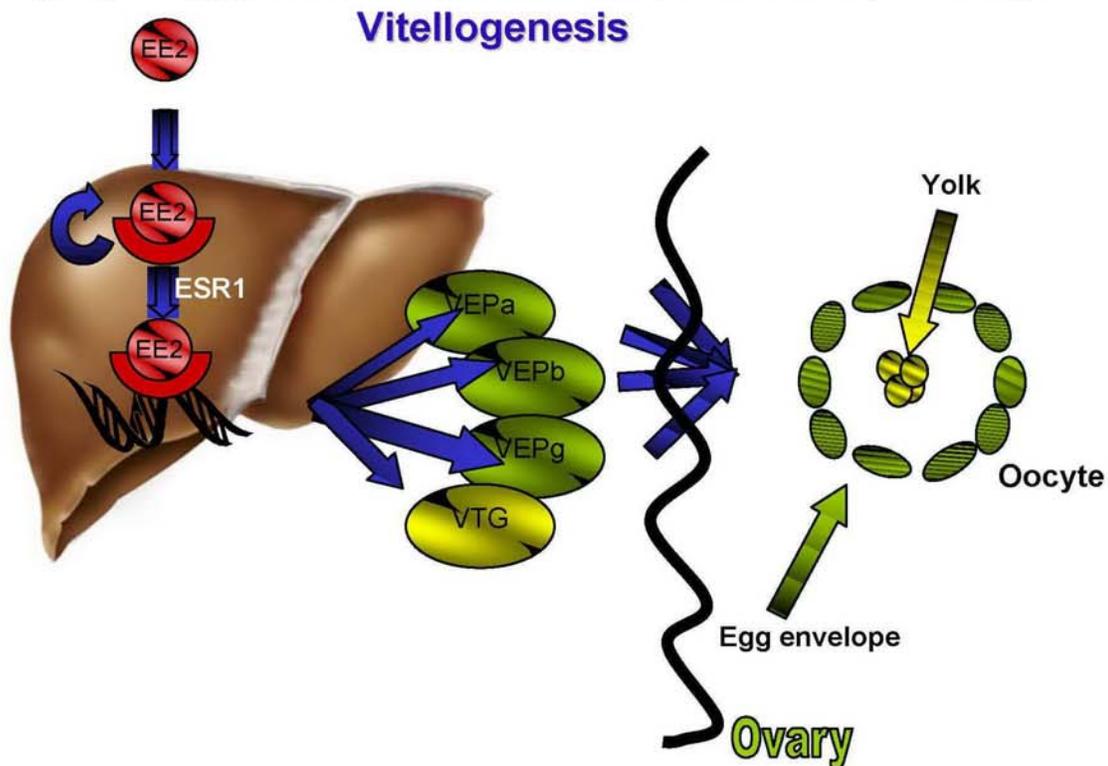
FIGURE 5 Vitellogenesis

estrogen. As depicted in Figure 5, estrogenic chemicals (e.g. EE2) bind to the estrogen receptor (ESRI) in the liver, inducing further synthesis of the receptor. The ligand-bound receptor interacts with estrogen-responsive genes leading to gene transcription of vitellogenin (VTG) and the vitelline envelope proteins (VEPa, VEPb, VEPg). The vitelline envelope proteins and vitellogenin are transported via the bloodstream to the ovary where the VEPs are incorporated into the egg envelope (egg shell) and VTG is incorporated into the egg yolk. The VEPs are synthesized before VTG. Testosterone is a precursor for estradiol synthesis and there is evidence that it participates in the feedback control of gonadotropin secretion by aromatization to estradiol (Giulio, R.T. et al, 2008).

Vitellogenin (VTG) is normally synthesized in the liver of female oviparous (egg-laying) fish in response to estrogen and is sequestered in developing oocytes where it acts as a nutrient reserve for the subsequent development of the embryo (Giulio, R.T. et al, 2008). The production of VTG is therefore usually restricted to sexually mature females, at least two years of age for Chinook and rainbow trout (Giulio, R.T. et al, 2008). Male fish also possess the VTG gene and will produce the gene transcript and protein when exposed to estrogens (Giulio, R.T. et al, 2008). Detection of VTG in male fish is therefore indicative of estrogen exposure (Giulio, R.T. et al, 2008).

Emerging Chemical Contaminants (ECC)

Emerging chemical contaminants (ECC) is a phrase used to describe either new families of compounds with potential toxicity, or known compounds with emerging concerns due to new information regarding fate, transport or effects of the compounds (Daughton, 2004). Municipal waste water effluent discharges have recently garnered worldwide concern because of the evidence of effects (including: endocrine disruption, physical deformities, organ damage, and tumours (CCME, 2006)) resulting from exposure to ECCs. Since the significant publication by D. Kolpin et al. (1999) regarding organic wastewater contaminants found in surface waters across the USA (thereby highlighting the possibility for compounds discharged in sewage to enter surface waters and cause effects on humans and wildlife), many major federal government departments in the USA and Canada, including the United States Geological Service (USGS) and Environment Canada, are studying ECCs in sewage. The Canadian Council of Ministers of the Environment (CCME) lists the following groups of compounds as the ECCs in Canadian sewage: Pharmaceuticals and Personal Care Products (PPCPs), endocrine disrupting compounds (EDCs) and brominated flame retardants (CCME, 2006).

PPCPs include: shampoos, perfumes, drugs, cosmetics, sunscreens, lotions, paints and cleaning agents. These products are ultimately incorporated into sewage by consumer ingestion or topical use or application to their pets and house interiors, followed by subsequent showering, excretion and flushing of these products. PPCPs are concerning because, by their very nature, they are bioactive for human use and potentially remain bioactive when discharged into the environment. PPCPs may be EDCs, but not all PPCPs are EDCs.

The greatest potential impact of sewage in the environment derives from the ECCs (ten, hundred or possibly several hundred compounds) that act as EDCs (Arcand-Hoy, 1998; Stubblefield, 2006). These EDCs may be present at very low concentrations in sewage but given that the daily discharge volume of sewage into the environment is high (thousands or tens of thousands of litres per hour from each sewage treatment plant worldwide), aquatic organisms are likely exposed to chronic, low-levels of these chemicals (Ma, 2005; Crane, 2006).

EDCs include a wide variety of compounds that affect the endocrine system. Endocrine disruption (ED) is a general term describing the adverse biological effects resulting from exposure to exogenous substances that interfere with the endocrine system and disrupt normal hormone function (Kime, 1998). The manifestations of ED are varied and dependent upon the properties of the compound or mixtures of compounds causing the abnormalities. ED effects in aquatic organisms include reductions or alterations in: hatching rate and success, behaviour and courtship, fertilization success and gonad development (Kime, 1998). ED effects are sometimes subtle, taking many years to manifest at the population level. Such effects have been noted in aquatic and wildlife populations, including snails (imposex due to tributyltin: Gibbs, 1986) and alligators (feminization due to DDT: Guillette, 1996). A complex mixture, like sewage, is the summation of all EDCs present - a balance of the additive, potentiating, antagonising and synergising activities of the compounds. Although EDCs can interfere with any portion of the endocrine system, worldwide study has focused on EDCs that are: androgenic, anti-androgenic, thyroidogenic, anti-thyroidogenic, anti-estrogenic, or estrogenic (Cheek, 2001; Andersen, 2002; Kirby, 2004; Guillette, 2006). Androgenic compounds stimulate or control the development of masculine characteristics. Anti-androgenic compounds prevent or inhibit the biological effects of the androgens. Likewise, thyroidogenic compounds act as thyroid hormones while anti-thyroidogenic compounds antagonize those actions. Estrogenic compounds mimic the behaviour of the female hormone estrogen while anti-estrogenic chemicals impair the activity of endogenous estrogens.

"Feminization of male fish" is a phrase frequently used to describe the phenomenon of male fish physiologically changing into female fish (in partial or in whole) in response to the presence of endogenous and exogenous estrogenic compounds (xenoestrogens) or complex mixtures of such compounds. Evidence of fish specifically undergoing estrogenic endocrine disruption downstream of a sewage treatment plant discharge point has been described by Jobling et al. (intersex roach; 1998 and 2002) in England and Batty et al. (reduced mosquitofish gonopodium; 1999) in Australia. Fish estrogenic endocrine disruption research often relies on physiological observations such as intersex gonads (Jobling, 1998; Batty, 1999; Afonso, 2002) and reduced male-sex characteristics (Rodgers-Gray, 2001; Parrott, 2005). These are highly useful observations that may be used to generate linkages between cause and effect in the organism and at the population level. However, observations of endocrine disruption at the physiological and/or population level may indicate irrevocable damage to the ecosystem. Therefore, m olecular level studies have been employed over the past decade to characterize the mechanisms underlying the physiological manifestations of endocrine disruption. The overall aim of

this research is to develop measures to predict and prevent the occurrence of endocrine disruption. Molecular level biomarkers such as the vitellogenin protein (Hansen, 1998; Larsson, 1999; Pait, 2003; Aerni, 2004; Jobling, 2004; Jimenez, 2007) have been extensively utilized to demonstrate estrogenic ED effects. In addition to the study of protein disruption (translational level), gene transcript (transcriptional level) alterations are being explored using toxicogenomic tools.

Toxicogenomics

Toxicogenomics is the study of the complex interaction between a cell's genes, chemicals in the environment and disease. Cells in all living organisms are continually activating or deactivating genes in response to exogenous (e.g. EDCs) and endogenous (e.g. hormones) signals. The central dogma of molecular biology states that DNA encodes RNA which can be translated into protein (DNA → RNA → Protein). DNA levels are predominantly immutable while both RNA and protein levels may be altered. When a particular protein is required by the cell, a gene transcript (mRNA) is transcribed (transcription) from the DNA template. That mRNA is then used as a template for the synthesis of a specific protein (translation).

When an organism's cells are exposed to a stress, drug or toxicant, they respond by altering gene expression (mRNA). The amount of mRNA produced does not always correlate with the amount of protein eventually synthesized; therefore, it remains valuable to assess the relationship between each mRNA transcript and the resulting protein when evaluating molecular data. The production of a protein encoded by a given gene may be increased, decreased or remain unchanged, depending upon the type of exposure and the cell's needs. Protein amounts are more difficult to measure than mRNA and alterations in mRNA levels are often the earliest detectable cellular events initiated in response to a potential toxin (Burczynski, 2003). As such, mRNA is one of the key toxicogenomic measures used to determine if a gene has been turned on (up-regulated) or turned off (down-regulated). Microarrays, Quantitative Polymerase Chain Reaction (QPCR) and QuantiGene Plex (QGP) are molecular-level assays that enable scientists to study many genes at one time.

cDNA microarrays

The complementary DNA (cDNA) microarray is a technology central to the field of toxicogenomics. The microarray has become an established toxicogenomic tool, having entered common usage over the past decade. As a consequence of the human genome project, microarrays have been highly utilized for medical purposes, particularly in the fields of cancer research and pharmaceutical discovery. In addition to medically-relevant organisms (humans, rats, mice, etc.), microarrays (small and large scale) have been produced for nearly all organisms of commercial, environmental, sentimental, scientific or domestic value. Microarray research has exploded due to the wealth of information it provides on transcript levels at a fixed point in time (Gatzidou, 2007). This quantitative data can be used to elucidate mechanisms and determine relationships between cellular events that were previously studied in isolation. Microarrays allow the collection of data in a very quick and efficient manner and since many genes are assessed at one time, a

comprehensive picture is generated. The current generation of microarrays is usually printed on glass slides, similar to microscope slides, coated with materials that bind DNA. Very small amounts (picolitres or nanolitres) of DNA fragments are printed in known arrangements on the microarray slide using a robotic instrument (Figure 6). The DNA fragments printed and affixed to the microarray slides are called the 'probes' (Figure 6). Hundreds to thousands of probes, representing a portion or all of an organism's genome, can be printed on one slide. The DNA fragments can be prepared from genomic DNA, but are more commonly prepared from cDNA (complementary DNA). The cDNA fragments are gene coding sequences and microarrays spotted with these fragments can be used for gene expression studies (studies that evaluate the amount of RNA produced from treated cells or organisms). As Figure 6 shows, fluorescently labelled 'target' cDNA (made from experimental samples) is applied to each microarray slide so that hybridization can occur between complementary strands of DNA in the 'probe' and the 'target'. Excess non-hybridized sample DNA is washed away (the probe DNA is affixed to the slide so it is not removed) leaving the fluorescently labelled 'target' bound to the 'probe' DNA. A microarray scanner uses lasers to excite the fluorescent dyes, reads the fluorescent signals and saves an image of the microarray for analysis (Figure 6).
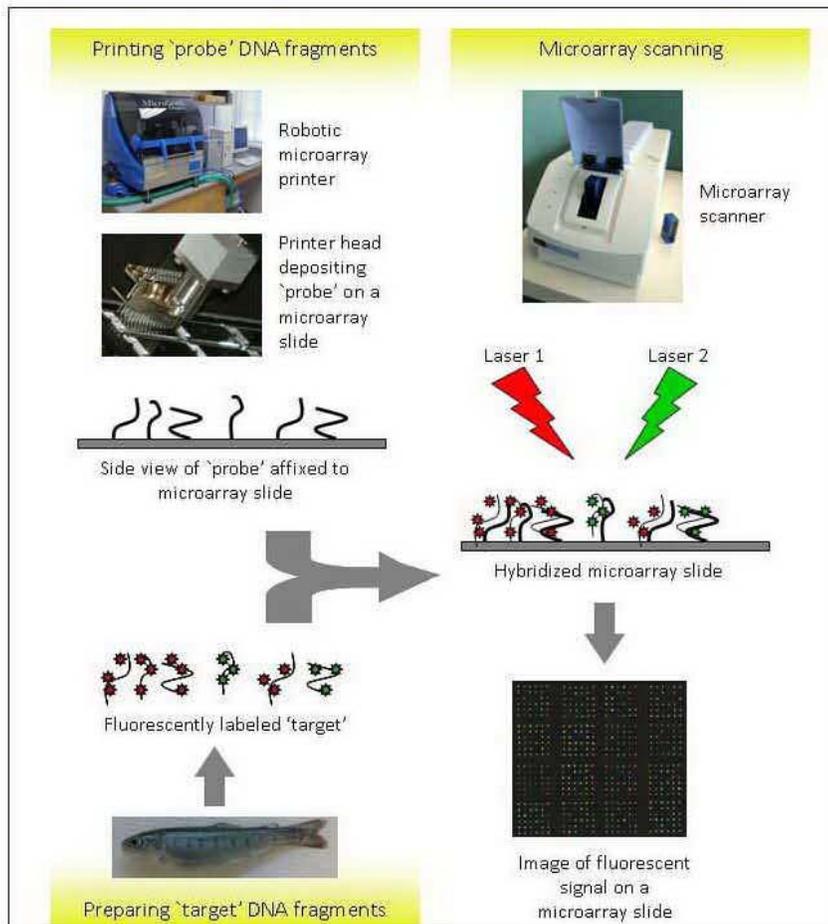

FIGURE 6 Overview of microarrays.

Traditionally, microarray experiments have compared the gene expression of a sample exposed to an experimental variable with the gene expression of a sample exposed to control conditions. Both samples are simultaneously applied to the microarray and are differentiated by labelling with unique fluorescent dyes. Fluorescent labels are incorporated into target cDNA during the reverse transcription reaction from isolated mRNA. The green-fluorescent dye is Cy3 and the red-fluorescent dye is Cy5. During hybridization the differentially-labelled cDNA targets will compete for binding to the probe cDNA on the array. The sample with the higher copy number will obtain a larger proportion of binding sites on the microarray and will generate greater fluorescence. For example, given that the control sample is labelled with the Cy3 (green) and the experimental sample is labelled with Cy5 (red), if a gene is down-regulated in the experimental sample then more Cy3 will bind to the probe and the spot will fluoresce green. If there is up-regulation in the experimental sample then the reverse will happen and the spot will fluoresce red. If the two samples have the same amount of transcript, then there will be equal amounts of Cy3 and Cy5 and the spot will appear yellow (Figure 6).

Rainbow trout cDNA Microarray

The PESC rainbow trout (*Oncorhynchus mykiss*) microarray is a "targeted microarray" containing 207 cDNA gene transcripts representing broad gene classes such as: binding and transport, embryogenic, endocrine, housekeeping, immune, metabolism, oncogene, proteolysis, signal transduction, structural and transcription (Figure 7). Two to four copies of DNA are spotted for each gene on the array. A complete list of cDNA fragment information (commonly referred to as genes) is available in Appendix 1. The microarray was constructed using highly conserved coding sequences from rainbow trout; however, material from any *Oncorhynchus* species (Pacific salmonids and rainbow trout) can be used due to the high level of conservation in coding sequences among the *Oncorhynchus* species (Rise, 2004; von Schalburg, 2005). The PESC microarray has previously been used to assess the potential of pulp mill effluents to exert xenoestrogenic effects on salmonids (Bruno, J., Mommsen, T., 2006). Since this rainbow trout microarray was a targeted, or designer, array intended for use in assessing the effects of contaminant exposure, a relatively large proportion (up to 25%) of the genes may be significantly altered. That proportion of altered genes is much higher than is typical for larger microarrays as those arrays are highly diverse, including cDNA pieces or ESTs (expressed sequence tags) from numerous tissues or development-stage libraries. Consequently, the majority of genes on large microarrays are unaffected by treatment, with only a small fraction showing significant alteration (Chua, 2006). For example, only 0.9% of the genes on the 16K GRASP (Genomic Research on All Salmon Project) array were significantly altered in the rainbow trout hepatocytes exposed to paraquat for 24 hours (von Schalburg, 2005, Finne *et al.* (2007)).
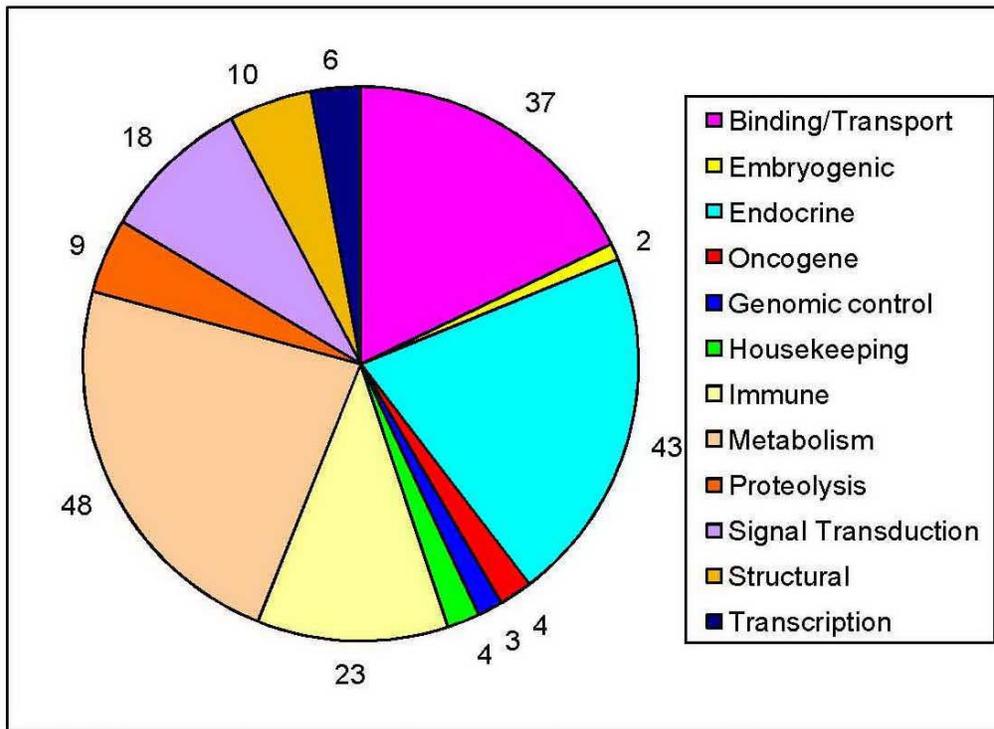
FIGURE 7 Gene Class representation on the rainbow trout cDNA microarray

Microarray Design Using Reference Material

A reference microarray experimental design allows for direct comparison between control and treatment gene expression patterns and is best employed in experiments with large numbers of microarrays (Bowtell, 2003). As shown in Figure 8, the reference design aims to create a signal for each gene in one of the channels (in this research, the green/Cy3 channel) so that a mathematical ratio can be generated between the experimental sample signals (here, the red/Cy5 channel) and reference signals for each gene on a slide. Mathematically, this ensures that a ratio can always be generated because the denominator will never be zero (in other words, the experimental sample signal is always divided by a value). The reference signal level can also be used as a diagnostic tool because it should remain constant across all treatments and all slides; therefore, an abnormal reference signal indicates a problem with the hybridization of sample materials or with the printing of that slide. The two most common materials used to generate reference signal are: Universal RNA Reference (URR; commercially available), or Universal DNA Reference (UDR; in-house produced). Figure 8 shows the use of UDR as a reference material. For microarray experiments utilizing fewer slides (perhaps one to three dozen for an entire experiment), alternative designs may be used, including: hybridizing treatment versus control on each slide, or the loop design where hybridization in a batch of slides occurs in a such a pattern: control versus treatment 1, treatment 1 versus treatment 2, treatment 2 versus treatment n, treatment n versus control (Bowtell, 2003).
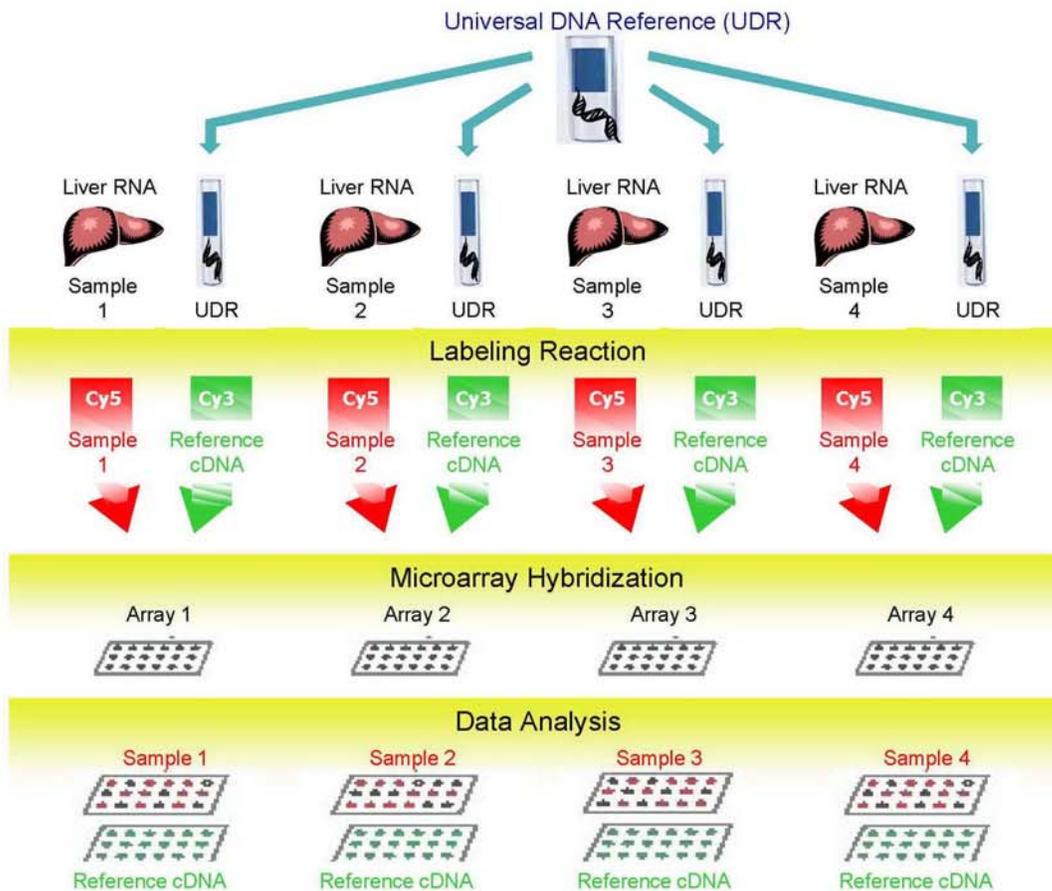
FIGURE 8 Microarray experimental design using a Universal DNA Reference (UDR) material in the green (Cy3) channel and experimental samples in the red (Cy5) channel. (Stratagene, 2007)

Quantitative Polymerase Chain Reaction (QPCR)

Quantitative Polymerase Chain Reaction (QPCR) is a highly accurate, quantitative PCR method that is commonly used in gene expression research because of its specificity, sensitivity, throughput and reproducibility (Burns, 2005; Schefe, 2006). The end result of QPCR analysis is information regarding up- or down-regulation of gene transcripts in treatments versus control. QPCR is a tool often used after initial microarray analysis to quantify specific gene expression changes (Morey, 2006). Whereas microarrays identify changes in hundreds or thousands of gene transcripts, QPCR quantifies the exact magnitude of change in a small subset of those transcripts. Due to the cost-effectiveness and high throughput of the QPCR method, a greater number of biological replicates (i.e. more individual organisms) can be evaluated, increasing the power of statistical analyses. In general, microarrays are used as a broad screen and QPCR is used for in-depth analysis.

QPCR differs from standard PCR in that it outputs quantitative data compared to presence or absence data. QPCR follows the same principles as PCR (see PCR in glossary), except that the QPCR reaction mixture contains a fluorescent dye that intercalates into double-stranded DNA where it is excited and quantified by a QPCR machine. There are numerous fluorescent dyes that can be used in QPCR, SYBR Green I (henceforth called SYBR) is the most common. Software is used to convert the fluorescence signal into quantitative data. Figure 9 describes the QPCR methodology (Schefe, 2006).

The QPCR instrument outputs data as C_t values: Cycle (C) at threshold ($_t$). This value is determined for each QPCR reaction and represents the value at which a target gene in a cDNA sample begins amplification (Figure 10). Threshold is a relative value calculated (usually 10 times above the background noise level) for each target gene during method development and then later applied to experimental samples to maintain consistency between QPCR runs. The C_t value is assigned when the amplification curve crosses the threshold (Figure 10).
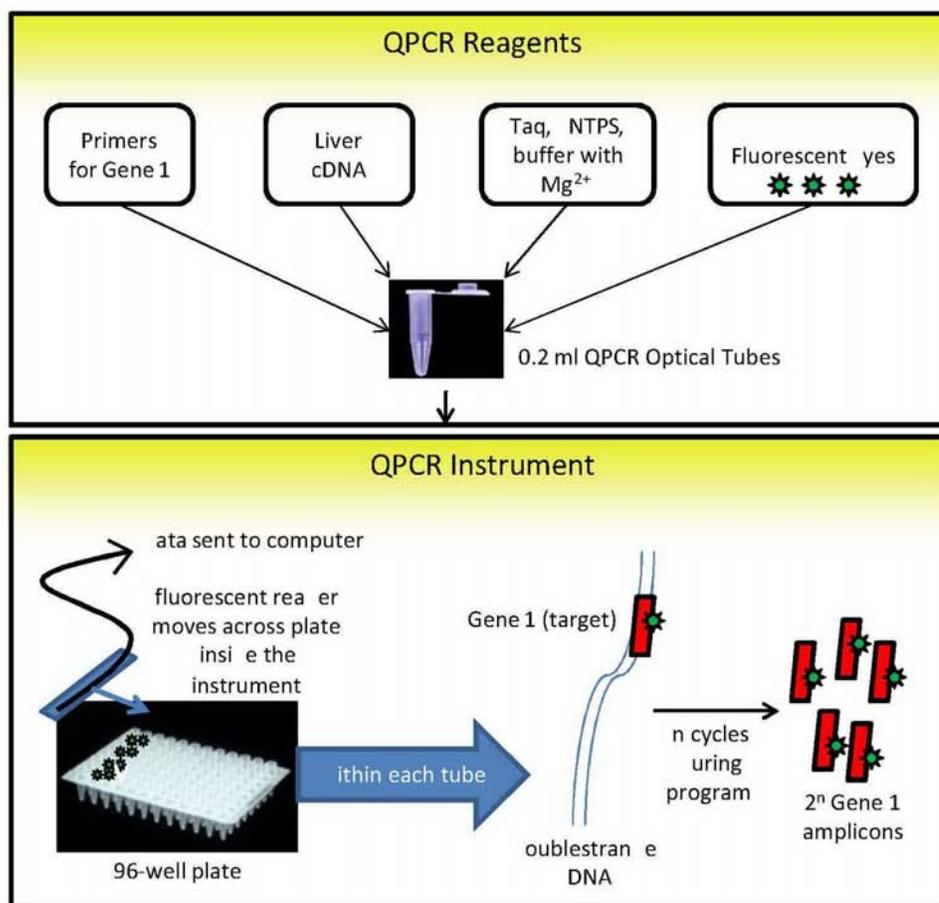
FIGURE 9 Overview of Quantitative Polymerase Chain Reaction (QPCR) reagents and the process that occurs during analysis on a QPCR instrument.

C_t values can be used to calculate copy numbers (the number of copies of a gene transcript in the original tissue). A lower C_t value indicates higher copy number in the starting material (i.e. samples with more starting material amplify quicker than samples with less starting material). C_t values are inversely proportional to the copy number.
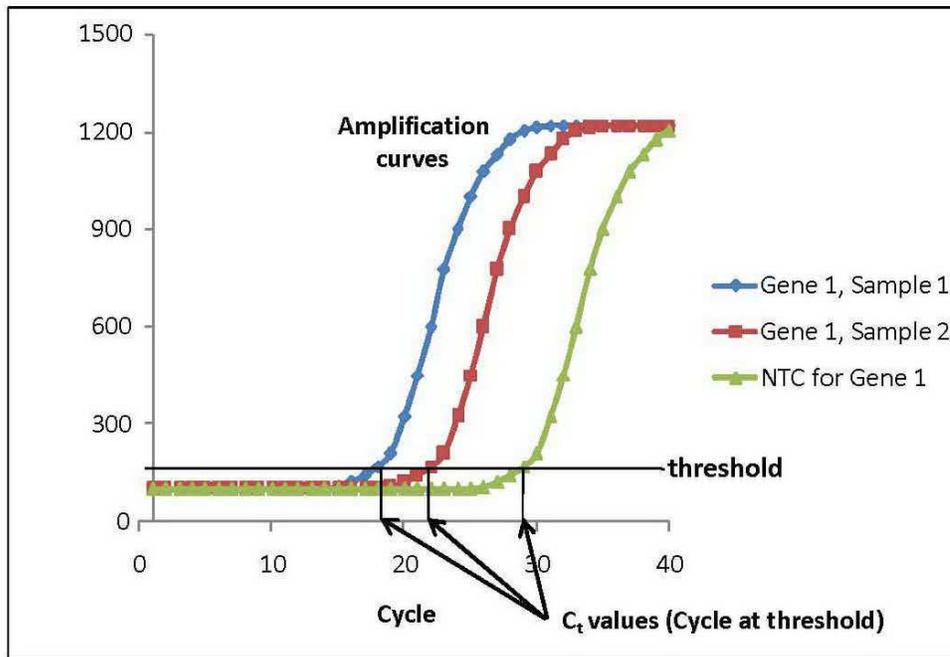
FIGURE 10 QPCR amplification curves indicating the location for each curve at which the instrument calculates a C_t value (the Cycle (C) at which the amplification curve crosses the threshold ($_t$)).

QuantiGene Plex Assays (QGP)

Reverse transcription amplification methods, such as QPCR, are highly quantitative but have low multiplex capabilities, while microarray technologies are useful as broad screens to conduct discovery research but are hampered by the relatively long experimental procedure and low sensitivity. Most current methods used to detect and quantify mRNA expression levels require RNA isolation, reverse transcription and often target amplification. Each procedural step can potentially introduce variability leading to reduced assay precision. It is therefore desirable to minimize the number of steps necessary to obtain data. The QGP assay fulfils this criterion by allowing quantification of RNA immediately following isolation from the target tissue (Figure 11). No RNA amplification or reverse transcription are required. Consequently, the assay is relatively quick to perform and suffers from fewer procedural variables.
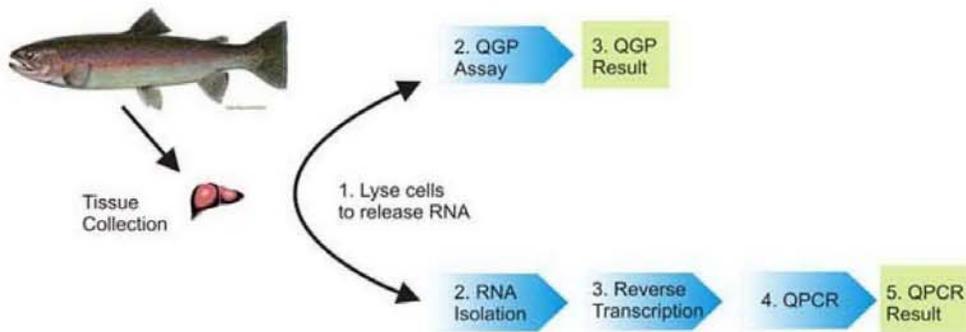
FIGURE 11 QuantiGene Plex assay workflow compared to QPCR (adapted from www.panomics.com).

The QGP assay (Panomics) combines branched DNA (bDNA) signal amplification technology and xMAP® (multi-analyte profiling) beads to enable simultaneous quantification of multiple RNA targets directly from target tissue (Figure 12). Branched DNA technology is a sandwich nucleic acid hybridization assay that amplifies the reporter signal rather than the sequence. By measuring the RNA at the sample source, the assay avoids variations or errors inherent to extraction and amplification of target sequences. The xMAP system, developed by Luminex Corp, combines flow cytometry, fluorescent-dyed microspheres (beads), lasers and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample.
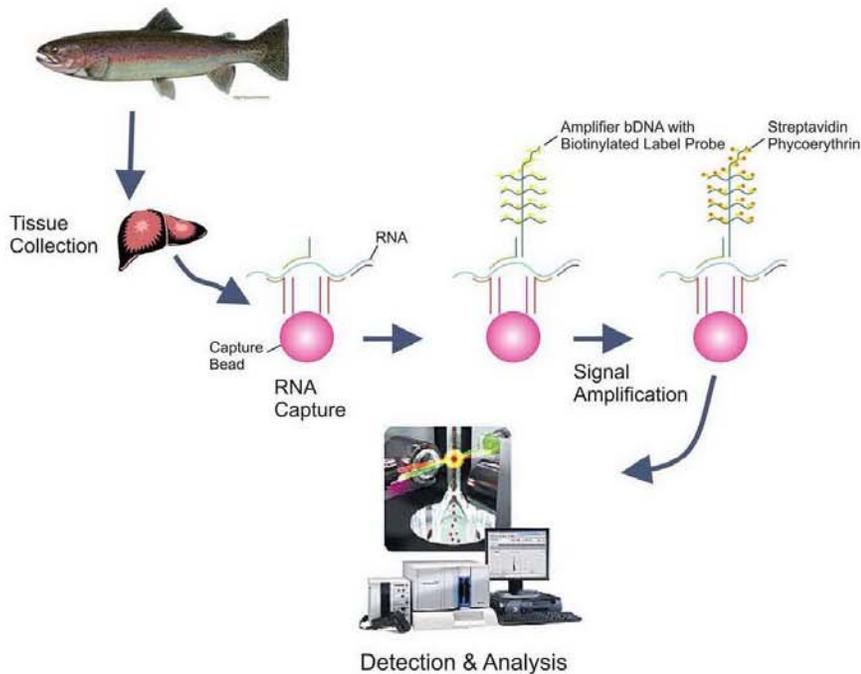
FIGURE 12 QuantiGene Plex assay (adapted from www.panomics.com).

MATERIALS AND METHODS

Effluent Sampling

For each study toxicogenomic exposure, sewage was collected in two batches to conform to Environment Canada's effluent storage policy, which states that effluent testing should begin within three days and must commence no later than five days after termination of sampling (Environment Canada, 1990). All effluents were collected within 5 days of study exposure. Effluents were collected in pre-rinsed, new five imperial gallon (20 litre) cube shaped plastic collapsible containers with plastic screw closures. Containers were made of nontoxic material and were filled to minimize any remaining air space. Upon collection, each sample container was labelled with sample type, source, date and time of collection. The sample containers were transported in coolers at 4°C to PESC. All but one collection, Clover 2004, were grab samples. Grab samples are meant to represent the MWWE stream at a given point in time. The one composite sample, Clover 2004, was collected over a 24 hour time period. Composite samples are more desirable as they represent effluent discharge over a broader timeframe, but they are more technically difficult to obtain; therefore only one composite sample was obtained in this study. Upon arrival at the laboratory, effluent samples were held at 4°C and then adjusted the night before the test to 15°C. Samples in the collection containers were agitated thoroughly just prior to pouring into 200 litre vats and the vat contents were mixed by hand stirring. Aliquots from this effluent vat, in conjunction with control/dilution water, were measured into another vat to make the desired effluent concentrations.

A 96-hour range-finding LC50 experiment (where mortality was the endpoint measured) was conducted prior to the definitive gene expression effluent test. The 96-hour test was used to establish NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) concentrations for the toxicogenomics experiments. Initially, the 96-hour range finding effluent test was conducted with a separate collection of effluent but as the study progressed the same effluent was used for both the LC50 and the initial toxicogenomic exposure. After the onset of the definitive test, another effluent sample was collected and used for the 80% solution refreshment on day 4.

GVRD Annacis Island Wastewater Treatment Plant

Fresh effluent was collected for each study. For each of the salmonid tests, effluent was collected within 3 days of starting or refreshing the test. Effluent was collected by GVRD staff and transported back to PESC by PESC Environmental Toxicology (ETOX) personnel (Table 2).

TABLE 2 Annacis Island Effluent Tests

Annacis	2004-1	2004-2	2005	2006
Collection Dates	19Jan04 (M); then WFM thereafter	1Mar04	14Jan05; 20Jan05	16June06; 22June06
Collection Type	grab	grab	grab	grab
Testing Dates	19Jan-23Feb04	1-9Mar04	17Jan-2Feb05	19June-5July06
Species	Coho/Ranid	Rainbow trout	Rainbow trout/Ranid	Chinook/ Daphnia
Test Type	Egg alevin/ tadpoles	Underyearling	Underyearling/ tadpoles	Underyearling
Exposure Period/Regime	35 days	8 days	8 days effluent/8 days recovery	8 days effluent/8 days recovery
Comments	Test ended early due to large numbers of dead	Fish in 100% effluent died after Day 1	Double exposure amount for UVic (B.Koop)	

CRD Clover Point and Macaulay Point Outfalls

Effluent was collected within 5 days of starting or refreshing the test. The effluent was collected by CRD staff and transported by personnel from PESC ETOX (Tables 3, 4).

TABLE 3 Clover Point Effluent Tests

Clover	2004-1	2004-2	2005	2006
Collection Dates	20May04	26May04; 3June04	27May05; 2June05	31Mar06; 6Apr06
Collection Type	grab	24hr composite	grab	grab
Testing Dates	21-25 May04	31May-16June04	30May-15Jan05	3-19Apr06
Species	Chinook	Chinook	Chinook	Chinook
Test Type	Underyearling	Underyearling	Underyearling	Underyearling
Exposure Period/Regime	96-hr LC50	8 days effluent/8 days recovery	8 days effluent/8 days recovery	8 days effluent/8 days recovery
Comments	LC50 for Clover 2004			

TABLE 4 Macaulay Point Effluent Tests

Macaulay	2004-1	2004-2	2005	2006
Collection Dates	15June04	18June04; 24June04	17June05; 23June05	10Mar06; 16Mar06
Collection Type	grab	grab	grab	grab
Testing Dates	16-20June04	21June-7July04	20June-6July05	13-29Mar06
Species	Chinook	Chinook	Chinook	Chinook
Test Type	Underyearling	Underyearling	Underyearling	Underyearling
Exposure Period/Regime	96-hr LC50	8 days effluent/8 days recovery	8 days effluent/8 days recovery	8 days effluent/8 days recovery
Comments	LC50 for Macaulay 2004			

Frequency of Collection

The collection and subsequent testing of the effluents was repeated several times over the three-year testing period in an effort to capture seasonal variations in effluent dispersion in the water column. The frequency and timing of effluent collection was determined in agreement between PESC and GVRD/CRD staff (Tables 2, 3, 4).

Test Organisms

This report discusses the underyearling fish exposures. Rainbow trout (*Oncorhynchus mykiss*) were used for the Annacis 2005 exposure. All remaining exposures discussed in this report used Chinook (*Oncorhynchus tshawytscha*). Chinook were the preferred species as they could be genetically sexed, unlike rainbow trout (Zhang, Q., *et al.*, 2001). All fish were obtained under Department of Fisheries and Oceans permit. For each effluent test, all fish were derived from the same population and source and were free from known diseases. Fish were acclimated and held in uncontaminated on-site well water according to Environment Canada Biological Test Method: Acute Lethality Test Using Rainbow Trout EPS 1/RM/9 July 1990 with May 1996 and May 2007 amendments (water (pH 6.0-8.5, dissolved oxygen 80-100% saturation, temperature 15± 2°C), lighting (lux ≤500, 16±1 hour light: 8±1 hour dark)). During holding and acclimation, fish were fed once daily to satiety with # 1 Crumb Nutra Plus Starter Feeds food (Skretting Canada, Vancouver, BC, Canada) for 7 days a week. Food was withheld 24 hour prior to test start.

Rainbow trout

Underyearling rainbow trout were acquired from SunValley Trout Park (Mission, BC) one month prior to use in effluent testing with Annacis 2005. Fish were approximately two months post-hatch at time of testing. The average wet weight of test fish was 1.5 g.

Chinook

Juvenile Chinook salmon were obtained from a variety of sources. Chinook from Chilliwack River Hatchery (Chilliwack, BC) were used in the Clover 2004, Clover 2005, Macaulay 2005 and Annacis 2006 exposures. Chehalis River Hatchery (Agassiz, BC) Chinook were used in the Macaulay 2004 exposures while Tenderfoot Creek Hatchery (Brackendale, BC) Chinook were used for the Macaulay and Clover 2006 exposures. Fish were held in well water at the Pacific Environmental Science Centre (PESC; North Vancouver, BC, Canada) following standard operating procedures (PESC, 2007a). For the marine discharging effluents, over the course of six weeks, fish were acclimated in stages to filtered saltwater which is a process normally undertaken at this lifestage (smoltification). At the start of the experiments, fish were approximately seven months post-hatch with an average weight of nearly 5 grams (Figure 13).
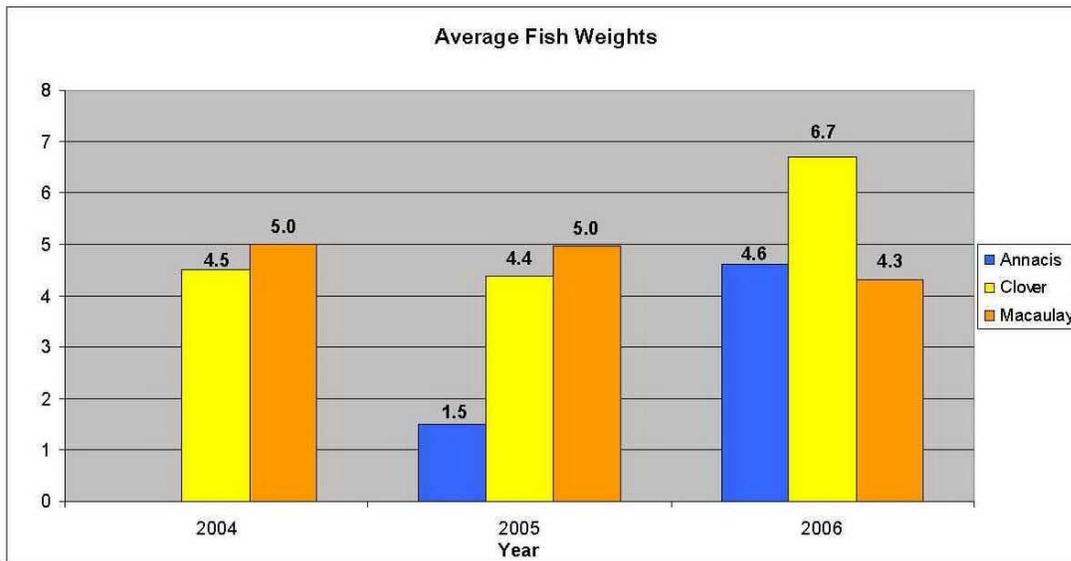

FIGURE 13 Average weights of fish used in effluent studies.

Effluent Testing

Toxicity Tests

The experimental design evolved over the course of the study, starting with salmonid early life stage tests (Coho eggs) and developing into underyearling salmonid 8-day MWWE exposures followed by 8-day recoveries in dilution water (see Appendix 2 for the complete list of tests conducted).

Salmonid toxicity tests were conducted at Environment Canada's Pacific Environmental Science Centre (PESC), Environmental Toxicology Section (ETOX), North Vancouver, B.C. The Environmental Toxicology Section falls under the Science and Technology Branch, Water Directive, and Operational Analytical Laboratories and Research Support, organizationally. The salmonid toxicity tests involved all members of the ETOX section. Member include: Graham vanAggelen (Head), Craig Buday, Michelle Linssen-Sauvé, Grant Schroeder, Heather Osachoff, Rachel Skirrow, Lorraine Yu and Joy Bruno.

All biological test methods followed are adaptations (species selection, salinity, duration) of Environmental Protection Series Biological Test Methods: Acute Lethality Test Using Rainbow Trout Report EPS 1/RM/9 July 1990 with May 1996 amendments and Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout Report EPS 1/RM/13 Second Edition- December 2000. The rainbow trout 96-hour bioassay is an Environment Canada standardized method for evaluating the acute lethality of effluents (Environment Canada, 1990) and is used nationally as a key component of regulatory and compliance monitoring.

Static 96-hour acute toxicity bioassays were conducted according to the methods outlined by Environment Canada (1990a & b) to determine the concentration series for the subsequent non-lethal toxicogenomic exposures. The data was analyzed statistically using CETIS™ (Comprehensive Environmental Toxicity Information System, Tidepool Scientific Software). The LC50 and its 95% confidence limits were derived by statistical analysis of mortalities using probit, Spearmann-Karber, moving average, or binomial methods. The LC50 is the median lethal concentration (i.e. the concentration of material in effluent that is estimated to be lethal to 50% of test organisms). The software was also used to compute NOEC/LOEC estimates. NOEC/LOEC estimates are the no-observed-effect concentration and lowest-observed-effect concentration, respectively. NOEC is the highest concentration of a test substance to which organisms are exposed that does not cause any observed and statistically significant adverse effects on the organism while LOEC is the lowest concentration of a test substance (to which organisms are exposed), that causes effects on the organism, which are detected by the observer and are statistically significant. The NOEC was the highest concentration used in the toxicogenomic evaluation series of effluent concentrations.

Control/Dilution Water

Freshwater tests used groundwater from the well on the PESC site for control/dilution water. The marine tests used sand-filtered saltwater from Burrard Inlet for control/dilution water. The salinity was adjusted to 28 ± 2 ppt by the direct addition of hypersaline brine to the effluent. Hypersaline brine is prepared by freezing uncontaminated natural seawater from Burrard Inlet (25-27 ppt) and then collecting the initial melt water to obtain a hypersaline brine of 90 ppt which is used to adjust the salinity of the effluent concentrations to the test range.

Test Volumes and Procedure

The final test solution volume was determined by the size and corresponding loading density (not to exceed 0.5 gram/L) of the fish. Salmonids (rainbow trout, Chinook) were acclimated a minimum of two weeks prior to testing. According to Environment Canada RM 13, the minimum mean weight should be at least 0.3 grams and should not exceed 2.5 grams following acclimation. The fish used in this study averaged from 1.5 to 6.7 grams in weight. Larger fish sizes were required to obtain sufficient liver tissue for toxicogenomic analysis and to allow for Chinook acclimation from freshwater to saltwater. Chinook are not acclimated to saltwater until their mean weight averages at least 3 grams.

The volumes were measured on a digital balance and transferred into aquaria. Oil-free compressed air was delivered to each test vessel at a rate of 6.5 ± 1 mL/minute by means of aquarium airline tubing and disposable borosilicate glass Pasteur pipettes. Each aquarium was covered with a Plexiglas lid. Test solutions were pre-aerated for 30 minutes prior to fish addition after which time the dissolved oxygen (D.O.) level was measured and the fish were added. All tests were conducted in a temperature-controlled environmental chamber set to maintain a temperature of $15 \pm 1°C$ and a photoperiod of 16 hour lightness and 8 hour darkness with a morning and evening transition time of 15 minutes. These conditions correspond with those in the fish acclimation/holding area of the laboratory. Further water quality parameters measured at the start of the test included: pH, temperature and conductivity. Mortality and behaviour were observed periodically throughout the test, more frequently during the first few hours and then daily until the test end. At each observation, numbers of dead fish in each test vessel were recorded and removed. Final water quality was taken at the end of the test.

Reference Toxicant Testing

Reference toxicant tests were used to assess, under standardized test conditions, the relative sensitivity of the population of test fish and the precision and reliability of data produced by the laboratory. Fish sensitivity to the reference toxicant was evaluated following acclimation and at least once a month thereafter. A 96-hour LC50 static test using reagent grade phenol was used to evaluate fish sensitivity. A warning chart was used to evaluate reference toxicant data; acceptable results were within ±2SD of values obtained in previous tests.

Quality Assurance/Quality Control

The PESC laboratory has a stringent Quality Assurance/ Quality Control program. Quality assurance (QA) is the overall program designed to ensure that the sample data meets data quality objectives (DQOs). This program includes: sampling plan, employee training, equipment maintenance and calibration procedures, quality control, corrective action plans, performance audits, data assessments, validation, storage, management and reporting. Quality control (QC) is the system of guidelines, procedures and practices designed to regulate and control the quality of products and services, ensuring that they meet pre-established performance criteria and standards. The QC system encompasses sample blanks, replicates, splits, equipment calibration standards, sample container size,

quality, use, and preservation amount. Quality control is part of the overall quality assurance program.

In addition, the Department of Environment requires that their laboratories have both national (CAEAL (Canadian Association for Environmental Analytical Laboratories)) and international accreditation (ISO/IEC 17025, International Laboratory Accreditation Cooperation (ILAC) and APLAC (Asia Pacific Laboratory Accreditation Cooperation)). Accreditation includes formal recognition of the competence of the laboratory to carry out specific tests requiring ongoing demonstration of performance through proficiency testing and biannual laboratory audits to maintain capabilities.

Toxicogenomic Exposures

The first test (Annacis 2004-1) included an early life stage test with Coho eggs but the Coho egg controls failed and invalidated the test so the method was abandoned. Given previous success with 96-hr underyearling rainbow trout exposures (BC Pulp Mill study), an 8-day underyearling rainbow trout exposure, with an 80% refresh of the effluent after four days (Annacis 2004-2), was undertaken. A 4-day refresh was chosen to limit effluent variability given that some chemicals, such as sterols, are quite stable over this period and should not degrade significantly. Due to the length of the test, feeding was also introduced into the experimental design. Fish were fed two hours prior to the water change on day 4. Ultimately, this exposure proved problematic due to fish mortality in the 100% effluent exposure. Neither of these two Annacis exposures were evaluated using toxicogenomic methods and will not be discussed in this report.

The study then grew to include tests with an 8-day exposure to effluent followed by an 8-day depuration/recovery period where the fish (still separated by sewage treatment group) were placed in new aquaria containing dilution/control water (Figure 14). The 8-day depuration was introduced to determine if molecular responses to exposure were permanent or transient in nature. Due to the length of the exposure, fish were fed at days 4 and 8, two hours prior to the water change. Fish were dissected on days 1, 4, 8 and 16.

Marine tests used saltwater acclimated Chinook salmonids, while freshwater tests used both rainbow trout and Chinook. Rainbow trout were used in the initial exposures but since they could not be genetically sexed, Chinook were used in the remaining freshwater tests.

Other than acute lethality and related data, Macaulay 2005 will not be discussed in this report as poor quality RNA prevented any evaluation by toxicogenomic analysis.
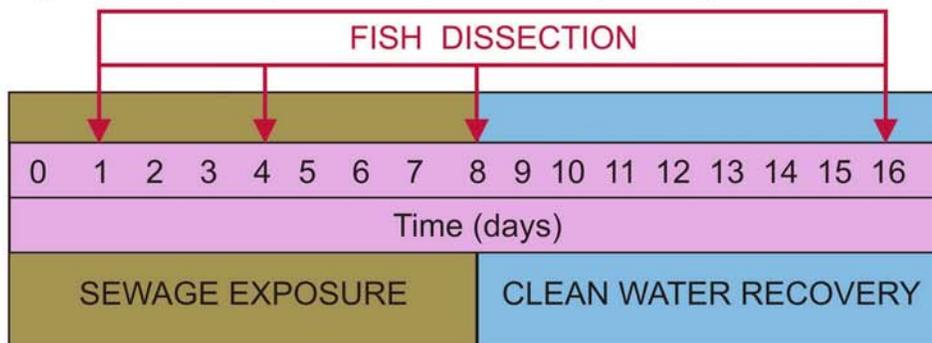
FIGURE 14 Exposure and sampling regime for 8-day sewage exposure and 8-day recovery.

Table 5 presents the concentrations used in the toxicogenomic tests. Concentrations were derived from the NOECs obtained from acute lethality tests as the highest concentration and concentrations provided by the GVRD/CRD (based on seasonal effects and relevant field concentrations). Due to the number of aquaria required for the longer tests, ammonia levels were not adjusted in these tests. Where physical space allowed, one or more positive controls were included.

TABLE 5 Concentration Series used in the MWWE toxicogenomic tests

Source/ Years	Annacis Island		Clover Point			Macaulay Point		
	2005	2006	2004	2005	2006	2004	2005	2006
# Treatments	5	6	6	6	6	6	6	6
Concentrations	Control	Control	Control	Control	Control	Control	Control	Control
	1.0%	1.0%	0.05%	0.05%	0.06%	0.06%	0.06%	0.05%
	10%	3.0%	0.10%	0.10%	0.15%	0.15%	0.15%	0.10%
	30%	10%	0.70%	0.70%	0.80%	0.80%	0.80%	0.70%
	60%	30%	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%
		60%	10.0%	10.0%	10.0%	10.0%	10.0%	10.0%

Positive Controls

Space permitting, a known EDC (e.g. estradiol) was used as a positive control during the MWWE toxicogenomic exposures. The liver from positive control fish was used for gene expression profiling to identify the gene fingerprint indicative of estrogen exposure.

Biological Sampling: Tissue Collection

Fish were dissected following PESC's standard operating procedures (PESC, 2007b) at each of the four timepoints (days 1, 4, 8 and 16). In summary, fish from one aquaria (8 to 10 fish) were euthanized using approximately 10 g/L MS-222 (tricaine methanesulfonate; TMS; Syndel Labs) and then dissected on ice. Each fish was assigned a unique identifier. Liver tissue from each fish was collected and preserved individually in RNALater (Qiagen or Ambion). Brain and muscle were collected separately and stored in RNALater for genetic sexing. Tissues were stored at 4°C for one day to allow permeation of the tissues by RNALater and then transferred to -80°C for storage until RNA extraction (liver) or genomic DNA extraction (muscle). The brain tissue was not used in the toxicogenomics analysis.

Chemical Analysis

The Chemistry Section at PESC performed all of the chemical analyses on the sewage samples. Table 6 outlines the sampling volumes and containers for chemistry collection. Nitrogen (ammonia, nitrate, nitrite), total metals, sterols, fragrances/musks, acidic drugs, phthalates, caffeine, PCBs, GCMS scan and pesticide chemistry were conducted on MWW effluents. Not all chemical analyses were performed on every effluent; the composition of the chemistry package varied as the study progressed.

It is recognized that the analytical chemistry suite available at PESC is not complete as it is impossible to analyze for all the chemical constituents found in MWWE. However, the chemistry data was useful in interpreting the gene expression data.

In order to rank the relative strength of an effluent, some parameters were scored on a high, medium, low scale, relative to the range for individual parameters.

TABLE 6 Effluent Sampling Volumes and Containers

ANALYSIS	NOTES	MDL	VOLUME	CONTAINER	STORAGE
Ammonia	Acute toxicant	0.005 mg/L	20 mL	plastic	4°C
Total metals ICP-MS, hardness	Some genomic endpoints are metal sensitive	element dependant	250 mL	metals bottle, 1 mL HNO₃	Acidified, 4°C
Sterols	Microarray has numerous EDC responsive genes	compound dependent	1L	amber glass	10 mL formaldehyde, 4°C
Fragrances/ Musks	Pharmaceuticals, Personal care products	compound dependent	1L	amber glass	4°C
Acidic Drugs	Pharmaceuticals, Personal care products	compound dependent	1L	amber glass	4°C
Phthalates	Plasticizers for plastics	0.2 ug/L	1L	amber glass	4°C
Caffeine	Marker for MWWE	0.2 ug/L	1L	amber glass	4°C
PCBs	Persistent organic pollutant	0.1 ug/L	1L	amber glass	4°C
GCMS identification scan	Identification of major organic constituents	compound dependent	1L	amber glass	4°C
Pesticides	Chemical marker for surface water pollution	0.01 ug/L	1L	amber glass	4°C

Nitrogen, Nitrate and Nitrite

Nitrogen, in the form of ammonia, is analyzed by automated phenolhypochlorite flow injection. In this method, the ammonia content refers to the sum total of ammonia and ammonium ion. Phenol, sodium nitroprusside and an oxidizing solution composed of sodium hypochlorite in a basic ethylenediamine tetraacetate (EDTA) solution are sequentially added to the sample. Under basic conditions, ammonia reacts with phenol and is oxidized by sodium hypochlorite to form an organic complex, indophenol blue. The blue colour formed is intensified with sodium nitroprusside and the absorbance of the resulting complex is measured at 630 nm. Sodium EDTA complexes calcium and magnesium ions and prevents precipitation of their respective hydroxides. A heating coil is used to accelerate and optimise the colour development.

Nitrate and Nitrite are analyzed by Cadmium/Copper Reduction, Flow Injection Analysis. The filtered sample is passed through a column containing granulated copper-cadmium which reduces nitrate to nitrite. Ammonium chloride is added to maintain quantitative reduction in the column. The nitrite that is originally present in the sample and the reduced nitrate then react with sulphanilamide under acidic conditions to form a diazo compound. This compound will then complex with n-1-naphthylethylenediamine dihydrochloride to form a magenta azo dye, the absorbance of which is measured at 520 nm.

Total Metals

Total metal samples were collected in pre-washed polyethylene bottles and preserved with nitric acid. These samples were digested in a blend of nitric and hydrochloric acids in an autoclave for two hours. Ten percent or more of the samples were duplicated as a quality assurance requirement. Metal scans were performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, using a Perkin Elmer Optima 4300 DV ICP instrument) as described in Environment Canada PESC WATMET method. Metals scans were also performed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, using a Perkin Elmer Elan 6000 ICP-MS instrument) as described in Environment Canada PESC WICPMS method which is based on EPA 200.8.

Sterols

Sterol analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were acidified to pH 3, spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred, using a Teflon-coated stir bar, for two hours. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample prior to derivatization. Once the concentrated sample extracts were ready for derivatization, they were cleaned on a silica column and acetylated with pyridine/acetic anhydride. Following derivatization, internal standard was added and the extract was further concentrated to 200 uL and analyzed by gas chromatography mass spectrometry (GCMS) for Sterols.

Fragrance/Musks

Fragrance/musks analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were spiked with surrogate and had 100 ml of dichloromethane added.

Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. Internal standard was added and the extract was further concentrated to 1 mL and analyzed by gas chromatography mass spectrometry (GCMS) for Musks.

Acidic Drugs

Acidic Drug analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were acidified to pH 2, spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. The extract was further concentrated to 1 mL and analyzed by liquid chromatography mass spectrometry (LCMS) for Acidic Drugs.

Phthalates

Phthalates analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. Internal standard was added and the extract was further concentrated to 1 mL and analyzed by gas chromatography mass spectrometry (GCMS) for Phthalates.

Caffeine

Caffeine analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. Internal standard was added and the extract was further concentrated to 1 mL and analyzed by gas chromatography mass spectrometry (GCMS) for Caffeine.

Polychlorinated biphenyls (PCBs)

PCB analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. The extract was further concentrated to 1 mL and analyzed by gas chromatography electron capture detection (GCECD) for PCBs.

GCMS Identification Scan

GCMS Identification analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) had 100 ml of dichloromethane added. Samples were then

magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. The extract was further concentrated to 1 mL and analyzed by gas chromatography mass spectrometry (GCMS) for GCMS Identification

Pesticides

Organochlorine Pesticides analysis was conducted using unfiltered effluent samples. Samples (typically 850 mL) were spiked with surrogate and had 100 ml of dichloromethane added. Samples were then magnetically stirred for two hours using a Teflon-coated stir bar. After stirring, the sample and solvent were transferred to a separatory funnel to remove the dichloromethane and concentrate the sample. The extract was further concentrated to 1 mL and analyzed by gas chromatography electron capture detection (GCECD) for Organochlorine Pesticides.

Genomic Approaches

Microarrays, QPCR and QuantiGene Plex were all used in the genomic analyses. Gene expression profiling using the salmonid gene microarray was the principal means of analysis, while QPCR and QGP were used to target specific genes to quantify their response to exposure. The purpose of this analysis was to discover which gene classes were affected by the exposure and to determine if those changes were permanent and dose-responsive.

RNA Isolation

Total RNA was extracted from the liver tissue of individual fish. Approximately 30 mg of liver was homogenized in 1 ml of Qiazol buffer (Qiagen) using a 3 mm tungsten-carbide bead (Qiagen) and a Mixer Mill 300 (Qiagen) at 20 Hz for 4 minutes (or until homogenization was complete, a maximum of 8 minutes). Total RNA was isolated from the homogenized liver tissue using a RNeasy Mini kit (Qiagen) which included on-column DNaseI digestion. The manufacturer's protocol was followed except RNA was eluted in two 35 μl volumes.
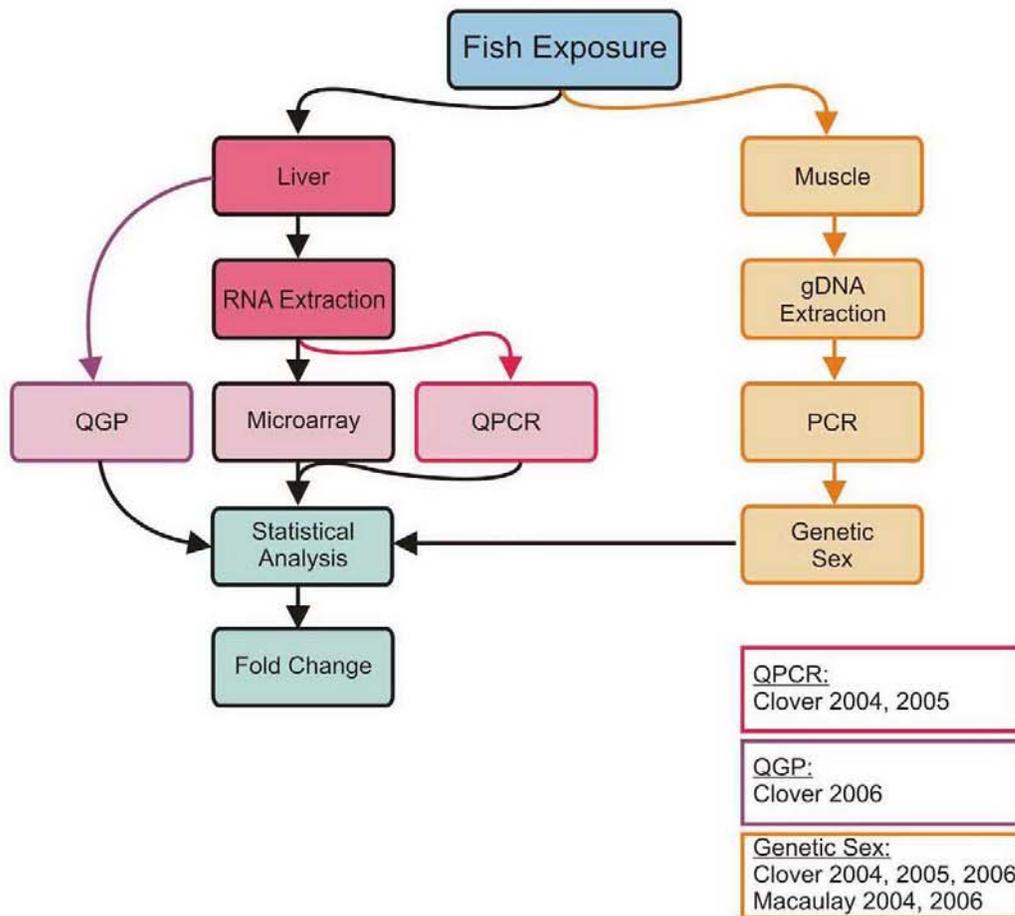
FIGURE 15 Flowchart of toxicogenomic analyses performed on salmonids exposed to MWWE.

Total RNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop) to measure the A260/A280 ratio. RNA integrity was evaluated using either a Bioanalyzer 2100 (Agilent Technologies Inc.) or agarose gel electrophoresis (Bio-Rad). Purified RNA was stored at − 80°C.

Microarrays

Four individual fish were evaluated for each concentration within a timepoint (one per microarray). Each exposure had 4 timepoints and 6 treatments (except Annacis 2005, which had 5 treatments) with 4 fish per treatment and timepoint, resulting in a minimum of 96 microarrays per experiment. `

Source and Printing

A total of 207 unique cDNA fragments were amplified by RT-PCR from total RNA isolated from rainbow trout liver, intestine, ovary, brain or kidney tissue. The cDNA

sequences for all genes on the microarray are available in GenBank (Appendix 1). Primers (17-21 nucleotides) were generally designed to amplify fragments between 450-550 base pairs in length using either the online primer design program Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA). The primer pairs were designed from the most conserved region of the cDNA sequence for each gene, based on sequence alignment with all available gene sequences including other species of fish. Primer pairs that consistently amplified only a single product, confirmed by separating on a 1% agarose gel stained with ethidium bromide, were used in the array construction. Primers were obtained from Alpha DNA (Montreal, QC, Canada).

PCR products were cloned into the Invitrogen pCR-Topo II vector (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol, except all reaction volumes were halved. Ligation of the PCR products into the vec tor was verified by PCR with the vector specific M-13 forward and reverse primers. Plasmids containing the desired PCR fragments were isolated using a Qiaprep Spin miniprep plasmid isolation kit (Qiagen). All plasmids were sequenced, for correct insert confirmation, at the Center for Environmental Health, at the University of Victoria (Victoria, B.C. Canada).

The cDNA pieces were prepared for printing as described by Wiseman *et al.* (2007). The Microarray Facility of The Prostate Centre at Vancouver General Hospital (Vancouver, BC, Canada) printed the microarrays on ez -rays™ aminosilane coated slides (Matrix Technologies Corp.) with a Biorobotics Microgrid II microarray printer (Genomic Solutions) equipped with Microspot 10K quill pins (Biorobotics) in a 48-pin tool that deposited ~ 0.1 ng cDNA pieces per spot. The cDNA fragments were spotted in 16 subgrids, with 36 spots per subgrid, and each subgrid included negative controls (empty spots and 3X SSC buffer-only spots) and corner markers (a 280 base pair cDNA fragment of GFP (Green Fluorescent Protein; GenBank accession # U17997) provided by the Microarray Facility of The Prostate Centre at Vancouver General Hospital).

Microarray Design Using Reference Material

This study incorporated a reference experimental design approach using an in-house produced Universal DNA Reference (UDR) material. The UDR was a fluorescently labelled (Cy3) mixture of all genes printed on the microarray. It was hybridized to every microarray slide along with the experimental sample (Cy5-labelled liver cDNA from exposed or control fish). The end result was a two-colour microarray having reference material in the green channel and fish liver material in the red channel. The reference design allowed for comparison between treatments and timepoints within an experiment as well as between the experiments. The reference signal level was also used as a diagnostic tool since it should remain constant across all treatments and all slides; therefore, an abnormal reference signal indicates a problem with the hybridization of sample materials or with the printing of that slide.

UDR was created by amplifying each gene fragment in ten PCR reactions (100 μl each or 1 ml total) using gene specific primers in the following PCR reaction: 1X Taq Polymerase buffer containing magnesium chloride, each dNTP at a concentration of 300

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µM, 3.75 U of Taq (Promega, Madison, WI, USA), 3 ng of plasmid and 0.6 µM of each specific primer. The following conditions applied: 35 cycles of 94°C for 30 s, appropriate annealing temperature (45 to 55 °C depending on gene specific primers) for 60 s, and 72 °C for 2 min followed by a final 12 min extension at 72°C. PCR products were visualized on a 1.2% agarose gel stained with 1 µg of ethidium bromide/ml to confirm correct size of amplicon as well as minimal primer dimer. The ten PCR reactions for each gene fragment were pooled and purified using the Qiagen QIAquick PCR Purification kit. The concentration of each gene fragment was determined via spectrophotometry, the samples were speed vacuum dried, resuspended in 3X SSC to a final concentration of 0.5 µg/ml for each gene fragment. The gene fragment was confirmed with a 1.2% agarose gel.

Preparation of Fluorescently-Labelled cDNA

A. Reverse Transcription of Liver RNA

Superscript Direct cDNA Labeling System kits (Invitrogen) were used to prepare Cy5-dUTP labelled cDNA from fish liver RNA. Labeled cDNA was prepared from 25µg of total RNA according to the manufacturers instructions. To remove unincorporated reagent from the Cy5-labelled cDNA samples, a Cyscribe GFX Purification kit (Amersham) was used as per the manufacturer's protocol except cDNA was eluted in two 60µl aliquots with 5-minute room temperature incubations for each elution step. The purified Cy5-labelled cDNA samples were stored at -20°C overnight in amber 1.5 ml tubes wrapped in aluminum foil.

B. Reference Material (UDR)

The UDR stock solution was prepared by pooling the purified cDNA pieces (in 3X SSC buffer). Stock UDR was prepared once and was stored at 4°C. For each microarray slide, a Cy3-labelled UDR cDNA sample was prepared using 600 ng of UDR stock material, 1 nmol of Cy3-dCTP dye (Amersham) and Ready-To-Go beads (Amersham), as per the manufacturer's protocol, except a 2-hour incubation at 37°C was used to maximize yield. The reaction was terminated by addition of 2µl of 0.5 M EDTA (pH 8.0) and purified using the Cyscribe GFX Purification kit (Amersham). The reactions were eluted from the Cyscribe GFX columns in two 60µl aliquots of elution buffer with 5-minute incubations at room temperature before each elution to maximize yield. Each Cy3-labelled UDR sample was eluted directly into a 1.5 ml amber tube containing one Cy5-labelled cDNA sample. The Cy3-labelled UDR samples and Cy5-labelled cDNA samples were combined at this step in order to co-precipitate the materials that were applied to each microarray slide. To precipitate the materials, the following reagents were added: 1µl of 20 mg/ml glycogen (beef liver; Fisher Scientific) in water, 0.1 volumes of 3 M sodium acetate, and 2.5 volumes of 98% ice-cold ethanol (aqueous; Commercial Alcohols). The labelled cDNA was allowed to precipitate for a minimum of 1 hour (maximum of overnight) at -80°C in aluminum foil wrapped tubes.

Array Hybridization and Washing

Preparation of Hybridization Material

The labelled nucleic acids were pelleted by centrifugation at 13000 x g at 4°C for 20 minutes, the supernatant removed, and 500μl 70% ethanol (Commercial Alcohols) prepared with DEPC-treated water (Ambion) was added. The pellet was washed by vortexing and then pelleted by centrifugation at 13000 x g for 5 minutes. The supernatant was removed and the pellet was air dried for 10 minutes at room temperature. The dry pellets were resuspended in 50 μl hybridization cocktail containing 25μl formamide (Fisher Scientific), 12.5μl 20X SSC (Fisher Scientific), 0.5μl 10% SDS (Fisher Scientific), 4μl 5 mg/ml polyA (Invitrogen), 4μl 2 mg/ml BSA fractionV (BioLynx) and 4μl 10 mg/ml yeast tRNA (Invitrogen). The pellets were resuspended by gentle vortexing and heating at 95°C for 3 minutes. The cDNA hybridization solutions were kept at 65°C (maximum 30 minutes) until ready for application to the prepared microarray slides.

Pre-Hybridization Slide Preparation

The microarray slides were washed with 0.1X SSC by inversion in coplin jars and then transferred to new coplin jars containing warm (48°C) pre-hybridization buffer (0.22μm filtered solution of 5X SSC (Fisher Scientific), 0.1% SDS (Fisher Scientific) and 0.2% BSA fraction V (Fisher Scientific)). The slides were incubated in the warm pre-hybridization buffer for 45 minutes in a 48°C waterbath. The slides were then transferred to a new coplin jar containing room temperature de-ionized water and mixed by inversion. This process was repeated and then the slides were allowed to cool for 8 minutes. The slides were dried by immersion in isopropanol (HPLC-grade, Fisher Scientific) followed by centrifugation at 1000 x g for 3 minutes in sterile 50 ml polypropylene centrifuge tubes (Sarstedt). After centrifugation, the slides were placed microarray-side up in Corning hybridization chambers (Fisher Scientific).

Hybridization and Washing

A silanized[1] Lifter-Slip (VWR) was placed in the center of each microarray slide and the pre-warmed cDNA-UDR mixture was added to each slide in 10μl aliquots. Immediately after the addition of the cDNA-UDR mixture, the hybridization chamber was assembled and placed in a 42.4°C hybridization oven. Microarrays were hybridized overnight (16 to 19 hours).

After overnight hybridization, slides were soaked in a pre-warmed (48°C for 10 minutes) solution of 1X SSC, 0.1% SDS to remove the LifterSlip. The slides were then transferred to a coplin jar containing a warmed solution of 1XSSC, 0.1% SDS and washed by gentle shaking for 4 minutes. The slides were transferred to a clean coplin jar containing room temperature solution of 1XSSC, 0.1% SDS 1 and washed by gentle shaking via vortex at room temperature for additional 4 minutes. This cycle was repeated twice more with

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[1] The cover slips were previously silanized with 10 μl of SigmaCote (Sigma) per slip.

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room temperature 0.1X SSC. The slides were dried by centrifugation at 1000 x g for 3 minutes in clean 50 ml polypropylene tubes. Dry slides were transferred to dark storage boxes and then scanned using a microarray scanner. If not immediately scanned, the slides were stored in a desiccator until they could be scanned.

Image analysis

Scanning

Dual-laser scanners by PerkinElmer were used to scan the microarray slides: laser 1 was 543 nm for Cy3 (green channel) and laser 2 was 633 nm for Cy5 (red channel). Microarray slides from Clover 2004 were scanned using a ScanArray Express (PerkinElmer) at 10μm resolution. The laser was set to 90% power and the PMT gain was 62 for the green channel and 66 - 68 for the red channel. The remaining slides were scanned on a ProScanArray (PerkinElmer) at 10μm resolution. The laser was set to 90% power, and the PMT gain was 51-70 for the green channel and 57-80 for the red channel.

Data analysis

Image TIFF files from the scanned microarrays were imported into ImaGene software version 6.1 (BioDiscovery) for quantification of the signal for each spot on the microarray. Spot finding was performed using the automated spot finding algorithm and each sub-grid was manually inspected to ensure the appropriate sub-grids and spots were selected.

ImaGene output files were imported into GeneSpring software version 7.3.1 for statistical analysis. Each timepoint for each experiment was treated as a unique block and analyzed independently of other timepoints. Four microarrays were hybridized for each treatment within a timepoint.

The array data was analyzed in GeneSpring by: (1) assigning values of zero to 0.01, to prevent mathematical calculations with a value of zero; (2) determining a ratio for each gene of the signal minus background in the red channel (Cy5-labelled cDNA) to the signal minus background in the green channel (Cy3-labelled UDR) (The background levels used in the ratio calculation were based upon the negative controls (empty spots and buffer-only spots)); and (3) normalizing the data to the 50th percentile of the median values for all genes across the microarrays within each timepoint. Normalized data was then analyzed by one-way ANOVA and Welch t-tests (variances not assumed equal) to identify significant changes ($p<0.05$) in gene expression between sewage and control treated tissue. GeneSpring data (fold changes and p-values) were exported to Excel software (Microsoft) for analysis.

The fold change data was graphed and R^2 values were obtained from logarithmic trend analysis[2]. An R^2 cut-off of 0.8 was applied to identify responsive trends. This cut-off was subjective and determined based on a visual assessment of linearity on the graphs.

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[2] Logarithmic trend analysis was used (not linear) since the sewage concentration series was logarithmic.

PESC employs microarray QA/QC. A DNA array experiment is a complex process requiring quality assurance and quality controls. Each step in the process is associated with a laboratory protocol that describes the substances, conditions and procedures used. The need to be able to reproduce and validate DNA experiments has lead to the creation of an international standardization society called Microarray Gene Expression Data (MGED) society. The MGED group developed the Minimal Information About a Microarray Experiment (MIAME) guideline (Nature Genetics, 29, 365-371; 2001). As a member of MGED, our data conforms to the MIAME guidelines.

Quantitative Real Time PCR (qRT-PCR)

Primer Testing and Optimization

The target genes included: vitellogenin (VTG), vitelline envelope proteins alpha, beta and gamma (VEPA, VEPB, VEPG), estrogen receptor A (ESR1) and ribosomal protein L8. The primer sequences and amplicon sizes are shown in Table 7.

VTG, VEPA, VEPB and VEPG were previously designed to produce rainbow trout amplicons for microarray printing as described by Wiseman *et al.* (2007). The ESR1 primers were described by Vetillard *et al.* (2005). L8 (GenBank accession #: AY957563) primer information was provided by Dr. Nik Veldhoen and Dr. Caren Helbing (University of Victoria, Victoria, BC; personal communication).

All primers were obtained from Alpha DNA. Stock primers were diluted with DEPC-treated water (Ambion) to a final concentration of 100μM. Fresh working primer dilutions (20μM concentration in DEPC-treated water) were prepared for each QPCR run to maintain primer quality. Primers were stored at -20°C.

Primers were designed to function at an annealing temperature in the range of 56 to 62°C. The specificity of the amplification at the target temperature was visually evaluated by agarose gel (i.e. no primer dimer or bands of the wrong size) and the clean band was subsequently cloned and sequenced to verify the identity of the target gene.

TABLE 7 Target gene list and associated primer information

Gene Symbol	Amplicon Size (bp)	Primer Sequence (5')	Primer Sequence (3')
VTG	488	ATGAGAGCAGTAGTACTTG	TCTTGCACACTCCCTGAGC
VEPA	490	CAACAGATACCCTACACCAAA	TAATCAGTGTCTCAACGGAAG

VEPB	537	CTTCTGTCTTTGCCATCTAC	TGGTCAGGTAACGGTCAT
VEPG	465	AGACTGCCCTATGACTGGA	TATCTGGGATTGGCGTTTA
ESR1	237	TCGCACGGGCTGTTAAGGAAG	AGATGACGTGGCTCTCCAGGG
L8	270	CAGGGGACAGAGAAAAGGTG	TGAGCTTTCTTGCCACAG

Cloning and Sequencing

PCR amplicons for each target gene were separated by electrophoresis on 2% agarose gels. Bands were excised from the agarose gel (based on expected sizes in Table 7) and the isolated DNA was purified from the agarose using the freeze-squeeze technique (Tautz, 1983 and M. Renz). Briefly, DNA was squeezed from the agarose by subjecting the gel cut-outs to six alternating 10 min incubations of -80°C and 37°C. The DNA solution was then spun at (12000 x g) for 10 min and 2 to 4µl of this liquid was cloned into Topo 2.1 plasmids using the TopoTA cloning kit (Invitrogen) as per the manufacturer's protocol. At least three isolated colonies per target gene were selected from LB/Amp/X-gal agar plates and grown separately in 5ml LB broths for 18 hours at 37°C with shaking at 200 rpm. M13 primers were used in a colony PCR reaction to confirm that the colony contained the correct amplicon. Each 15µl PCR mixture contained: 0.35 µl of broth, 0.5 U of Taq DNA Polymerase and 1X Taq incubation buffer with MgCl₂ (Fisher Scientific; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton-X, 0.2 mg/ml BSA, pH 9.0), 0.25 mM of each deoxynucleotide (Sigma), 0.4 pmoles each of M13 forward and reverse primers (Invitrogen), and DEPC-treated water (Ambion). PCR program parameters were: 94°C for 10 min, then 30 cycles of 94°C for 30s, 55°C for 45s and 72°C for 60s, followed by a 12-min step at 72°C and a final cooling at 4°C for 30s. Plasmids were isolated from two amplicon-containing cultures using a QiaPrep plasmid purification kit (Qiagen) following the manufacturer's protocol. Plasmids were sequenced in both directions using M13 primers by the Microarray Facility at the Prostrate Research Centre of Vancouver General Hospital. Sequence identities were confirmed by alignment with GenBank sequences using BLAST. One correct plasmid for each target gene was chosen to generate a standard curve.

Standard Curves

Dilutions of each amplicon-containing plasmid were used to generate standard curves for each target gene using the Mx3000P QPCR System (Stratagene). Plasmids were freshly diluted in water to generate the following number of molecules per reaction: 250000, 50000, 25000, 10000, 5000, 2000, 1000, 400. The final volume of each QPCR reaction was 15µl, 2µl of which was the plasmid dilution and 13µl was a Master Mix (MM)

containing the following reagents: 7.5μl of Superscript III QPCR 2X Supermix (Invitrogen), 0.6μl of ROX (Invitrogen), 0.6 pmoles of primer 1, 0.6 pmoles of primer 2, and nuclease-free water (Ambion). The PCR program parameters were: 50°C for 2 min, 95°C for 2 min, then 35 cycles of 95°C for 20s, 58°C for 30s, two plate reads (collection of data) and 72°C for 40s, followed by a dissociation curve analysis. Each dilution was run in quadruplicate and the Ct values were used to generate standard curves. The Ct values obtained from those reactions were plotted against the log copy number of each plasmid and the resulting linear equation was used to determine the copy number of each gene.

A standard curve was deemed acceptable if it met the following criteria: (1) the correlation coefficient (R^2) of the line was greater than or equal to 0.95, (2) the efficiency (calculated from the slope of the line) was between 70 – 120 % and (3) the threshold value was less than 0.020. The threshold was unique to each target gene and was calculated based on the signal between cycles 4 to 12. The calculation used a factor of 10 to create the threshold, which ensured that the threshold was set to at least 10 times above the background noise levels.

cDNA Synthesis

Total liver RNA (2 μg) was used to generate cDNA (complementary DNA) using Superscript III First Strand Synthesis kit (Invitrogen) as per the manufacturer's protocol, except that the recipe was doubled to generate 40 μl of cDNA.

The quality of the cDNA was evaluated by amplifying beta-actin using a PTC-200 (MJ Research) or Tetrad2 (BioRad) PCR machine. The PCR reaction recipe per tube contained 1 μl of each cDNA sample and 24 μl of a PCR reaction solution. The PCR reaction solution consisted of: 1.5 U of Taq and 1.3 X Taq incubation buffer with $MgCl_2$ (Fisher Scientific; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X, 0.2 mg/ml BSA, pH 9.0), 0.25 mM of each deoxynucleotide (Sigma), 0.2 pmoles of each salmonid beta-actin primer (designed by Dr. Ellen Busby; synthesized by AlphaDNA; forward primer sequence = GTCAGGCAGCTCGTAGCTCT, reverse primer sequence = CTGACCCTGAAGTACCCCATT) and DEPC-treated water (Ambion) up to a final volume of 24 μl. The following program was run: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s, followed by 72 °C for 10 min and a cooling step to 4 °C for 2 min. Presence or absence of amplicon (540 bp) was determined by agarose gel electrophoresis. Absent or poor quality beta-actin bands indicated a failed cDNA synthesis reaction. The stock cDNAs were diluted 15-fold with nuclease-free water (Ambion) to create working dilutions for use in the QPCR reactions.

Performing QPCR Runs

QPCR runs were conducted in 96-well format. Set-up dependent variability was minimized by including control and all treatments for a single timepoint of an experiment on a single plate. All cDNA samples were analyzed in quadruplicate, with the replicates spread over four plates which were run sequentially.

A QPCR Master Mix (MM) with sufficient reagent for quadruplicate plates was prepared for each target gene(s). The final QPCR reaction volume was 15μl, with 2μl of 15-fold diluted cDNA and 13μl of MM used for each target gene listed in Table 2. Each reaction included 7.5μl of Superscript III QPCR 2X Supermix (Invitrogen), 0.2μl of ROX (Invitrogen), 0.6 pmoles of primer 1, 0.6 pmoles of primer 2, nuclease-free water (Ambion). A No Template Control (NTC) was included on each plate as a negative control for each primer pair (i.e. target gene). The NTC reaction mixture contained 2μl of nuclease-free water (Ambion) and 13μl of MM.

All QPCR runs utilized the same program except that one out of four of the replicate plates included a dissociation curve to verify the specificity of the reactions. The PCR program parameters were: 50°C for 2 min, 95°C for 2 min, then 35 cycles of 95°C for 20s, 58°C for 30s, two plate reads (collection of data) and 72°C for 40s. Data was collected by the instrument for ROX dye (ROX filter) and SYBR dye (FAM filter). The reference ROX dye was used to adjust for small differences in reaction volumes. The instrument read the SYBR signal as the gene expression data and output the data as C_t values.

QPCR Data Analysis

Determining Ct values and Copy Numbers by Absolute Quantification

The threshold value (Table 8) for each gene's standard curve (unique for each target gene) was used to determine C_t values. The adjusted C_t values for each QPCR reaction were exported as text files to Excel software (Microsoft) where the data was organized by timepoint. There were no inter-plate comparisons because the layout included all cDNA samples from a single timepoint on one plate. C_t quadruplicates were evaluated for outlying values using the first outlier checking test suggested by Burns *et al.* (2005). Quadruplicate C_t values were averaged after outlier removal.

The No Template Controls (NTCs) for each primer pair were used to evaluate the difference in C_t values between samples and NTC. A difference of at least two C_t values from the average sample C_t values (with outliers removed) for all fish in a treatment was required. This step ensured that the sample C_t values were derived from target amplification and not from primer dimer or other non-specific products.

Target gene copy numbers were then calculated (Absolute Quantification analytical method) from the averaged C_t values using the standard curve equations (y = mx +b equations) shown in Table 8.

TABLE 8 Standard curve equations, correlation coefficients (R^2), efficiency, and threshold values for target QPCR genes at an annealing temperature of 58°C.

Gene	Equation	R^2	Efficiency (%)	Threshold
VTG	y = -3.6215x + 36.658	0.98	89	0.0062
VEPA	y = -4.2574x + 38.257	0.99	72	0.0048
VEPB	y = -3.54x + 36.364	0.97	92	0.0053

VEPG	y = -3.9872x + 44.275	0.97	78	0.0031
ESR1	y = -3.0781x + 37.265	0.97	111	0.0059
L8	y = -2.9174x + 31.826	0.99	120	0.0029

Outlier testing

Outlier analysis was conducted on technical replicates and biological replicates using the Graphpad Software QuickCalcs online Grubb's test (http://www.graphpad.com/quickcalcs/Grubbs1.cfm) to remove a small number of gene transcripts from downstream analysis. For Clover 2005 samples, 2.6% to 3.9% of the normalized copy number values were discarded as outliers and the maximum number of outliers found in a treatment for a particular gene was 3. Therefore, in Clover 2005, the minimum value of n for a treatment was 13 (55% of the time, n = 16; 39% of the time, n = 15; 3% of the time, n = 14, 2% of the time, n = 13). For the complete list of n values for each experiment, see Appendix F.

Normalization

These genes were evaluated as potential normalizers for QPCR data: histone H3, alpha-actin, beta-actin, ribosomal protein S5, ribosomal protein S6, 60S ribosomal protein, and ribosomal protein L8. Each gene was amplified by PCR in control liver tissues and visualized by agarose gel electrophoresis. Gene quality was scored based on signal intensity on the agarose gel and based on the C_t values of the amplification curve (target C_t range: 15-33). Ribosomal protein L8 best met the criteria. To determine if L8 was invariant under the experimental parameters, a thorough analysis with highly controlled RNA input was performed using liver cDNA from Clover 2004 (approx. 176 samples). There were no significant differences (Welch t-test, $p < 0.05$) in L8 expression between any treatments within a timepoint.

The invariant L8 reference gene was used as a normalization factor (NF) to account for variations in cDNA input and instrument runs. The NF for each fish was calculated using the following equation:

Example: NF for fish 1 in sewage concentration 1 of Experiment 1, day 1:

$$NF_1 = \frac{\text{average L8 copy number for every fish in Experiment 1, day 1}}{\text{L8 copy number for fish 1}}$$

$$NF_1 = \frac{100000}{77000}$$

$$NF_1 = 1.3$$

Statistical Analysis

Welch's t-tests were performed on the normalized copy numbers (outliers removed). Within a timepoint, each sewage treatment was compared to control and significance was denoted when $p < 0.05$. Fold change in the sewage treatment versus the control was determined by generating the following ratio: average copy number for sewage treatment/average copy number for control (within each timepoint).

Genetic Sex Determination

Genetic sex was determined from previously isolated muscle tissues using primers specific to a y-linked growth hormone pseudogene identified by Devlin *et al.* (1993: other *Oncorhynchus* species; 2001: Chinook) as male-specific.

Extraction of Genomic DNA

Genomic DNA (gDNA) was extracted from approximately 10 mg of muscle tissue using a DNeasy Mini kit (Qiagen). The manufacturer's protocol was followed except the proteinase K digestion was conducted for 16-19 h and the final elution was performed with 200µl of elution buffer.

Polymerase Chain Reaction with Male-specific Primers

Polymerase Chain Reaction (PCR) was performed on muscle gDNA using sex-specific male primers published by Afonso *et al.* (2002). Each 50 µl PCR reaction volume contained: 1.25 U Taq (Fisher Scientific), 1.8X Taq incubation buffer with $MgCl_2$ (Fisher Scientific; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton-X, 0.2 mg/ml BSA, pH 9.0), 0.2 mM of each deoxynucleotide (Sigma), 0.4 pmoles of GH5 primer, 0.4 pmoles of GH6 primer, and 2µl of gDNA. The PCRs were conducted on either a PTC-200 (MJ Research) or Tetrad2 (BioRad) machine. The conditions of the PCR program were: $94^{o}C$ for 3 min, 30 cycles of: $94^{o}C$ for 1 min, $52^{o}C$ for 1 min, $72^{o}C$ for 1 min; $72^{o}C$ for 10 min, $4^{o}C$ hold.

Agarose Gel Electrophoresis

Each PCR sample (9µl) was mixed with 1 µl of 10X loading buffer (30% sterile glycerol, 0.25% bromophenol blue, 60 mM EDTA, 40 mM tris-acetate) and separated by

electrophoresis using 2% agarose gels at 60V for 50-60 min in Sub-cell GT electrophoresis boxes (BioRad). If a bright, clear 240 bp band was present, the sample was a genetic male fish (Afonso, 2002).

QuantiGene® Plex Assay

Liver samples from Clover 2006 were analyzed using a custom 9-gene QuantiGene Plex 1.0 assay created by Panomics Inc (Table 9) and used according to the manufacturer's instructions (Panomics). Nine individual fish liver samples were analyzed in duplicate per concentration per timepoint. Liver samples from fish exposed to control, 0.05%, 0.7%, 2% and 5% sewage were analyzed. Day 1 and day 8 samples were analyzed on a Bio-plex 200 System (Bio-Rad) and the day 4 and 16 samples were analyzed on a LiquiChip 100 Workstation (Qiagen). Median fluorescence intensity (MFI) data was exported to Excel where it was normalized by subtraction of the bead background signal. Signals below the limit of detection were omitted from analysis. The limit of detection was set as the background signal + 3 times the standard deviation of the background replicates. The technical replicate MFI for each gene was averaged and normalized to the corresponding L8 MFI. Biological replicates were averaged for each treatment per timepoint and divided by the control treatment values to obtain fold change data. Statistical significance between gene expression in control and treated tissues was determined by Welch t-test.

TABLE 9   Gene targets in 9-gene custom QGP 1.0 Plex.

Gene symbol	Accession #	Biomarker Use
L8	AY957563	Normalizers
60Srp	AF281334	
VEPB	AF231707	Estrogenic
ESRA	AJ242740	
VTG	M27651	
THRB	AF302246	Thyroid
DIO2	AF312396	
CYP1A1	AF059711	Toxicant
MT1A	M18103	Metal

RESULTS

Remove these when we know these have been covered off, Anticipated results:
- Relating gene profile(s) to mechanistic effects (e.g. vitellogenin (Vtg)). The microarray contains a number of gene transcripts that are responsive to exposure to xenoestrogens. An example of linking gene expression to mechanistic effects is the vitelline envelope proteins and vitellogenin. These genes are primarily responsible for the maturation of oocytes and are expressed in the liver in response to estrogen. Under normal conditions, these genes are not present in juveniles and do not become expressed until sexual maturation. Expression can

be artificially induced in juveniles and males by exposure to xenoestrogenic compounds. Based on the degree of expression, the clustering of related genes and the greater than 2-fold expression difference from the control group, we will be able show those organisms that are experiencing abnormal and potentially deleterious molecular effects as the result of exposure to the effluent.

- Chemical analysis data will provide a profile of the EDC's in the effluent.

Effect definition:
- An "effect" is something brought about by the organisms exposure to the effluent as compared to the organisms exposed to the control water.
- Criteria for significance of an effect:
  For gene expression profiling an effect is either a doubling or halving of the gene transcript being expressed as compared to the control (Seta, K.A. 2004; Simon, R., Radmacher, M.D., and K. Dobbin, 2002). Many researchers consider this expression change to be too conservative such that biologically important genes that have smaller fold changes are completely missed (Larkin, P., Sabo-Attwood, T., Kelso, J. and N.D. Denslow, 2003; Spruill, S.E, Lu, J., Hardy, S., and B. Weir, 2002).

Acute Lethality of Municipal Wastewater Effluent to Rainbow Trout

The results of the acute lethality of the effluents toxicity tests are summarized in Tables 10, 11 and 12. These same effluents were used to initiate the toxicogenomic studies, except Clover 2004-1 and Macaulay 2004-1 which had effluents collected uniquely for the 96 hr LC50 tests and Annacis 2006 refresh which was the effluent used for the 80% solution renewal on day 4 of the toxicogenomic test. The dissolved oxygen, pH and temperatures were within the acceptable ranges during the effluent exposures. All MWW effluents exhibited some mortality in the effluent exposures except the freshwater discharging Annacis MWWE pH controlled tests. Research at PESC with Annacis MWWE has shown that lethality due to ammonia toxicity can occur if the pH is not controlled during Rainbow trout testing. Ammonia toxicity is largely dependent on pH. As the pH increases, the toxicity of the ammonia increases since the relatively benign NH_4^+ is converted to much more toxic NH_3 form of ammonia (Environment Canada, 2008). In side by side testing (pH v. non-pH controlled) with Annacis MWWE (n = 18), 28% of the non-pH controlled tests (n = 5) had greater than 50% mortality at full strength. None of the 18 tests had greater than 20% mortality when the pH was controlled.

TABLE 10 96 hr LC50 of Salmonids to Annacis Island MMWE

	GVRD Annacis Island MWWE		
	2005	**2006**	**2006 refresh**
Collection	14Jan05	16June06	22June06
96 hr LC50 (not pH controlled)	ND	77.5% (65.9%-91.0%) Trimmed Spearman Karber	54.0% (44.8%-65%) Trimmed Spearman Karber
96 hr LC50 (pH controlled)	ND	>100%	>100%
100% effluent LT50	72 hrs	>1.33 < 24 hrs	> 1.33 < 19 hrs

ND= not done

TABLE 11 96 hr LC50 of Salmonids to Clover Point MWWE

	CRD Clover Point MWWE		
	2004-1	**2005**	**2006**
Collection	20May04	27May05	31Mar06
96 hr LC50 (not pH controlled)	13.4% (10%-18%) Non-Linear Regression	10% (6.4%-14.2%) Probit	5.9% (4.6%-7.5%) Trimmed Spearman Karber

TABLE 12 96 hr LC50 of Salmonids to Macaulay Point MWWE

	CRD Macaulay Point MWWE		
	2004-1	**2005**	**2006**
Collection	15June04	17June05	10Mar06
96 hr LC50 (not pH controlled)	24% (19.5%-29.5%) Probit	24% (18%-32%) Binomial	18.79% (14.7%-24.2%) Probit

The graph below (Figure 16) illustrates the predicted rainbow trout ammonia LC50 versus the actual ammonia concentration in effluents. The points on the graph represent the intersection of the ammonia and pH from the highest concentration used in the toxicogenomic tests. This figure provides visual evidence that ammonia should not cause toxicity at these concentrations.
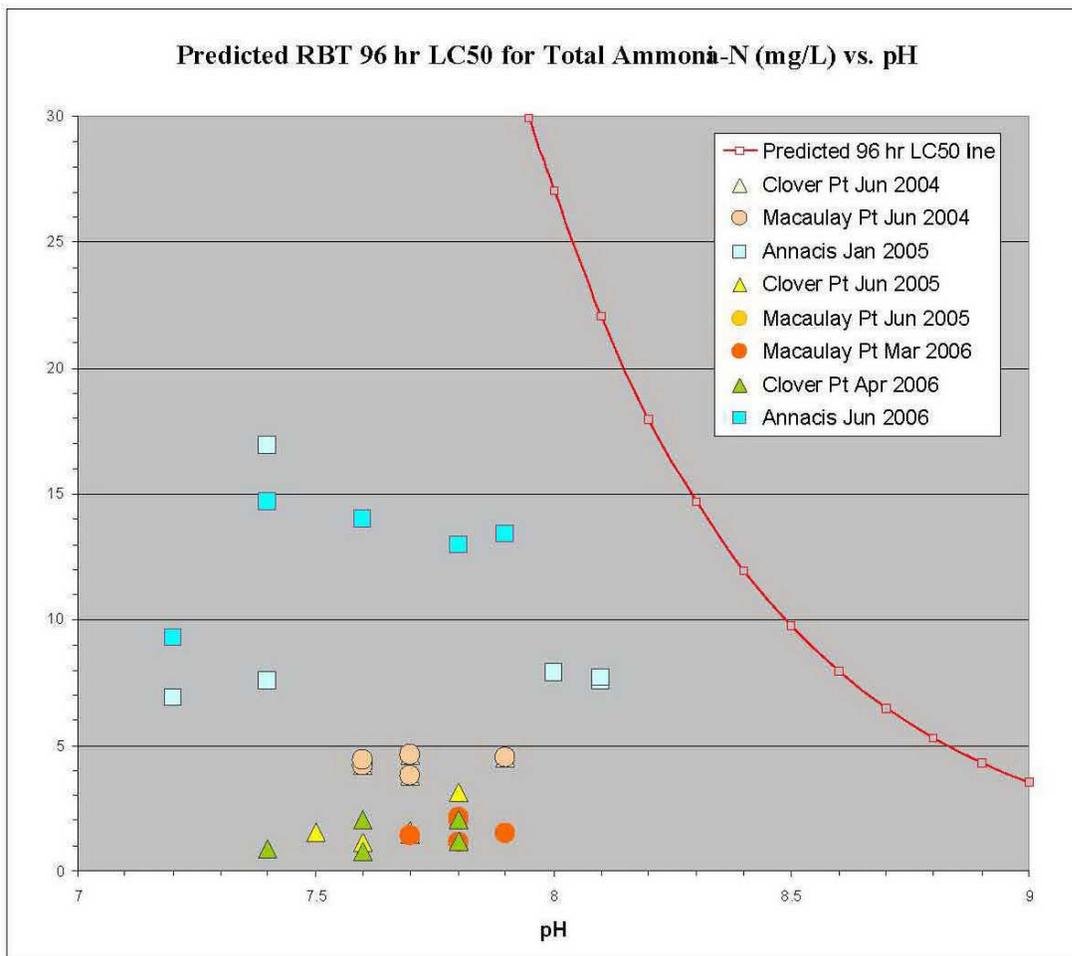
FIGURE 16 Predicted Rainbow Trout Acute Ammonia LC50 versus the Actual Ammonia Concentration Measured in MWWE Effluents.

(1) Is chemistry data predictive of biological effect?

Municipal Wastewater effluents are a complex mixture of chemicals and their composition changes with demographics (industrial, urban, residential) and effluent treatment. The samples collected for this study represent a "snapshot" of the effluent at the time of collection and may not be representative of the sewage treatments plant's typical effluent. The numbers presented in the chemistry analysis represent the average value of the two full strength (100%) effluent collections (from Days 0 and 4) for each toxicogenomic exposure.

Environment Canada   Environnement Canada   GVRD and CRD MWWE Study

Inorganic Chemistry

Metals

Metal content is an important aspect of chemistry data since some metals can be toxic to fish. Cadmium, copper and zinc are known to induce metallothionein expression in rainbow trout (Gagné, F. *et al.* 1990). Metallothionein proteins have the capacity to bind both physiological and xenobiotic heavy metals and can therefore be used as an indicator of metal exposure. The metal content was evaluated according to the aquatic life guideline levels from the Canadian Council of the Ministers of Environment (CCME) and BC freshwater aquatic life criteria. To simplify the interpretation of the chemistry results and to compare the effluents, the relative 'strength' of the effluents was ranked on a scale from high to low (Table 13). The metals were scored either one, if the metal concentration was at or above the guideline value for that metal, or zero, if the metal concentration was below the guideline concentration for that metal. The scores from each effluent were then summed and categorized as high (>5), medium (4 or 5) or low (<3) depending on the total. This categorizing was developed in consultation with BCMOE, University of Victoria and PESC and was previously used to rank metals in BC pulp mill effluents (Bruno, 2007).

TABLE 13 Ranking of Effluent Based on Metal Score. Statistically significant upregulation of metallothionein (MT1A) gene expression is denoted as +; X denotes no MT1A induction and ND indicates no data for MT1A gene expression.

(1 = at or above guideline value, 0 = below median value)

METAL	Annacis Island		Clover Point			Macaulay Point		
	Jan 2005	June 2006	June 2004	June 2005	Apr 2006	June 2004	June 2005	Mar 2006
Aluminum	1	0	0	0	0	0	0	0
Arsenic	0	0	0	0	0	0	0	0
Cadmium	1	0	0	1	1	1	1	1
Copper	1	1	1	1	1	1	1	1
Lead	1	1	1	1	1	1	1	1
Molybdenum	0	0	0	0	0	0	0	0
Nickel	1	1	0	0	0	0	0	0
Selenium	1	0	1	0	0	1	1	1
Silver	0	0	0	0	0	0	0	0
Thallium	0	0	0	0	0	0	0	0
Zinc	0	0	0	0	0	0	0	0
SUM	6	3	4	4	4	5	5	5
RANKING	high	low	med	med	med	med	med	med
Metallothionein (MT1A)	+	X	X	+	+	+	ND	+

There was considerable consistency in the metal composition in all the effluents. Clover Point and Macaulay Point MWWE ranked as medium, while the Annacis effluent ranked as high and low for 2005 and 2006 respectively.

This generalized ranking did not entirely correlate with expression of the metallothionein

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Environment Canada   Environnement Canada   GVRD and CRD MWWE Study   PESC

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gene transcript in the livers of fish exposed to the effluents (Table 13). Metallothionein was detected in fish exposed to effluents from five of the eight effluents: Annacis 2005; Clover 2005, Clover 2006; Macaulay 2004 and Macaulay 2006. Metallothionein was not detected in fish exposed to Annacis 2006 or Clover 2004. Macaulay 2005 was not examined by toxicogenomic testing due to poor quality RNA. Cadmium was the only metal common to all five metallothionein-positive effluents and not found in the two metallothionein-negative effluents.

Organic Chemistry

The organic chemistry profiles for each of the 8 effluents collected were assessed for relatedness by Euclidean distance mapping (Figure 17). The two Annacis effluents were found to be most similar while the Clover and Macaulay effluents showed a higher degree of variability between each other and over the three collection years. Most organic chemicals measured in this study were present in low amounts. The most abundant organic chemicals measured in all effluents were the fecal sterols: coprostanol, epicoprostanol, cholestanol, cholesterol, stigmasterol, campesterol, 24-ethylcoprostanol and beta-sitosterol. The composition of these sterols can be used to differentiate the source of fecal contamination from humans, herbivores, carnivores and birds (Shah, V.G. *et al.*, 2007). The NSAIDs salicylic acid, naproxen and ibuprofen were most abundant following the fecal sterols. Except for galaxolide and tonalide, most of the musks were least represented in the sewage.
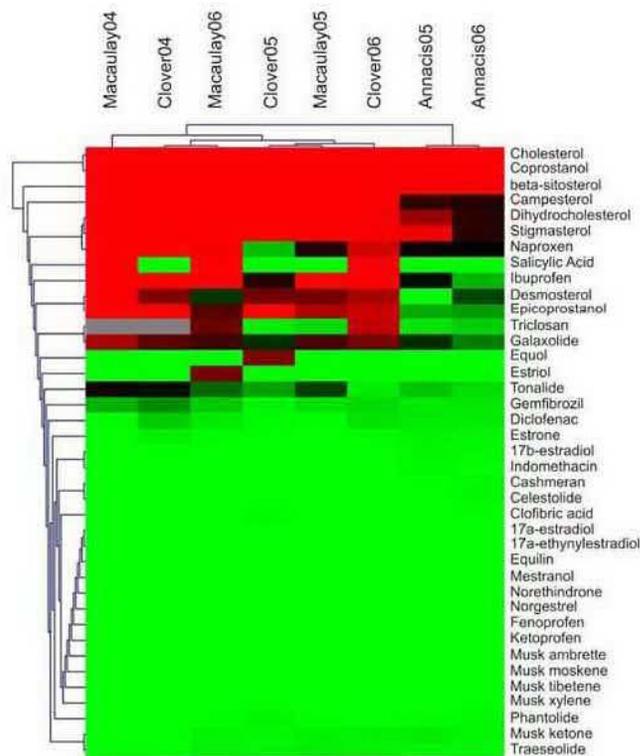
FIGURE 17 Euclidean Distance Tree for Organic Chemistry. Red denotes high abundance and green denotes low abundance.

Sterols

Sterols were analyzed because they are known to exert estrogenic effects in fish and exposure to these compounds may result in an induction of estrogenic genes in rainbow trout. One consideration is the bioavailability of the sterols since sterols in sewage may not be immediately available to the fish during the exposure. As shown in Table 14, the June 2004 effluent sampling from Macaulay had the highest concentration of β-sitosterol and the amount was quite variable between the three samplings of that effluent. In contrast, the β-sitosterol levels for both Annacis and Clover were consistent between the samplings. β-sitosterol is a phytosterol, a naturally occurring sterol in plants. Only Annacis had detectable levels of 17α-estradiol. Animal estrogens, such as 17α-estradiol and estrone, and natural phytosterols (e.g. β-sitosterol) are xenobiotics - chemicals that may be found in an organism but which are not normally produced or expected to be present in it. Equol and estriol were only measureable in the effluents collected for Clover 2005 and Macaulay 2006 respectively. Estrone was quantifiable in all Annacis and the Clover 2004 collections. Equol is an isoflavandiol, metabolized by intestinal bacterial flora, and is a nonsteroidal estrogen. Estriol is one of the three main estrogens produced by the human body.

TABLE 14 Concentrations (μg/L) of Top Estrogenic Sterols in Effluent. Estrogenicity was scored according to the number of estrogenic genes significantly upregulated in the livers of fish exposed to each of the effluents. Five gene transcripts were assessed (ESR1, VEPA, VEPB, VEPG and VTG). ND denotes no data.

STEROL	Annacis Island		Clover Point			Macaulay Point		
	Jan 2005	June 2006	June 2004	June 2005	Apr 2006	June 2004	June 2005	Mar 2006
-sitosterol	9.3	6.3	194	193	192	345	170	122
17a-estradiol	0.03	0.02	MDL	MDL	MDL	MDL	MDL	MDL
Equol	MDL	MDL	MDL	3.0	MDL	MDL	MDL	MDL
Estriol	MDL	MDL	MDL	MDL	MDL	MDL	MDL	3.0
Estrone	0.04	0.03	0.06	MDL	MDL	MDL	MDL	MDL
Estrogenicity	5	5	3	4	3[A]	4	ND	2

[A] Assessed by QGP assay, which included only 3 estrogenic markers: ESR1, VEPB, VTG.

All sewage effluents were functionally estrogenic to exposed fish, as determined by the expression of five known estrogen-responsive gene transcripts (ESR1, VEPA, VEPB, VEPG and VTG). The Annacis effluents were the most estrogenic according to both chemistry and toxicogenomic analysis. Five out of five estrogenic gene transcripts were significantly induced in response to Annacis effluent exposure. Likewise, this effluent had detectable levels of three estrogenic chemicals: β-sitosterol, 17α-estradiol and estrone. The Annacis effluent had the lowest concentration of β-sitosterol but it was also the only effluent to display a measureable amount of 17α-estradiol, which may have been a more important contributor to the overall estrogenicity of the effluent. It is also important to note that most animal estrogens are excreted as biologically inactive glucornide and sulphate conjugates. These conjugates are converted into biologically active hormones during secondary sewage treatment processes. The Annacis effluent was also the only sewage in this study to undergo secondary treatment (Belfroid, A.C. *et al*, 1999). The chemical methods used in this study did not include analysis of conjugated estrogens.

Acidic Drugs

The acidic drugs differ in their intended biological function and chemical structure and are classified as "acidic" only because they are extractable at pH 2 or less. The top acidic drugs found in MWWE were: salicylic acid, ibuprofen, naproxen, triclosan and gemfibrozil.

Three non-steroidal anti-inflammatory drugs (NSAIDs) were measured in the study: salicylic acid, ibuprofen and naproxen. Most NSAIDs act as non-selective inhibitors of

the enzyme cyclooxygenase. Cyclooxygenase catalyzes the formation of prostaglandins and thromboxane from arachindonic acid. Prostaglandins act as messenger molecules in the process of inflammation. NSAIDs are broadly characterized by their chemical structure. Aspirin is a salicylate and both ibuprofen and naproxen are 2-arylpropionic acids (profens).

Aspirin (acetylsalicylic acid) was the first-discovered member of the NSAID family. It has analgesic (pain reliever), antipyretic (fever reducer), anti -inflammatory (NSAID) and anti-platelet (anti-clotting) properties. It is also one of the most widely used medications in the world; an estimated 120 billion aspirin tablets are consumed each year (Warner, T.D. and J.A. Mitchell, 2002). As shown in Table 15, the aspirin metabolite, salicyclic acid, was not detected in Annacis effluent and was only measureable in one of the Clover collections (Clover 2006) and two out of three Macaulay collections (June 2004, March 2006).

Ibuprofen is another NSAID used for relief of symptoms of arthritis, primary dysmenorrhea, fever and as an analgesic. It also has antiplatelet activities but is relatively mild and short-lived compared to aspirin. Ibuprofen levels averaged 8.0 µg/L in both CRD effluents, which was 13.3 times higher than the amount found in Annacis Island effluent (0.6 µg/L). The highest amount was found in Clover 2006 effluent (14 µg/L) and the lowest was in Annacis 2006 effluent (0.28 µg/L).

Naproxen is an NSAID commonly used for the reduction of moderate to severe pain, fever, inflammation and stiffness. Concentrations of this drug varied between all three sites with Macaulay Point having the most on average, followed by Clover Point and Annacis Island. The highest amount was found in Macaulay 2004 (46 µg/L) and the lowest in Annacis 2006 (0.99 µg/L).

Salicylate has been shown to disrupt steroidogenic acute regulatory protein (StAR) and liver glucocorticoid receptor (NR3C1) in rainbow trout, impairing the stressor-mediated plasma cortisol response and the associated liver metabolic capacity (Gravel, A. and MM Vijayan. 2007a). Both salicylate and ibuprofen have also been shown to disrupt the heat shock response in rainbow trout (Gravel, A. and MM Vijayan. 2007b). Consequently, microarray analysis was used to assess the liver transcriptomic impact of sewage exposure on the heat shock protein gene, HSPA1A, and the stress-response genes, STAR and NR3C1. The greatest disruption was seen following exposure to Annacis 2005 sewage with all three gene transcripts showing significant downregulation in response to exposure (Table 15). The chemistry data was not predictive of this effect as the Annacis 2005 effluent had no detectable salicylic acid and low levels of both ibuprofen and naproxen. Effluent from Annacis 2006 had the second highest impact on gene expression, affecting two out of three markers. The chemistry profile for that effluent was similar to the Annacis 2005 effluent having no detectable salicylic acid and low levels of ibuprofen and naproxen. Effluent from Clover 2004 had no detectable effect on expression of any of the three marker genes and yet that effluent had higher levels of ibuprofen and naproxen than either of the Annacis effluents. It is possible that expression of the three biomarker genes is more strongly induced by low levels of NSAIDs but this

cannot be assessed in this study as dose-response exposures with pure chemical standards were not performed.

TABLE 15 Effluent NSAID Concentrations (ug/L) and Gene Expression of 3 Known NSAID-Responsive Genes: HSPA1A, STAR, NR3C1. ND denotes no data. "-" indicates downregulation and "+" indicates upregulation.

Chemical/ Gene	Annacis Island		Clover Point			Macaulay Point		
	Jan 2005	June 2006	June 2004	June 2005	Apr 2006	June 2004	June 2005	Mar 2006
Salicylic acid	MDL	MDL	MDL	MDL	37	12	MDL	15
Ibuprofen	0.95	0.28	8.5	1.6	14	6.6	5.3	12
Naproxen	1.2	0.99	5.5	0.24	4.3	46	1.6	4.8
HSPA1A	-	-		+	ND		ND	
STAR	-				ND	-	ND	
NR3C1	-	+			ND		ND	+

Triclosan is a potent wide-spectrum antimicrobial agent that inhibits the growth of bacteria, viruses and fungi. There are concerns over widespread triclosan usage and the development of antibacterial resistance (Health Canada, 2002). One of the other concerns regarding triclosan is the potential for ultra-violet conversion into the more harmful dioxin form (Son, H.S., *et al*, 2007). Triclosan was not measured in the earlier collections (Clover 2004 and Macaulay 2005) but ranged from 0.06 µg/L in Clover 2005 to 4.1 µg/L in Clover 2006, 0.12 µg/L in Macaulay 2005 to 2.7 µg/L in Macaulay 2006 and 0.09 µg/L in Annacis 2005 to 0.18 µg/L in Annacis 2006 (Table 16). In all cases, the concentration of triclosan in effluent from each of the three sites increased in the second year of testing. Triclosan concentrations increased 68-, 23-, and 2-fold in the Clover, Macaulay and Annacis effluents respectively. There are currently no known associations between triclosan exposure and gene expression in rainbow trout.

TABLE 16 Effluent Concentrations (ug/L) of Triclosan.

ACIDIC DRUGS	Annacis Island			Clover Point				Macaulay Point			
	Jan 2005	June 2006	Avg	June 2004	June 2005	Apr 2006	Avg	June 2004	June 2005	Mar 2006	Avg
Triclosan	0.09	0.18	0.14	n/a	0.06	4.1	2.1	n/a	0.12	2.7	1.4

Gemfibrozil belongs to a class of lipid-lowering drugs known as fibrates. These drugs function to increase the activity of peroxisome proliferator-activated receptor alpha

(PPARα), which increases the synthesis of lipoprotein lipase (LPL), to clear triglycerides. GEM has also been shown to increase gene expression of APOA1 (Berthou, L., *et al.* 1995) and glutathione peroxidase (GPX2) (Mimeault, C., *et al.* 2006) in goldfish liver. On average, the CRD sewage effluents contained 2.5 times more GEM than the Annacis effluent with Clover 2004 having the highest level at 0.45 μg/L. Gene expression of three fibrate-responsive gene transcripts (APOA1, LPL, GPX2) was examined by microarray analysis but no strong correlation was evident between chemical concentration and gene expression.

TABLE 17 Concentration (μg/L) of gemfibrozil (GEM) measured in the 8 sewage effluents and gene expression data for 3 known fibrate-responsive gene targets: APOA1, LPL, GPX2. ND denotes no data. "-" indicates downregulation and "+" indicates upregulation.

Chemical/ Gene	Annacis Island		Clover Point			Macaulay Point		
	Jan 2005	June 2006	June 2004	June 2005	Apr 2006	June 2004	June 2005	Mar 2006
Gemfibrozil	0.07	0.08	0.4	0.02	0.1	0.3	0.1	0.2
APOA1		-	+	-	ND	-	ND	
LPL	-				ND		ND	+
GPX2	-				ND		ND	

In all cases, the two CRD effluents had higher acidic drugs levels, ranging from as little as 2.5 times higher with the gemfibrozil (both sites) and sixteen times higher for naproxen (Macauley).

Fragrance/Musks

The two most prevalent fragrance/musk compounds, galaxolide and tonalide, are shown in Table 18. Polycyclic fragrance/musks are more chemically generic than acidic drugs and are typically used as scent components for many personal care and cleaning products. Consequently, their release into the aquatic environment is primarily through STP discharges. Musks tend to accumulate in the fatty tissues and have been shown to bioaccumulate in fish (Gatermann et al., 1999). Galaxolide is the most commonly used product followed by tonalide. The concentrations of both musks did not differ dramatically between the CRD and GVRD effluents. Compared to Annacis Island effluent, galaxolide was three and four times greater in Clover and Macauley respectively, while tonalide was 2.5 and 4 times greater. Macaulay 2004 had the highest amount of these two chemicals (3.7 μg/L galaxolide and 1.25 μg/L tonalide) and Annacis 2006 had the least of either chemical (0.51 μg/L galaxolide and 0.14 μ/L tonalide). The effect of either musk on gene expression in the rainbow trout is largely unknown. There is some evidence to suggest an anti-estrogenic effect (Schreurs, R.H. *et al.* 2004). However, with respect to sewage exposure, any anti-estrogenic activity is effectively masked by the overwhelming estrogenic character of the mixture.

TABLE 18 Effluent Concentrations (ug/L) of Galaxolide and Tonalide.

Musks	Annacis Island	Clover Point	Macaulay Point

	Jan 2005	June 2006	Avg	June 2004	June 2005	Apr 2006	Avg	June 2004	June 2005	Mar 2006	Avg
Galaxolide	0.83	0.51	0.7	2.5	0.825	2.86	2.1	3.7	2.19	2	2.6
Tonalide	0.23	0.14	0.2	0.95	0.37	0.06	0.5	1.25	0.76	0.6	0.9

The chemical detection of known estrogens can be predictive of a biologically-relevant estrogenic effect but the chemistry data was not predictive of a biological response to metals, NSAIDs or fibrates. Because sewage is a complex mixture, analysis of individual components may not be entirely useful in predicting the impact of the effluent on a living organism. Therefore, the relatedness of the organic chemistry profiles was compared to the relatedness of the gene expression profiles using Euclidean distance mapping (Figure 18A).
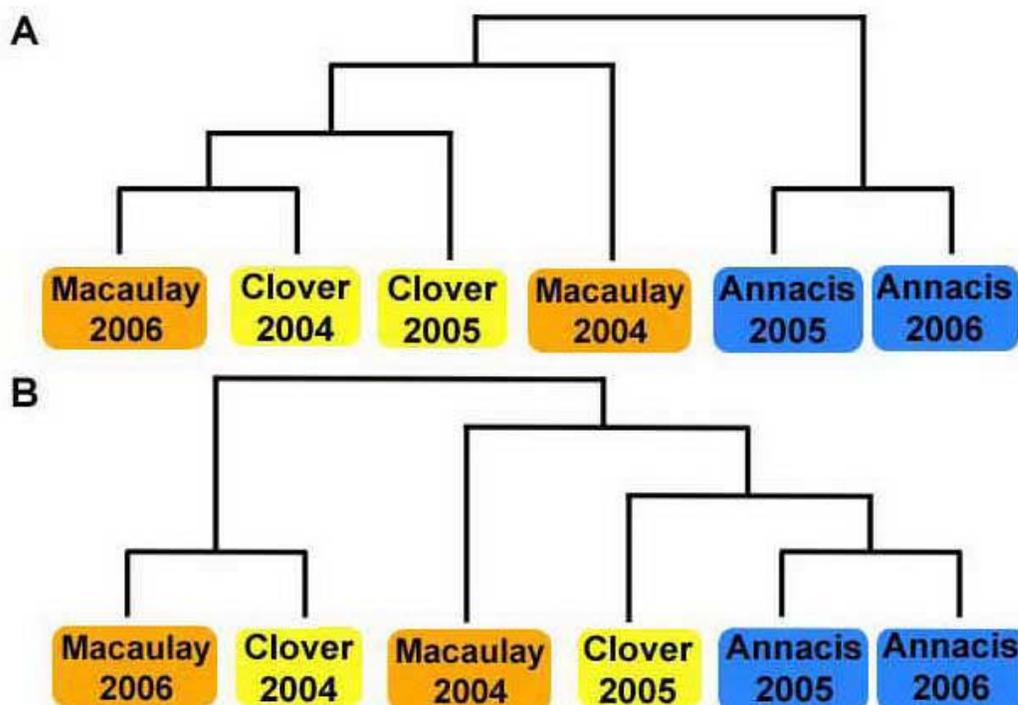

FIGURE 18 Euclidean distance tree of (A) organic chemistry data and (B) gene expression profiles.

Transcriptomic data was not available for Macaulay 2005 or Clover 2005 and therefore those effluents were not included in generating the chemical Euclidean distance tree shown in Figure 18A. The Annacis effluents were most chemically similar to each other, while the CRD effluents showed no site-specific or time-dependent similarity. Clover 2004 was most similar to Macaulay 2006 while Clover 2005 was most similar to Macaulay 2005 and Clover 2004/Macaulay 2006. Gene lists describing the genes responding in a statistically significant manner to effluent exposure were likewise compared for each of the six conditions (Clover 2004, Clover 2005, Macaulay 2004, Macaulay 2006, Annacis 2005, Annacis 2006). Gene expression data clustered in a similar manner to the chemical data with the two Annacis effluents showing greatest

similarity and the CRD effluents showing no site-specific or time-dependent similarity (Figure 18B). The Macaulay 2006 effluent was most similar to the Clover 2004 effluent, while the Clover 2005 effluent was most similar to the two Annacis effluents and the Macaulay 2004 effluent was most similar to Clover 2005 and the two Annacis effluents. Both chemistry and gene expression data showed a high degree of similarity between batches of Annacis effluent collected during two separate calendar years and between Macaulay 2006 and Clover 2004 effluents. Gene expression data and chemistry data correlated for 4 of the 6 conditions.

(2) Does sewage exposure cause measurable changes in gene expression within the liver of exposed salmonids?

Gene Classes

The microarray was designed as a screening tool and therefore contained select genes from significant molecular pathways and not all genes in a pathway. The selected genes were from main biological processes and categorized into 11 gene classes (ordered from the least to the most genes represented on the microarray): embryogenic, housekeeping, oncogene, transcription, proteolysis, structural, signal transduction, immune, binding/transport, endocrine and metabolism. Using a targeted microarray, a relatively large proportion of the genes was expected to change which was indeed the case; the percentage of genes changing in an exposure ranged from 35% (Macauley 2006) to 71% (Macaulay 2006) (Figure 19). The two Annacis effluents affected 49 and 60% of genes on the array for the 2005 and 2006 effluents, respectively, while the 2 Clover effluents affected 38 and 54% for the 2004 and 2005 effluents, respectively.

Clover effluent affected a similar percentage of genes at each timepoint (Figure 19A). Clover 2004 affected 73 genes in total and Clover 2005 affected 102 genes. Both batches of effluent affected roughly 40% of those responsive genes at each timepoint. The Macaulay effluents were more variable with the 2004 effluent affecting 131 genes and the 2006 effluent affecting 65 genes. More genes (66%) responded to the Macaulay 2004 effluent at day 4 compared to the response at day 1 (44%) and day 8 (32%). Response to Macaulay 2006 effluent differed in that the day 8 exposure affected the greater percentage of genes (61%) compared to day 1 (33%) and day 4 (27%). The two Annacis effluents both affected a similar number of genes (92 and 114 for Annacis 2005 and Annacis 2006, respectively) and both showed a time-dependent increase in gene expression. Annacis 2005 effluent affected only 23% of responsive genes at day 1, increasing to 75% by day 8. Annacis 2006 effluent affected 39% of responsive genes at day 1, increasing to 52% by day 8.

The two Clover effluents showed slight dose-responsiveness with the percentage of responsive genes increasing from 29% and 27% for the lowest doses of Clover 2004 and 2005 effluents, respectively, to 42 and 44% for the highest doses of each effluent (Figure 19B). The two Macaulay effluents were more variable with the fourth highest dose (2%) of the Macaulay 2004 effluent affecting the greater percentage of responsive genes (50%) and the 2nd highest dose (0.15%) of the Macaulay 2006 effluent affecting 56% of the

responsive genes. The two Annacis effluents were equally variable with the lowest dose of Annacis 2005 (1%) affecting the greatest percentage of responsive genes (57%) and all doses of Annacis 2006 affecting a similar percentage of genes (31-44%).
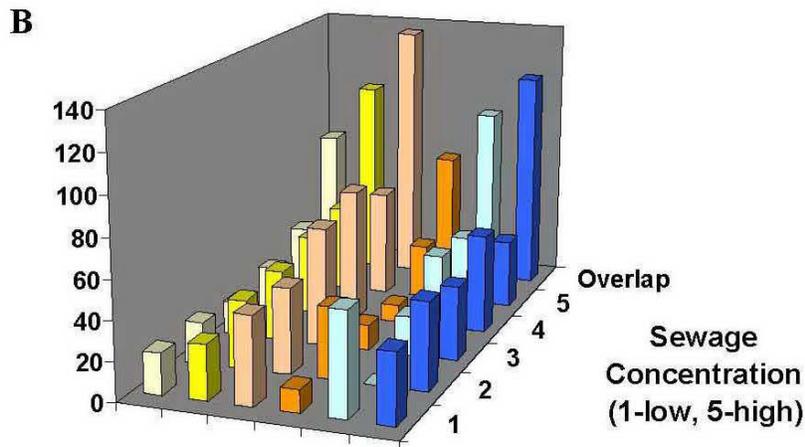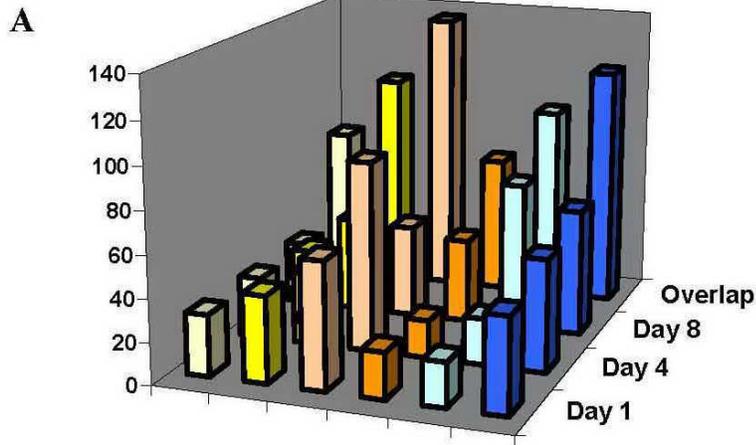
Between all 6 exposures, 98% of the genes responded at some timepoint and
concentration (Figure 20); however, most of these genes did not appear consistently
across exposures.

The gene classes were not equally represented on the array; therefore, it might be
expected that the classes most affected by sewage exposure would reflect their relative
abundance on the array. The top five gene classes on the array included: metabolism
(24%), endocrine (22%), binding/transport (17%), immune (11%) and signal transduction
(10%) (Figure 20). The same five classes were affected by sewage exposure proportional
to their representation on the array between all 6 exposures (2 years, 3 sites); however,
the proportion of genes from each class varied for each site and date of exposure (Figure
21).
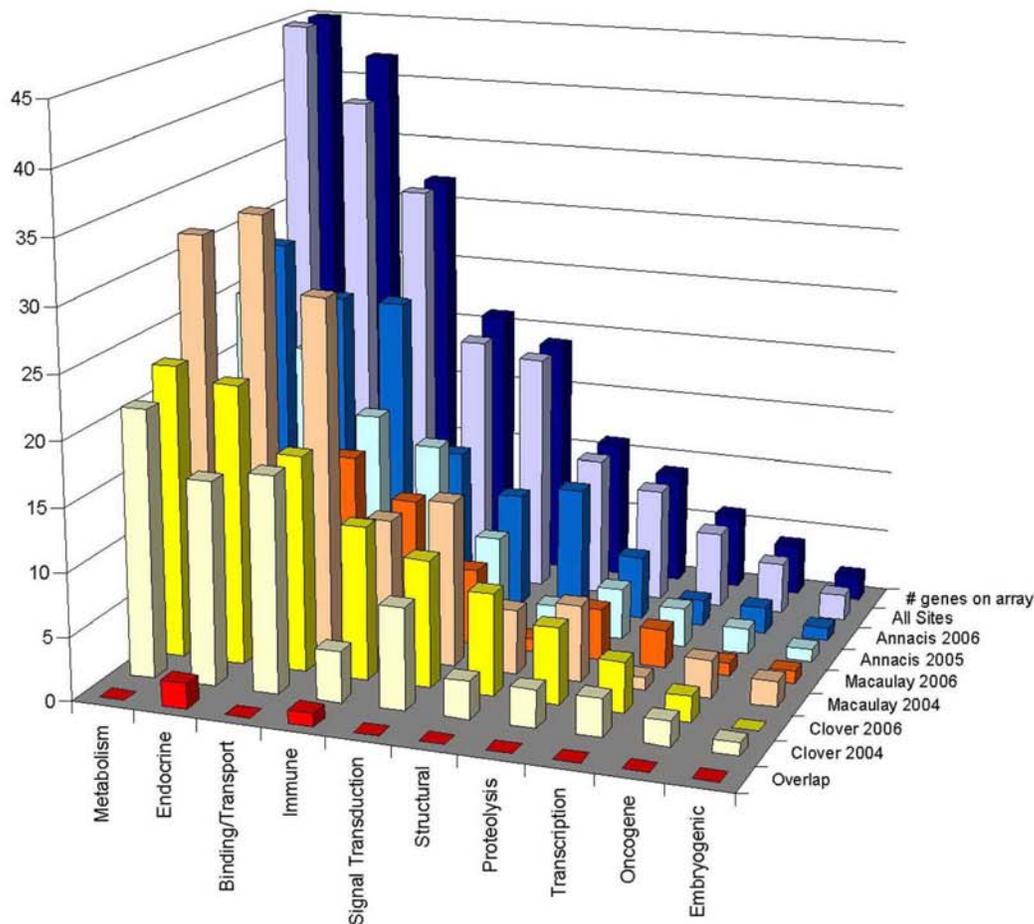
FIGURE 20 Representation of the Gene Class Distribution on the Array and the Impact of
Sewage Exposure on the Expression of Genes within those Classes.

For Clover 2004, 73 genes responded to sewage exposure at some time point and concentration during the exposure. The greatest proportion of those genes derived from the metabolism class (26%), while 22% of the genes derived from the binding/transport class, 19% from endocrine, 11% from signal transduction and 5% from immune. Clover 2005 differed from the 2004 exposure in that 102 genes were altered at some point during the exposure with 22% from the metabolism class, 21% from the endocrine class, 17% from binding/transport, 12% from immune and 10% from signal transduction. Thirty-eight genes commonly responded to exposure to Clover effluent from both years (overlap). The greatest proportion of these genes derived from the metabolism class (26%), 18% from the endocrine class, 16% from binding/transport, 11% from signal transduction and 8% from the proteolysis class. Genes from the proteolysis class represent only 5% of the genes on the array, gene class 7 out of 11.

For Macaulay 2004, 131 genes responded to sewage exposure with 26% of those genes deriving from the endocrine class, 23% from the metabolism class, 19% from binding/transport, 10% from signal transduction and 8% from immune. The Macaulay 2006 exposure differed dramatically with only 65 genes responding to exposure and 22% of those genes deriving from the binding/transport class, 20% from metabolism, 18% from endocrine, 15% from immune, and 9% from signal transduction. Forty-nine genes responded to Macaulay effluent during both years of exposure with 32% of those genes deriving from the binding/transport class, 26% from the endocrine class, 21% from metabolism, 16% from immune, and 11% from proteolysis.

Response to the Annacis effluents was most consistent between the two disparate years of exposure. Ninety-two genes responded to Annacis 2005 effluent, with 25% of genes from the metabolism class, 23% from endocrine, 16% from binding/transport, 15% from immune, and 8% from signal transduction. The first 4 gene classes were conserved between Annacis 2005 and Annacis 2006 exposures. Exposure to Annacis 2006 effluent affected 114 genes with 23% of those genes deriving from the metabolism class, 21% from endocrine, 20% from binding/transport, 11% from immune, and 9% from structural. The structural genes make up 6% of the total microarray, gene class 6 of 11. Sixty-three genes commonly responded to Annacis effluent between the two years of exposure with 27% from the metabolism class, 22 % from endocrine, 19% from binding/transport, 14% from immune and 5% from proteolysis. The proteolysis gene class was the fifth most commonly affected gene class for each of Clover, Macaulay and Annacis overlaps, while never appearing within the top five for any of the single exposures, suggesting a moderate, but consistent response within this class across effluents.
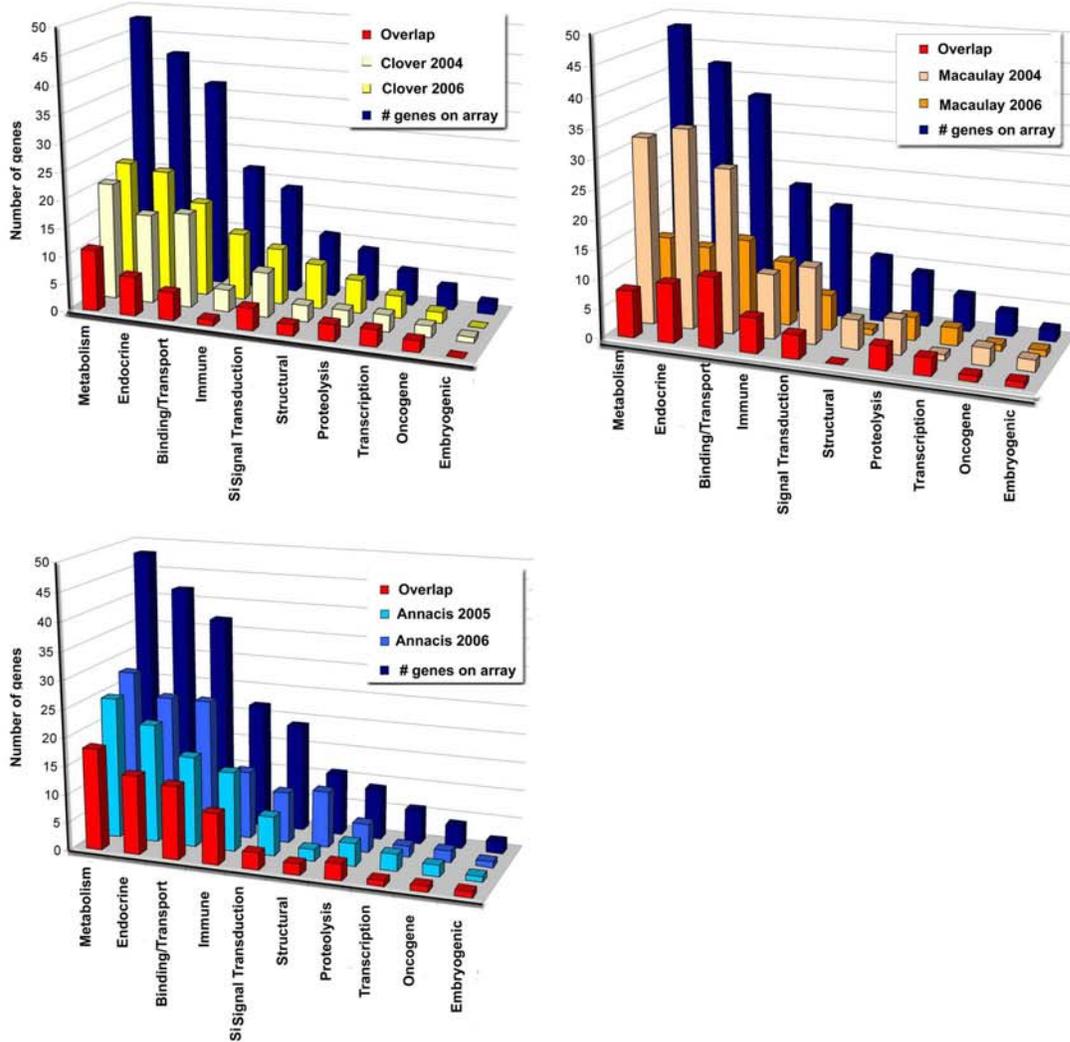
FIGURE 21 Impact of Sewage from Clover, Macaulay and Annacis on Fish Liver Gene Expression, by Class.

Gene responses within the top five classes have been shown in previously published studies of fish exposed to MWWE. In 2008, Garcia-Reyero *et al* showed that the gonads of fathead minnow exposed to water downstream of a water treatment plant had significant changes in transcript levels for 33 GO category biological processes, including signal transduction, five classes relating to metabolism, and four classes relating to immune response. Wang *et al* (2007) saw alterations in liver gene expression for binding/transport, metabolism and immune gene classes in goldfish resident to a lake receiving MWWE. The endocrine response was also expected, considering the amount of previously published data documenting fish endocrine responses to sewage exposure in other geographic regions (Hansen, 1998; Nichols, 1998; Larsson, 1999; Aerni, 2004; Jobling, 2004; Burki, 2006).

The gene class representation for each site and date of sewage collection was remarkably similar (Figure 22). There was no obvious bias in classes between either the location or collection date. No location generated a unique gene class "fingerprint". However, each site did generate a list of genes that were unique for each location (Figure 23). Exposure to Annacis sewage affected 63 genes with 35 of those genes unique to the Annacis effluent. Exposure to Clover sewage affected 38 genes with 19 of these genes unique to the Clover effluent. Exposure to Macaulay sewage affected 49 genes with 25 of those genes unique to the Macaulay effluent. Three genes were common to all three sites.
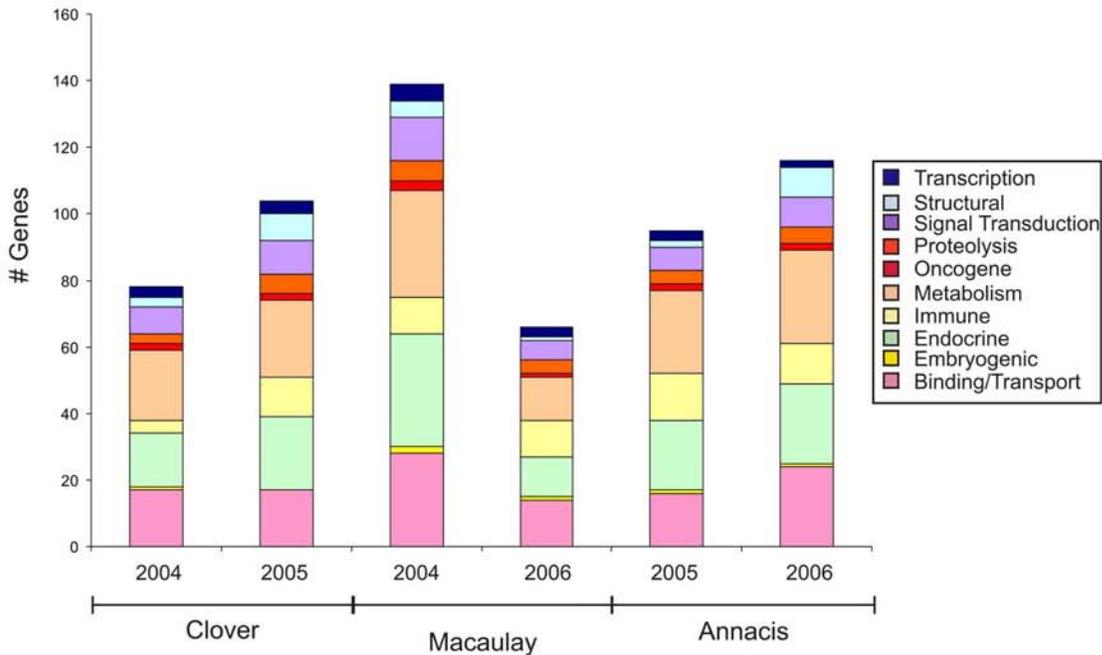

**FIGURE 22**   Distribution of Gene Classes Between Sites.
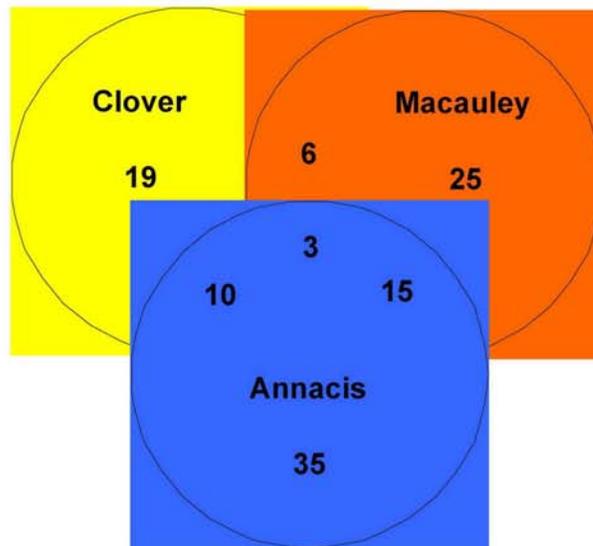
FIGURE 23   Genes Affected By Sewage Exposure Between Sites.

While the immune, signal transduction and binding/transport classes were most affected by sewage exposure, specific genes within the endocrine and metabolism classes were more consistently affected across most exposures (Table 19). Three genes (C1R, GNRHR2, and VEPB) were significantly altered by exposure to all six sewage effluents and 16 genes were significantly altered in five out of six sewage exposures. These genes constitute possible biomarker candidates whose expression should be verified by QPCR or QGP analysis.

TABLE 19 Genes Consistently Significantly Affected by Sewage Exposure. Genes highlighted in red were validated by QPCR analysis (see Figure 24).

Gene Name	Gene Class	Count	Not Sig In
GNRHR2	Endocrine		
VEPB	Endocrine	6/6	
C1R	Immune		
ATP1A1C	Binding/Transport		Clover 2005
TF	Binding/Transport		Clover 2004
VLDLR	Binding/Transport		Clover 2005
THRA	Endocrine		Clover 2005
VEPA	Endocrine		Macauley 2006
VEPG	Endocrine		Macauley 2006
BF	Immune		Clover 2004
MHC1	Immune	5/6	Clover 2004
CYP1A2	Metabolism		Macauley 2004
CYP2K1	Metabolism		Macauley 2006
GLUD1	Metabolism		Macauley 2006
GLUL3	Metabolism		Clover 2005
HSD3B1	Metabolism		Annacis 2005
KRAS	Oncogene		Annacis 2006
HSPA8	Signal transduction		Annacis 2006
HSPCA	Signal transduction		Annacis 2006

To date, QPCR and QGP have been used to validate the expression of the estrogenic genes: VEPA, VEPB, VEPG, VTG, and ESR1. The microarray was the least sensitive platform, identifying significant changes in gene expression 10 times over three days, while QPCR identified 40 changes and QGP identified 24 (Figure 24). Most significant changes in gene expression occurred at day 4 with microarrays identifying 7 changes, QPCR identifying 12 changes and QGP identifying 13 changes. The fewest changes occurred at day 1, with the microarray identifying 3 changes, QPCR identifying 12 changes and QGP identifying 3 changes. VEPA was most frequently identified as significantly altered by sewage exposure. VEPA was identified 3 times by microarray analysis, 12 times by QPCR analysis and 7 times by QGP analysis. VEPB was the second most frequently identified target with 3 identifications by microarray analysis, 11 by QPCR and 6 by QGP. VTG, VEPG and ESR1 were sporadically identified. VTG was never identified by microarray or QGP, but was identified 10 times by QPCR. VEPG was not detected on day 1 and day 4 by QPCR, but was identified by microarray and QGP on day 4. ESR1 was most identified by QGP (5 times), while it wasn't

identified by microarray at all and only identified 3 times by QPCR. Based on three toxicogenomic platforms, VEPA and VEPB are the best estrogenic indicator genes for inclusion in an endocrine biomarker screening panel.
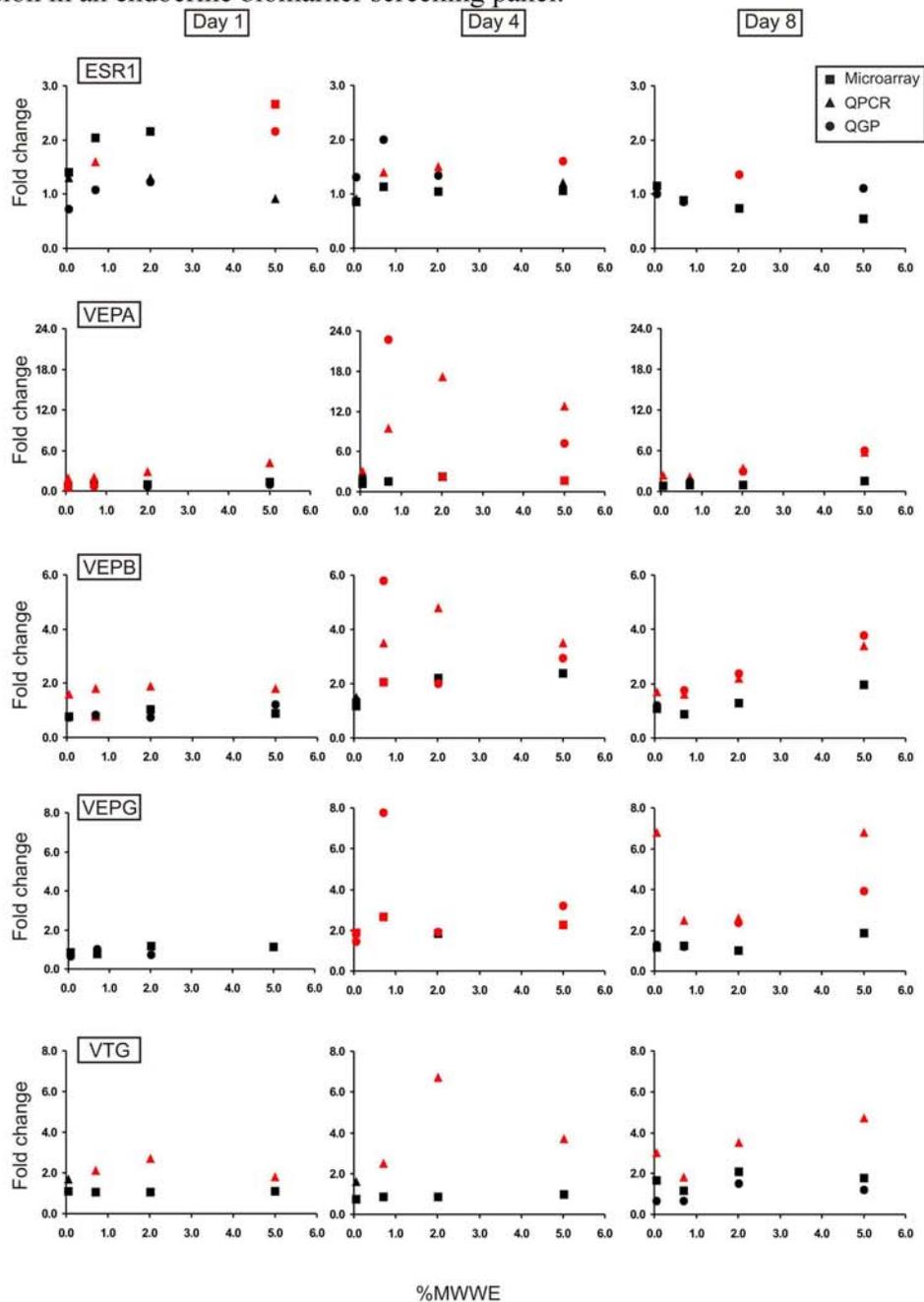

FIGURE 24 Correlation between Microarray, QPCR and QGP Data. Gene expression data for Clover 2005 day 1, day 4 and day 8 exposure for the estrogen-response genes ESR1, VEPA, VEB, VEPG, and VTG. Red symbols are significantly different from control levels (p<0.05, Welch T-test).

(3) Are the gene expression changes permanent?

Sewage exposure caused alterations in many different gene targets in the salmonid liver, but the importance of those changes is tempered by their longevity. As shown in Figure 25, most genes recovered to baseline levels after an eight day depuration, ranging from 55.2% of genes recovering from exposures to Annacis effluent in 2006, to 82.9% of genes recovering from exposure to Macaulay Point effluent in 2006 (Day 16). Macaulay 2004 is not shown as there was no depuration phase associated with that exposure. The text needs to match the figure, also add newly infront of altered in figure. Y-axis should be % genes.

	Clover 2004	Clover 2005	Macauley 2006	Annacis 2005	Annacis 2006
■ Recovered	69	97	64	86	98
■ Did not recover	6	11	1	15	25
■ Altered	38	28	22	23	22
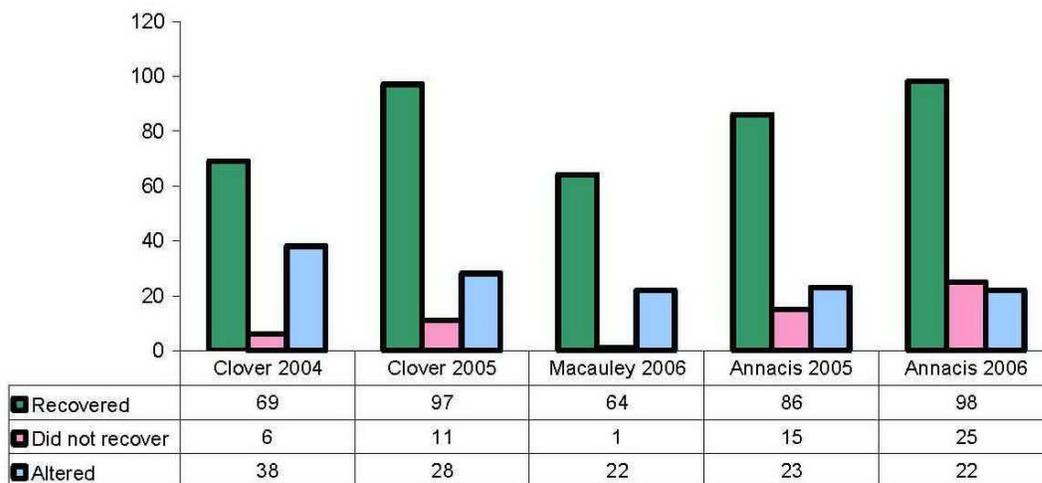
FIGURE 25 Gene Transcript Recovery at Day 16.

Most genes recovered. The percentage that failed to recover in at least one concentration ranged from 0.6% for Macaulay 2006 to 19.8% for Annacis 2006. All but one gene (CRH) recovered in both Clover exposures, while four genes (BF, CYP1A, RBP4, VEPB) failed to recover in both Annacis exposures. Only RHO did not recover in the Macauley 2006 exposure. A greater percentage of genes were newly altered in at least one concentration at the day 16 timepoint, ranging from 16.4% for Clover 2005 to 31.9% for Clover 2004. Few genes were consistently changed at day 16 alone (Figure 26). At the day 16 timepoint, five genes altered following exposure to both Clover effluents, eight genes were altered following exposure to Macaulay 2006 effluent and six genes

were commonly altered after exposure to both Annacis effluents.
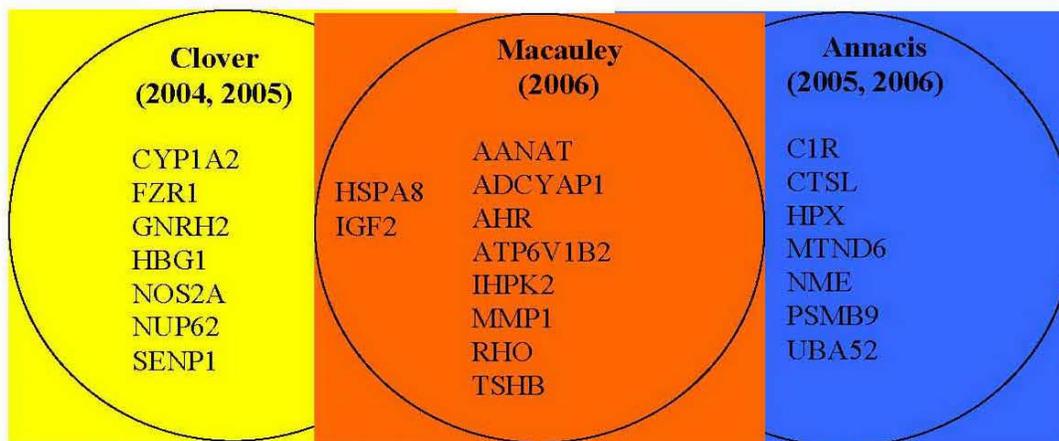
FIGURE 26 Genes that were newly altered in at least one concentration of each experiment at day 16. Clover: 9 genes; Macauley: 13 genes; Annacis: 10 genes.

(4) Does the lowest sewage concentration cause significant changes in gene expression?

The sewage concentrations used in these exposures ranged from environmentally-relevant to supra-environmental. The high concentrations were tested to clearly identify and characterize the effects of sewage on fish, while the low concentrations were tested to determine if fish in the wild could be affected by exposure to sewage discharge. In almost every case, the highest concentration in an exposure series induced the greater response in fish tissue; however, the lowest concentrations also affected a large number of genes (Figure 27). The Annacis 2005 effluent did not follow this trend, with the lowest concentration affecting 55 genes and the highest concentration affecting only 38 genes. Gene class representation between low and high concentration exposures within a site was often similar. However, some gene classes had increased representation following exposure to the higher sewage concentration. The proteolysis and endocrine gene classes were most consistently affected by the high concentration of sewage with four of the six exposures showing increased representation in the high dose vs the low dose. By contrast, the low dose affected more genes in the immune and signal transduction classes with four of the six exposures showing increased representation.
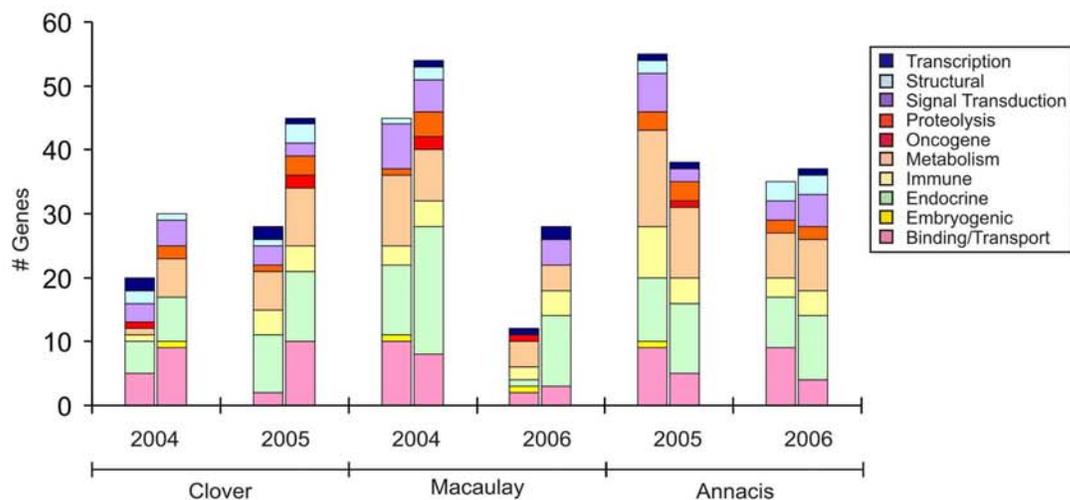
FIGURE 27 Class Distribution for Genes Responding to Low and High Concentrations of Sewage.

The genes responding to low levels of sewage were distinct from the genes responding to high levels, with few overlapping genes. Table 20 describes the number of genes shared between low and high concentration exposures within an exposure, the number of genes unique to each concentration and the relative percentage of each. Macaulay 2006 had the lowest correlation between low and high exposure gene lists, with only 3% commonality. By contrast, Macaulay 2004 had the greatest correlation between low and high exposure gene lists with 22% commonality.

TABLE 20 Comparison of Gene Lists from Fish Exposed to the Lowest and Highest Sewage Concentrations Used in this Study.

Site/date	# Shared Genes	# Unique Genes	Total # Genes	% common	% unique
Clover2004 low	7	36	43	16%	84%
Clover2005 high	13	50	63	21%	79%
Macaulay2004 low	18	64	82	22%	78%
Macaulay2006 high	1	38	39	3%	97%
Annacis2005 low	14	69	83	17%	83%
Annacis2006 high	8	56	64	13%	88%

Five genes were consistently affected by exposure to low levels of Clover effluent for both years of study (FGF6, GNRHR2, MYH1, MYOD1, VEPA); four genes (CBLN4, GLUL2, TF, MT1A) responded to low level exposure to Macaulay effluent; and 12 genes (ATP1A1C, BF, CCK, DBT, DIO2, HSPA1A, MMP1, MYH6, NR3C1, PPARB, SLC2A4, TF) responded to low levels of Annacis effluent. There were no genes common to both concentrations across all three sites (Figure 28). More genes were consistently affected by exposure to high levels of effluent within all three sites. Nine genes

(CYP2K1, GNRHR2, HBA1, HBB, MMP1, RHAG, VEPA, VEPB, VEPG) responded to exposure to high levels of Clover effluent; 10 genes (CYP2K4, DRD2, GLUL2, GNRH1, GNRHR2, INSRA, NR3C1, SLC5A1, SLC8A1, VEPB) responded to high levels of Macaulay effluent and 13 genes (CYP1A1, CYP1A2, CYP2K1, GLUL3, PKLR, MHC1, TF, THRA, THRB, VEPA, VEPB, VEPG) responded to high levels of Annacis effluent. One gene was common to Clover and Macaulay alone (GNRHR2); three genes were common to Clover and Annacis alone (VEPA, VEPG, CYP2K1) and one gene was common to all three effluents (VEPB).
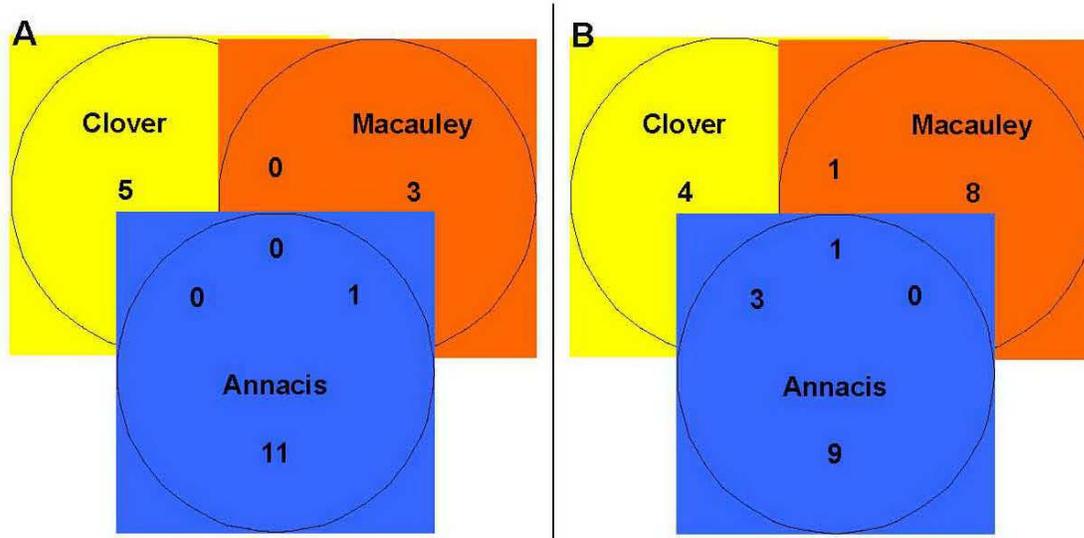

FIGURE 28 Genes Common to Each Location Following Exposure to either Low (A) or High (B) Sewage Concentration.

There were only two genes (GNRHR2 and VEPA) common between low and high exposure gene lists for both Clover 2004 and Clover 2005; only one gene (GLUL2) common between all four Macaulay lists; and only one gene (TF) common between all four Annacis lists (data not shown).

(5) Are any gene expression changes dose-responsive?

Gene dose-responsiveness was assessed by trend analysis (linear regression $R^2 > 0.8$) when assessing gene expression fold change as a function of sewage concentration (Table 21). Dose-responsiveness was further classified as either directly proportional (D) or inversely proportional (I) to sewage concentration. There was no obvious trend regarding proportionality. Within each site, the percentage of genes responding directly or inversely varied from year to year. The number of genes responding in a dose-responsive manner also varied between years. Exposure to Annacis sewage generated the greatest number of genes responding in a dose-responsive manner with 31 genes in 2005 and 14 genes in 2006. Only two of those genes (PPARB and VEPB) responded in both 2005 and 2006. There were no genes common between the two Clover exposures or the two Macaulay exposures.

TABLE 21 Genes Responding to Sewage Exposure in a Dose-Responsive Manner. %D indicates the percentage of genes whose response was directly proportional to

sewage concentration and %I denotes the percentage of genes whose response was inversely proportional to sewage concentration.

Site	Year	# Genes	%D	%I
Clover	2004	5	40%	60%
	2005	15	67%	33%
Macaulay	2004	20	75%	25%
	2006	2	50%	50%
Annacis	2005	31	35%	65%
	2006	14	71%	29%

There was little correlation between dose-responsiveness and gene class; however, the endocrine class showed a greater dose-responsiveness frequency across all sites with a 25% response-rate (Table 22). The immune, metabolism, signal transduction and binding/transport classes all had similar representation with 17%, 16%, 14% and 13%, respectively. The proteolysis, housekeeping, oncogene and transcription classes showed the least dose-responsiveness with 6%, 3%, 2% and 2%, respectively.

TABLE 22   Gene Class Distribution for Dose-Responsive Genes.

Class	Clover		Macaulay		Annacis		Total	%
	2004	2005	2004	2006	2005	2006		
	0	0	1	0	0	1	2	2%
Signal transduction	3	1	2	0	3	3	12	14%
Proteolysis	0	1	1	0	2	1	5	6%
Oncogene	0	1	1	0	0	0	2	2%
Metabolism	1	1	2	0	9	1	14	16%
Immune	0	3	4	2	4	2	15	17%
Housekeeping	0	1	1	0	1	0	3	3%
Endocrine	0	6	6	0	6	4	22	25%
Binding/Transport	1	0	2	0	6	2	11	13%
# Genes	5	15	20	2	31	14	87	100%

(6) Does sewage differentially affect gene expression in males as compared to females?

QPCR analysis was conducted to determine if there was any gender bias in gene expression of the estrogen-responsive genes VEPA, VEPB, VEPG, VTG, and ESR1. QPCR was used for this assessment rather than microarray analysis as there was an insufficient sample size to compare gender-based response by microarray analysis. Due to the labour-intensive nature of QPCR analysis, only a small subset of genes was assessed. QPCR analysis was conducted on samples from Clover 2005 (Table 23). The male and female fish responded differently to sewage exposure; although, that difference

was rarely statistically significant. Yellow-coloured cells denote gene expression changes that are statistically different (Welch T-test, $p < 0.05$) from gene expression in gender-matched fish exposed to control seawater. All significant changes in gene expression were positive (i.e. gene transcripts were upregulated). Both genders showed a similar number of significant changes in gene expression when exposed to the Clover 2005 sewage. Gene expression in females was significantly altered from control levels in 31 of 56 instances and male gene expression was altered in 29 of 56 instances. Males showed more significant gene changes on day 1 than females, having 10 significant gene responses as compared to the six changes seen in female fish. By day 4, both genders showed equal impact with each having 10 significant changes in gene expression. At day 8, female fish had 9 significantly altered genes while male fish had 7 gene alterations. Overall, most significant changes in gene expression occurred at day 4 with 20 significantly altered genes between the two genders while both day 1 and day 8 each had 16 alterations. Cells outlined in red indicate gene expression changes that were statistically significant between genders. There were only two out of 56 cases where gene expression differed between genders: VTG at day 8 for 5% sewage and VEPA at day 1 for 5% sewage.

TABLE 23   QPCR Fold Change Data for VTG, VEPA, VEPB, VEPG, and ESR1 in Fish Exposed to Clover 2005 Effluent for 1, 4 and 8 Days. Cells outlined in red denote expression levels that are significantly different ($p < 0.05$) between male and female fish. Cells highlighted in yellow denote gene expression changes that are significantly different ($p < 0.05$) from control expression levels. ND = not detected.

	%MWWE	Day 1		Day 4		Day 8	
		F	M	F	M	F	M
VTG	0.05	1.7	1.5	1.4	2.1	2.1	5.0
	0.7	1.2	3.6	2.2	3.0	1.8	1.8
	2	4.6	2.5	7.5	7.5	3.1	4.5
	5	1.6	2.1	3.7	4.0	2.3	6.9
VEPA	0.05	1.8	2.1	2.5	4.5	2.5	2.4
	0.7	2.0	2.1	6.8	15.6	1.8	3.0
	2	3.2	2.6	23.9	16.6	2.5	5.0
	5	2.4	4.9	10.8	18.1	4.3	8.0
VEPB	0.05	1.2	2.1	1.3	2.0	2.1	1.3
	0.7	1.5	2.2	2.8	4.9	1.7	1.5
	2	1.5	2.5	6.3	4.8	1.8	2.5
	5	1.0	2.6	3.3	4.1	3.7	3.3
VEPG	0.05	ND	ND	0.4	0.7	8.1	4.7
	0.7	ND	ND	0.6	1.4	2.3	2.7
	2	ND	ND	1.0	1.4	3.4	1.8
	5	ND	ND	1.1	1.4	8.6	6.3
ESR1	0.05	1.5	2.2	1.2	1.2	2.0	1.3
	0.7	1.6	3.1	2.3	2.0	2.0	1.4
	2	2.3	3.1	2.5	1.7	1.6	1.6
	5	1.8	4.0	2.1	1.5	1.0	1.1

Discussion

Start with STP
Exposure
Toxicogenomic approach
Discussion of results, cover off these points
1-chemistry predictive of biology?
2- sewage impact on gene expression
3- permanence of gene expression changes
4- impact at environmentally relevant concs?
5- dose reponse?
6- gender bias.

3- permanence of gene expression changes
Most gene expression changes returned to baseline levels after an eight day depuration phase (fig 25)