PROTEOMIC RESPONSES IN THE GILL OF ZEBRAFISH FOLLOWING EXPOSURE TO IBUPROFEN AND NAPROXEN

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Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most abundant environmental pharmaceutical contaminants. In this study, a proteomic analysis was conducted to identify proteins differentially expressed in gill tissue of zebrafish (Danio rerio) after a 14-day exposure to the NSAIDs ibuprofen or naproxen. A total of 104 proteins with altered expression as indicated by 2-dimensional electrophoresis were analyzed by liquid chromatography with ion trap mass spectrometry (MS/MS). A total of 14 proteins fulfilled our requirements for identification which included consistency among replicate gels as well as successful MS/MS ion searches with the MASCOT database. The most prominent feature of the differential protein expression observed after NSAID exposure was an up-regulation of proteins belonging to the globin family which are involved in the transport of oxygen from gills and availability of heme molecules required for synthesis of cyclooxygenase. Differential expression was observed at exposure concentrations as low as 1-10 µg/L indicating that altered gene expression may occur in fish subjected to environmentally realistic levels of NSAID exposure.

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ABBREVIATIONS

2-DGE	2-D gel electrophoresis
AA	Arachidonic acid
ACN	Acetonitrile
CID	Collision-induced dissociation
COX	Cyclooxygenase
EPA	Eicosapentaenoic acid (20:5n-3) Or 5, 8, 11, 14, 17 eicosapentaenoic acid
ESI	Electrospray ionization
FA	Formic acid
HETE	Hydroxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized protein gradient
LC	Liquid chromatography
MS	Mass spectrometry
NSAID	Non-steroidal anti-inflammatory drugs
PG	Prostaglandin
pI	Isoelectric point
TX	Thromboxanes

CHAPTER 1

OVERVIEW

Non-steroidal anti-inflammatory drugs (NSAIDs) comprise a widely used class of therapeutic agents that target proteins of various kinds with an ultimate objective of reducing pain with few side-effects (McGettigan and Henry 2000) . Identification and characterization of differential abundance of proteins in association with drug metabolism provide clues for better understanding of the mechanisms of the drug's actions, side effects and possible risks associated with widespread environmental release. The primary objective of this research was to establish a base proteomics capability within the University of North Texas aquatic toxicology group by examining alterations of protein expression in the zebrafish, a model aquatic vertebrate. Our general aims were (1) examine 2-D protein profiles from the gills of zebrafish exposed to varying concentrations of ibuprofen or naproxen (2) identify the proteins that are significantly over-expressed or under-expressed, and (3) interpret the possible significance of the altered expression patterns in terms of known properties of protein function, derived largely from mammalian literature, and potential risks for environmental exposure of aquatic vertebrates.

The main body of the dissertation is organized in 5 chapters. Chapter 2 introduces the background and scope of this research. The biology of NSAIDs in general, and ibuprofen and naproxen in specific, are introduced in context of human pharmacology. The role of the zebrafish as a model organism is briefly described. Finally, techniques for protein extraction, separation, quantification and identification are reviewed.

Chapter 3 explains the experimental design, materials and methods in detail. The methods include exposure of zebrafish, isolation of gills and extraction of proteins, 2-D gel

electrophoresis, and comparison of gel images using PDQuest software, tandem mass spectrometry and the protein identification.

Chapter 4 presents the results of the alteration in protein expression seen with exposure to various concentrations of the NSAIDs ibuprofen and naproxen. Representative 2-D gel images of control and exposed gill tissue proteins in 4-7 and 7-10 pI ranges are presented along with summaries of total numbers of protein spots observed, numbers significantly altered in expression and results of tandem mass spectrometry protein identifications. Where available, function of identified proteins is provided.

Chapter 5 discusses possible biological implications of the changes in abundances of the proteins identified using proteomic tools and reference section lists the references cited.

CHAPTER 2

BACKGROUND

2.1 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Human defense mechanisms are activated upon exposure to potentially harmful agents, such as pathogens and chemicals. The result is mobilization of cells belonging to the immune system and production of inflammatory intermediates which not only aids in eliminating the infecting agents but also causes swelling and discomfort. Various types of immunosuppressant and anti-inflammatory drugs, including glucocorticoids and anti-histamines, have been developed over the years to alleviate these symptoms. NSAIDS are, however, the most widely used anti-inflammatory drugs today (National Prescription Audit (2000)). Since the synthesis of acetylsalicylic acid and the discovery of its anti-inflammatory actions in the late nineteenth century, a large number of new NSAIDs have been developed. The NSAIDs are non-narcotic and possess not only anti-inflammatory, but also analgesic and anti-pyretic activity (fever reduction) and are used to reduce pain, fever and inflammation. The term "non-steroidal" distinguishes these drugs from steroids, having a similar eicosanoid-depressing and anti-inflammatory action. They include more than 50 different types of over-the-counter medications like aspirin, Advil, Motrin (ibuprofen), Aleve (naproxen) and newer, expensive prescription drugs with reduced side effects, such as, Celebrex (celecoxib), Diclofenac and Vioxx (rofecoxib). Among them, the most popular are aspirin, ibuprofen, and naproxen. In the United States alone, over 100 million prescriptions are written annually for NSAIDs, comprising 4% ofthe U.S. prescription volume (National Prescription Audit (2000)). The molecular structures of ibuprofen, naproxen and two primary metabolites related to their mode of action are shown in Figure 1.

2.2 NSAID Effects on Cyclooxygenase

NSAIDs are thought to act at normal doses predominantly via inhibition of one or more cyclooxygenase (COX) isozymes (COX-1, 2; also known as prostaglandin H synthases) which catalyze the initial committing step in the synthesis of prostaglandins (PG), thromboxanes and prostacyclins from arachidonic acid resulting in the inhibition of production of these inflammatory products (Dannhardt and Kiefer 2001). These enzymes are capable of both cyclooxygenase and peroxidase activity. The cyclooxygenase function converts arachidonic acid to the highly unstable hydroperoxide (PGG_2) which is then reduced with the peroxidase function to the ultimate products of COX activity. COX enzymes reside in the endoplasmic reticulum (ER) and nuclear membrane with the substrate-binding pocket precisely orientated to take up the released arachidonic acid (Hyllested, et al. 2002).

The first form of COX (COX-1) has been described as a constitutively expressed enzyme that is present in most cells and tissues, and is involved in cellular housekeeping functions. It is important in physiological homeostasis and mucosa protection, and is responsible for production of low basal levels of prostanoids (Bertolini, et al. 2001; McGettigan and Henry 2000). On the other hand, COX-2 is an inducible enzyme which is expressed in a more limited range of cell types in response to signals such as cytokines and growth factors and is important in inflammatory processes, and produces larger amounts of PGs even from relatively low levels of arachidonic acid (McGettigan and Henry 2000). The structure of COX-2 has been shown to be similar to that of COX-1 with the notable distinction being an amino acid difference that allows for a larger “side-pocket” for substrate access in COX-2 (Smith, et al. 2000). COX-2 enzyme is induced upon inflammation and produces prostaglandins found in various organisms including fish, amphibians, birds and many invertebrates. In vertebrates, these compounds serve a crucial

role in defense mechanisms and inflammatory processes by vasodilating blood vessels, increasing vascular permeability, and sensitizing neuronal pain fibers to inflammatory mediators such as serotonin and bradykinin (Linhart, et al. 2003).

Over expression of inducible COX-2 leads to excess production of prostanoids, mainly PGE2, which are important mediators in pain, fever, chronic and acute inflammation, neurodegenerative diseases and are inhibitors of insulin secretion in the pancreatic islets of diabetics (Linhart, et al. 2003; Rowley, et al. 2005). COX-2 is over expressed in premalignant and malignant cancer tissues of the colon, liver, pancreas, stomach, breast, head and neck (Mittal, et al. 2008). Increased production of PGE_2 has been implicated in various cancer-promoting effects such as an alteration in cell adhesion to extra cellular matrix, up-regulation of VEGF (vascular endothelial growth factor) and apoptosis resistance (Cha, et al. 2006). PGE2 has been shown to decrease host immunity, stimulate the progression of metastases, mediate the expression of proliferative oncogenes, and promote angiogenesis. Surgical removal of brain tumor reduced high levels of PGE2 and the prostaglandin pathway is being evaluated as a novel target for the treatment of various cancers, including gastrointestinal cancer (Yung, et al. 2009). In contrast to an increased risk of cancer due to COX-2 overexpression, some prostanoids inhibit COX-2 activity and induce apoptosis, inhibit proliferation and exhibit anti-tumor activity in human cancer cells (Abbate, et al. 2006; Subbaramaiah and Dannenberg 2003). So, consumption of NSAIDs could have various beneficial effects such as antitumor activity, reduced risk of breast cancer, induction of apoptosis, and inhibition of angiogenesis (Bank, et al. 2008; Davies, et al. 2000; Grösch, et al. 2006; Marnett and DuBois 2002).

In addition to COX-1 and COX-2, several other isoenzymes have been described, most notably COX-3 which is produced by the COX-1 gene but retains intron 1 in its mRNA and is

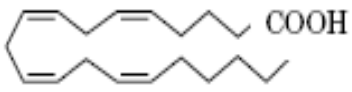
believed to be the most abundant COX in the cerebral cortex and heart in humans (Mittal, et al. 2008). Different members of NSAIDs have different mechanisms to inhibit COX enzymes. Aspirin inhibits COX through a covalent modification and is a selective irreversible inhibitor of COX-1 at low dose but a nonselective irreversible inhibitor of COX at high dose. Flurbiprofen and indomethacin inhibit COX via ionic interactions between their carboxylic acid groups and an arginine residue. They are slow, time-dependent, reversible, nonselective inhibitors of COX. Vioxx (rofecoxib) and celebrex (celecoxib), are very weak competitive inhibitors of COX-1, and are slow, time-dependent, irreversible inhibitors of COX-2. Ibuprofen and naproxen are carboxylic acid derivatives and compete with the substrate of COX at the catalytic center to block the hydrophobic channel of COX active site for arachidonic acid, in a reversible manner (Capone, et al. 2007).

2.3 NSAIDs in the Environment

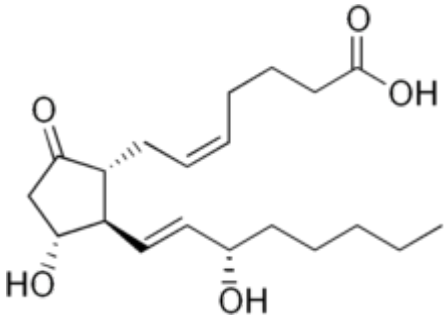
NSAIDs are among the most abundant of environmental pharmaceutical contaminants. Once released into the environment, NSAIDs are transported and distributed into air, water, soil or sediment. Widespread appearance of NSAIDs in the aquatic environment is due to their high volume of consumption and their incomplete removal during wastewater treatment. NSAIDs enter into surface water through various routes and may be a contributing stressor in the aquatic environment (Cleuvers 2004). One of their common routes to the surface water is through their oral administration, where they are absorbed, metabolized and then excreted to the sewer system (Gravel and Vijayan 2007). Other minor routes of entry to surface water include emissions to air and disposal of unused medicines and containers (Spurgeon, et al. 2010). The degree of distribution of NSAIDs primarily depends on their sorption behavior in soils, sediment-water systems and treatment plants and their susceptibility to biodegradation. NSAID biodegradation

products may have pharmacological activities similar to their parent compounds (Cleuvers 2004).

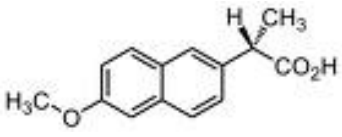
NSAIDs may be found in the effluents of sewage treatment plants (STPs) at concentrations of >1 μg L^{-1} and in lower concentrations in surface waters (Cleuvers 2004). The effectiveness of STP removal of NSAIDs can vary depending on the chemical characteristics of the NSAID. Removal of ibuprofen and naproxen has been reported at rates varying from 40% to 90 % (Ashton, et al. 2004; Gagné, et al. 2005). Nonetheless, the contribution to the receiving waters by extensive use from human population remains significant, as it has been estimated up to 10 kg of ibuprofen per day is contained in the final effluent of a treatment plant that processes the wastewater of a city with a population of 1.5 million inhabitants (Gagné, et al. 2005; Schwaiger, et al. 2004).



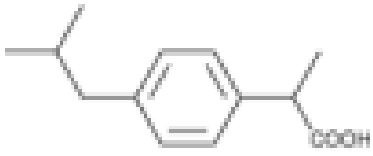
Arachidonic Acid



PGE2



Naproxen



Ibuprofen

Figure 1. Molecular structures of COX substrate (arachidonic acid), a primary product (PGE2) and pharmacological inhibitors ibuprofen and naproxen. (Rowley, et al. 2005).

2.4 Zebrafish as a Model Organism

Zebrafish has become a model organism for the study of vertebrate biology and genetics that is a useful and cost-effective alternative to mammalian models, such as, rodents, dogs and pigs (Cerón, et al. 2005). The zebrafish genome is completely described and its proteins typically display high levels of homology to their human orthologues (Langheinrich 2003). It is reported that some of the phenotypes identified from screening of chemical mutagenesis are reminiscent of human diseases. For example, the mutation of the novel basic helix–loop–helix transcription factor gridlock which results in a stenosis of the aorta and may be analogous to coarctation of the aorta in humans (Grosser, et al. 2002). It is also reported that cardiovascular, anti-angiogenic and anti-cancer drugs elicit comparable responses in the embryos of zebrafish and mammalian systems (Cerón, et al. 2005). Similar observations have been reported for a variety of pharmaceutically active compounds affecting a range of processes in exposed zebrafish (Cerón, et al. 2005). Studies also showed that numerous human diseases are mimicked by zebrafish and several drugs known to inhibit the function of human proteins showed the same effects on zebrafish (Dooley and Zon 2000; Zon and Peterson 2005). Zebrafish pharmacological assays have specific advantages compared to in vitro cell culture studies and in vivo experiments using mice, complementing these assays to give valuable guides for future tests of new drugs for human therapy (Cerón, et al. 2005). Zebrafish embryos are also being used as a model for environmental risk assessment (Shi, et al. 2009). Its value as a model organism for drug target discovery, target validation, drug-finding strategies and toxicological studies is widely recognized (Belyaeva, et al. 2009; Zon and Peterson 2005). Thus, zebrafish not only is appreciated as a suitable model for several human diseases and basic mammalian physiological processes, but has also become a model organism for different kinds of toxicological and

pharmacological approaches (Pomati, et al. 2007; Zon and Peterson 2005). This backdrop of detailed functional information on genetics, proteomics, physiology and pharmacology makes the zebrafish an appropriate choice for our studies aimed at understanding possible risks of environmental exposure of aquatic vertebrates to NSAIDs.

2.5 Proteomics

Proteomics is the systematic analysis of complex protein mixtures and refers to the entire complement of proteins of a certain type of cell or organism. Protein expression level is affected by post-translational processing and modification, such as, phosphorylation, acetylation, ubiquititation, or glycosylation, and cannot be deduced from the DNA or RNA sequence or levels (Champion, et al. 2003). So, a genomic study indicates which protein could be or might be expressed, but not necessarily the status of the proteins that are expressed or their post-translational modifications (Anderson, et al. 2000; Champion, et al. 2003). Proteomics directly measures protein expressions, their modifications, and thus is a more meaningful way to study biological functions of proteins than the genomic approach alone (Anderson, et al. 2000). However, proteomic analysis is proving to be far more challenging than genomic analysis, not only because of the diverse nature of proteins from membrane-bound to soluble state in different cellular compartments, but also post-translational modifications leading to a multiplicity of protein and peptide structures with similar physicochemical properties, such as, p*I*, hydrophobicity and molecular size, all of which makes analyte separation and identification more difficult (Hancock, et al. 2002). Moreover, analytical methods of high sensitivity need to be developed to allow accurate quantification of the identified proteins and the levels of site-specific modifications of individual protein molecules, as there is no comparable amplification scheme as is available for nucleic acids (Oda, et al. 1999). The prospects of the knowledge to be

gained on how living things work, makes proteomics with all its challenges, an important area of investigation (Haynes and Yates 2000).

2.5.1 Comparative Proteomics and 2-D Gel Electrophoresis (2-DGE)

Comparative proteomics characterizes differences between different protein populations such as from control and altered states (Anderson, et al. 2000)**.** The most frequently adopted approach both for determining changes in protein expression and for separating proteins prior to their identification is 2-DGE (Bosworth, et al. 2005). Two-dimensional gel electrophoresis is a combination of two types of gel electrophoresis: isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bosworth, et al. 2005). IEF separates proteins by their ioselectric points, the pH of protein with zero net charge, using a pH gradient across a high electric potential. In this technique, the protein sample is first dissolved in a small volume of rehydration buffer that includes nonionic detergent, reducing agent, such as, dithiothreitol (DTT), and denaturing reagent, such as urea and thiourea, and Bio-Lyte and trace amounts of bromophenol blue (BPB). The rehydration buffer solubilizes, denatures and dissociates disulfide bonds of the proteins but leaves the intrinsic charge of the proteins unchanged. The charged protein moves to the position of its isoelectric point and becomes stationary on immobilized pH gradient (IPG) gel strips made from acrylamide derivatives having different acidic and basic side chains on them (Baek, et al. 2010). Reproducible and commercially available IPG gel strips of various pH ranges are made by polymerization of different mixtures of acrylamide derivatives, and have a resolution of up to 0.01 pI units (Gygi, et al. 2000). The use of narrow range immobilized pH gradients gels for the first dimension increases resolving power and can help detect low-abundance proteins.

The second dimension in 2-DGE is SDS-PAGE, where the IPG gel strip containing the separated proteins is subjected to electrophoresis again in a direction at a right angle to the first dimension and proteins are separated according to their molecular weight. As a result of the two dimensional separation techniques, 2-DGE can provide protein mixtures as arrays of spots on a polyacrylamide gel, and, as many as 5000 different proteins can be separated falling in a window of pIs ranging from 3.5 to 10 and molecular weight (*M*r) ranging from 6 kDa to 300kDa, in a single experiment (Shen, et al. 2004). Such ability of 2-DGE, aided by efficient image analysis software, is useful to estimate concentration changes in one or more individual proteins of interest between samples from control and treated organisms.

Even though this technique is appreciated due to various merits, it is not without limitations. Some of the limitations of this technique include poor reproducibility, labor intensiveness, and difficulty in separation for very large or very small and highly acidic or highly basic proteins, unless the gel format is varied (Encarnación, et al. 2005). The method alone can have difficulty revealing low-abundant proteins in the presence of other, high-abundance proteins unless it is aided by various prefractionation methods, such as, centrifugal sub-cellular fractionation and/or various types of chromatography (Capone, et al. 2007; Champion, et al. 2003; Gao, et al. 2005; Zuo and Speicher 2000). Furthermore, a comparative analysis of stained gels is not sensitive enough to detect small changes in protein concentrations and variations in spot intensity and patterns have been observed even for identical spots on gels run in parallel, making it difficult to distinguish subtle quantitative differences.

However, techniques are being developed to minimize the limitations involved in the comparative analysis of different 2D gels. In such an effort to minimize the experimental variation during first dimension, a minimum of triplicate samples are run simultaneously using

similar conditions including the same lysis buffer composition, sample preparation and solubilization procedure. Similarly, to minimize the gel-to-gel variation during the second dimension, samples having the same concentration of all reagents including solubilizing, reducing and alkylating reagents are being used. Furthermore, running all the member gels simultaneously under identical electrophoretic conditions (co-electrophoresis) also helps to minimize experimental variation during second dimension run. Nowadays, the use of multiple narrow-range pH gradients before running the second dimension is being appreciated for better resolution of less abundant proteins, including difficult membrane and hydrophobic proteins (Champion, et al. 2003; Corthals, et al. 2000). However, profiling exceedingly hydrophobic membrane proteins using 2-DGE remains difficult and loss of protein occurs due to their poor solubility and their inherent chemical characteristics and membrane compartmentalization (Blomqvist, et al. 2006).

2.5.2 Staining Second Dimension Gel

The protein spots in SDS-PAGE can be visualized by several different gel staining methods, such as, silver staining, fluorescent staining, and Coomassie blue staining (Candiano, et al. 2004). Silver staining is rapid and sensitive and is able to detect as little as 1 ng of protein (Blomqvist, et al. 2006; Quadroni and James 2007). It is based upon saturating gels with silver ions and the formation of metallic silver on the protein surface (Merril and Pratt 1986; Shevchenko, et al. 1996). However, this method is quite complex, and requires multi-step procedures that must be stopped at some arbitrary time point in order to avoid over development. So, poor gel-to-gel reproducibility with variations in spot intensity and the large variation between different protein types of the bands, to the amount of applied molecules makes this stain

inappropriate for quantification purposes (Blomqvist, et al. 2006; Lopez, et al. 2000; Schwaiger, et al. 2004).

Staining with fluorescent dyes such as SYPRO Red, SYPRO Orange and SYPRO Tangerine dyes can detect as little as 8 ng of proteins (Lauber, et al. 2001). This is a simple single step technique which requires only 30-60 min. of staining time and avoids a destaining step. SYPRO Ruby dye is a ruthenium-based metal chelate stain, and is as sensitive as silver stains (Lauber, et al. 2001). Its linear dynamic range is three orders of magnitude, a better range than silver and Coomassie blue stains, and its compatibility with proteolytic digestion of excised protein spots and MS analysis, makes this the most commonly used fluorescent protein stain (Choi and Nesvizhskii 2007; Lauber, et al. 2001). Another fluorescent gel stain, Flamingo ^{TM}, is a recently developed non-specific protein stain with potential utility in proteomics research due to its high sensitivity and wide dynamic range. It is reported that Flamingo ^{TM} and SYPRO Ruby resulted in similar overall performance when used with LC-MS-MS for protein identification. Furthermore, staining with Flamingo ^{TM} results in more complete sequence coverage by MALDI tryptic peptide mass analysis than did SYPRO Ruby, as it resulted in a lower incidence of oxidative amino acid modifications than did SYPRO Ruby where the mass spectra showed a greater incidence of peaks and are shifted from expected peptide masses by the mass of one or two oxygen atoms (Zhang, et al. 2000).

Though a range of organic dyes has been used to visualize proteins in polyacrylamide gels, Coomassie blue dyes (R and G types) are the most popular and commonly used due to their low cost, ease of use and good compatibility with mass spectrometry (Candiano, et al. 2004). The proteins are stained in proportion to the amount of their basic and aromatic amino acids and the amount of sample in the spot. Coomassie blue R-250 dye, approaches a detection limit

similar to that of silver staining, and is also called as “blue-silver” (Candiano, et al. 2004). In this case, the background has minimum staining as the affinity of dye to proteins is higher than that to gel matrix (Encarnación, et al. 2005). With the identification of improved Coomassie dyes, the main limitations of poor detection sensitivity and small linear dynamic range, associated with conventional Coomassie blue have been reduced. However, lengthy staining and destaining steps slow down the procedure and limit its throughput, and the extensive washing steps can lead to protein losses. Still, the proteins can be visualized if abundant enough (about 50 ng) and can be subsequently analyzed by mass spectrometry as long as any excess Coomassie is carefully removed with additional long washing steps even after the protein spot has been excised (Encarnación, et al. 2005; López 2007).

2.5.3 Second Dimension Gel Image Analysis

The complicated process of quantitative comparison of protein bands in multiple 2-D gels is made possible by the development of image analysis software. Computer software can make a digitized image of inspected bands off a 2-D gel and can compare protein differential expressions in replicate gels of sample running under similar sample preparation conditions. These replicate gels minimize the problems with gel-to-gel inconsistency and enable quantitative analysis based on the relative amounts of the protein in the gel spots of concern. The protein spots in the replicate gels corresponding to control and treated samples are compared and quantified after subsequent analysis of stained gels using image analysis techniques such as PDQuest software developed by Bio-Rad Inc. These spots are verified manually and/or automatically and excised for subsequent proteolytic (trypsin) digestion. The tryptic digest peptides are extracted, concentrated and identified using different types of mass spectrometry including LC-MS.

2.5.4 High Pressure Liquid Chromatography Mass Spectrometry (HPLC-MS)

The trypsin digested peptide mixture is injected into HPLC system for a high efficiency separation of the peptides. The LC-MS analysis enables a sequential presentation of groups of peptides with similar retention times for mass determination and identification of the corresponding peptide (Grosser, et al. 2002). The peptides are eluted at different retention times according to their hydrophobicity using a reverse phase (RP) column and gradient solvent system. The peptides separated on the reverse phase column are introduced in solution in the LC eluent and transferred into the mass spectrometer for analysis. The eluent is introduced via atomization into the spray chamber at atmospheric pressure and bathed in hot nitrogen dry gas which results in evaporation of the solvent molecules and allows desolvated, ionized peptide molecules to enter the MS for analysis. The key features of an efficient RP-HPLC separation include good resolution, high recovery, and the minimization of background contaminants. For the efficient separation of peptides, a method has to be developed which is evaluated frequently using a commercial standard peptide digest such as bovine serum albumin (BSA).

2.5.5 Method Development in LC/MS

Method development steps of LC/MS are crucial to achieve the goal of molecular mass determination and identification of proteins. One of the most important initial steps during method development is to find the right column and elution method. A common practice is the use of reverse phase hydrophobic capillary column (C-18), and gradient elution, to separate proteins and peptides. The second step during method development is to optimize the gradient conditions, which is achieved after several elution trials using various percentages of two solvents: inorganic solvent A (typically water) and organic solvent B (typically acetonitrile). An elution profile of peptides from a standard protein, such as, BSA is used to optimize the MS

spectral signal and chromatography of individual peptides. Traditional LC techniques for protein and peptide identifications were performed at low pH by adding an ion-pairing agent, such as TFA, in the mobile phase, which binds to the basic residues of proteins and peptides and forms protein/TFA complex (Grosser, et al. 2002). This complex helps to achieve good chromatographic peak shapes. However, it suppresses the peptide ion response in MS analysis. Techniques have been developed to displace TFA from the sample and still get good chromatographic peaks. In one of such attempts, the effluent is exposed post-column, to a higher concentration of a weaker acid, such as, 5% acetic acid or mixture of propionic and isopropanol in 3:1 ratio (not used in our study). In addition, the Agilent mass spectrometer is configured with an orthogonal flow to improve the efficiency of sample ion transfer and reduce the amount of solvent entering the MSD. As a result, there is a marked increase in signal strength. In another attempt to optimize the signal, one can use a lower TFA concentration. The TFA concentration in the mobile phase can be reduced from 0.1% to 0.01% or even less and still result in good chromatographic results as well as improve the MS spectral signal. As the concentration of TFA is replaced or reduced, the strength of the mass spectral signal increases, the chromatographic resolution and peak shape improve and sample recovery and MS sensitivity remain high. In our current method, we replace TFA with a 0.1% (v/v) concentration of the formic acid which volatilizes in the spray chamber (mobile phase A = 0.1% formic acid in water and mobile phase B = 0.1% formic acid in acetonitrile) at 4 uL/min using a capillary column and pumping system to elute the separated peptides and proteins. The gradient proceeds from 3% B to 100% B over the course of approximately one hour. Peptides elute primarily between 18 and 35 minutes (see Chapter 3 Methods and Materials).

2.6 Mass Spectrometry

Mass spectrometry is a high-throughput method to determine the accurate mass of chemicals including biological macromolecules, such as, proteins, and is also called mass spectroscopy. Mass spectrometry is an analytical technique by which chemical and biochemical substances are identified by sorting of gaseous ions in electric and magnetic fields according to their mass/charge (m/z) ratios, and operates on the principle that moving ions may be deflected by means of electric and magnetic fields in a vacuum (Takáts, et al. 2004). A mass spectrometer has five primary components: inlet, source of ions, mass analyzer, detector, and recorder. In gas chromatographic mass spectrometry (GC/MS), a sample is introduced into the ion source under high vacuum and ionization occurs *via* electron impact. In the case of electron impact ionization, a beam of electrons collides with a neutral analyte molecule and displaces another electron and results in a positively charged ion. The ionization process can either produce a molecular ion, which will have the same molecular weight and elemental composition of the starting analyte, and/or it can produce fragment ions that correspond to smaller pieces of the analyte molecule. In chemical ionization, a reagent gas, such as, methane is ionized by a beam of electrons at a high reagent gas pressure and produces reagent gas ions and neutrals. The product ions react with analyte molecules and produce analyte ions dominated by intact molecular ions.

Mass spectrometry of liquid chromatographic eluants relies on ionization of the analytes at atmospheric pressure prior to entry into the vacuum of the spectrometer. This class of interface is generally referred to as Atmospheric Pressure Ionization (API). A combination of thermal and pneumatic means desolvate the ions as they enter the ion source. Peptide mapping is frequently conducted using pneumatically assisted electrospray API (or simply ESI) in which the peptides arrive in the interface in an ionized state in the liquid chromatographic eluant. The

addition of a low concentration of volatile organic acid (0.1% formate in our procedure) promotes formation of protonated peptides predominantly with 1-3 charges.

Matrix-assisted laser desorption/ionization (MALDI) is another popular technique to generate ions from nonvolatile macromolecules, such as, proteins or peptides. In MALDI, a small amount of analyte is placed on the laser target along with a co-crystallized excess of matrix having a chromophore such as sinapinic acid or dihydroxybenzoic acid (Wang, et al. 2001). The matrix absorbs the energy from a laser pulse and produces localized plasma that results in vaporization and ionization of the analyte. When the laser strikes the crystals on the target surface, the excitation energy of the crystals is transferred to the analytes volatilizing the analytes and leading to the formation of predominantly singly charged $(M+H)^+$ type ions. Sometimes multiple charged ions, dimers and trimers can also be formed (Nedelkov, et al. 2000). MALDI has been used in conjunction with a variety of mass spectrometers including quadrupole, ion trap, and time of flight (TOF) mass spectrometers. MALDI-TOF is commonly used for protein and peptide "fingerprint" analyses based on peptide molecular weights but lacking the daughter ions formed during the collision induced dissociation (CID) characteristic of tandem MS/MS techniques (Nedelkov, et al. 2000).

The approach used in this study was RP-HPLC with ESI and ion trap mass spectrometry. A more detailed explanation of this approach follows. In the ESI method, sample solution is sprayed across a high potential (a few kilovolts) difference from a needle into an orifice in the interface (Papayannopoulos 1995). Heated gas (nitrogen) flows are used to desolvate the ions existing in the sample solution (formed in the LC mobile phase) resulting in the formation of naked desolvated ions with the likelihood of multiple charged ions increasing as the molecular weight increases (Grosser, et al. 2002; Takáts, et al. 2004). This technique is used in the positive

ion mode and, because of the formation of multiply-charged species, can be used to estimate molecular weights as high as several hundred thousand Daltons for intact proteins. Nanospray is a refined version of electrospray and uses a needle with much smaller diameter than that used in capillary electrospray with nl/min flow rates (rather than uL/min) resulting in improved sensitivity (Ong and Mann 2005). ESI has been successfully used with a variety of mass spectrometers, including triple quadrupole, quadrupole ion trap and quadrupole time of flight (Ong and Mann 2005). In both capillary and nonspray ESI, the ion trap mass analyzer is able to accumulate and release the ions in a batch mechanism and acquire the resulting (MS/MS) fragmentation spectra through successive cycles of fragmentation (MS^n) and these techniques have become leading MS technologies for proteomic analysis (Kim, et al. 2004). When supported by bioinformatics software and other techniques that exploit the unique capabilities of ion trap technology, the sequence and structural information derived from MS/MS spectra of digest peptides can be employed to determine the identity and structure of the precursor protein(s) in a single run.

2.7 Protein Identification by ESI-MS/MS

Identification of proteins by knowing the amino acid sequence of various numbers of short peptides is made possible for model organisms with complete genome sequence information (Ong and Mann 2005). To identify such short peptide sequences, the protein in solution form or excised from gel pieces is digested in a suitable proteolytic enzyme. Trypsin has become the most popular enzyme to use in MS/MS analysis and cleaves next to the C-terminal of basic amino acid residues, arginine (R) or lysine (K), and is therefore well suited for positive ionization mass spectrometric analysis. The digest mixture produces a complex spectrum from which the molecular weights of the proteolytic fragments can be read. This spectrum, with its

molecular weight information, forms a peptide map. If the protein already exists in a database, then the ms/ms spectral information is often sufficient to confirm the identity of the protein. Mass spectra generated by the fragmentation of precursor peptide ions by low collision induced dissociation (CID) energy are dominant fragment ions resulting from cleavage at the amide bonds, as shown in a schematic of peptide fragmentation by low CID in Figure 2. If the positive charge in association with the parent peptide remains on the amino-terminal side of the peptide fragment, it is termed a *b* ion and if the charge remains on the carboxyl terminal side of the broken amide bond, it is termed a *y* ion (Papayannopoulos 1995). The spectra collected from the product ions, are transferred to powerful search engines like that developed by Matrix Science MASCOT (http://www.matrixscience.com) which reveals the sequence information of the precursor peptide. The amount of sequence information generated will vary from one peptide to another. Some peptide sequences will be confirmed totally, while others may produce a partial sequence of, say, 4 or 5 amino acid residues which may also be sufficient to search a protein database to confirm the identity of the peptide. The confident identification of the protein of interest requires enough peptide mass presented to the MS to produce the necessary number of ions required to produce high ion scores and enough sequence coverage to predict a protein after searching the protein sequence database using various search engines to achieve statistical significance (Ong and Mann 2005).

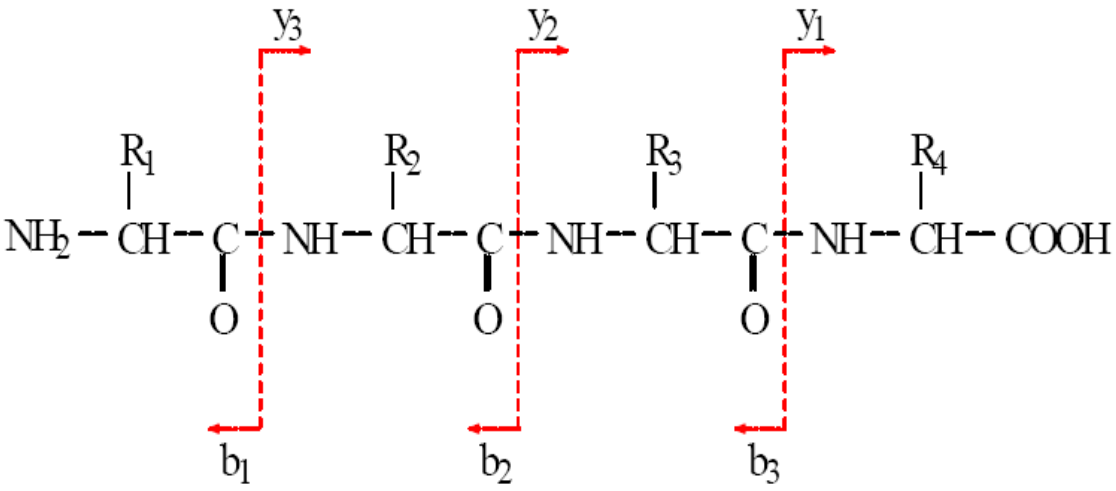


Figure 2. Schematic of peptide fragmentation by low collision energy CID.

CHAPTER 3

EXPERIMENTAL DESIGN, METHODS AND MATERIALS

To achieve the goals described in the overview chapter of this dissertation, the proteomics approach of using 2-DGE and mass spectrometry was used. This approach uses IPG strips of two pI ranges. Our preliminary experience using broad range pI (4-10) IPG strips resulted in protein spots with low resolution and overlapping of low abundance proteins by highly abundant proteins. We improved this separation with narrower pI range IPG strips, 4-7 and 7-10 which have been successful in other similar applications (Champion, et al. 2003). Adult male zebrafish were exposed to known concentrations of ibuprofen in a flow-through exposure system for 14 days. Ibuprofen effects were evaluated at exposure concentrations of 1 ppb and 10 ppb in pI 4-7 range, and 10 ppb and 100 ppb in 7-10 pI range.

To determine the similarity of response to a second NSAID, male zebrafish were also exposed to 10 ppb and 100 ppb of naproxen in a similar flow-through system for 14 days. The exposure concentrations for naproxen in both pI range of 4-7 and 7-10 were 10 ppb and 100 ppb.

3.1 Materials

Ibuprofen and naproxen were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). ReadyPrep Total Protein Extraction Kit, ReadyPrep 2-D Cleanup Kit, ReadyPrep 2D-Starter Kit, ReadyStrip IPG Strips (pI 4-7 & 7-11), 11 cm long, SDS-PAGE gel (8-16%, Tris-HCl, 1.0 mm, 11 x 8 cm), tris/glycine/SDS buffer, Precision Plus Protein Kaleidoscope standard marker, Bio-Safe Coomassie (Coomassie G250 Stain), and tributylphosphine (TBP),reduction and alkylation kit were purchased from Bio-Rad Co. USA (Hercules, CA). Protease inhibitor cocktail and sequencing grade modified trypsin were purchased from Promega Corporation (Madison, WI). DMSO, Ammonium bicarbonate, Bradford reagent, acetonitrile, protease

inhibitor cocktail and trifluoroacetic acid (TFA) were purchased from Sigma Chemical Co (St. Louis, MO). Glass tissue homogenizers for extraction of protein from tissue were purchased from Kontes (Vineland, NJ). The deactivated glass inserts were purchased from Agilent Technologies (Santa Clara, CA). Protean IEF cell first dimension electrophoresis and dodecyl SDS-PAGE gel runner was purchased from Bio-Rad Co. USA (Hercules, CA).

3.2 Methods

3.2.1 Fish Maintenance and Exposure to Ibuprofen

Five- to six-month-old male zebrafish were kept in 15-L glass aquaria maintained at room temperature (20-25 ^{0}C) with de-chlorinated city of Denton tap water delivered through a flow-through system as shown in Figure 3. Water chemistry was performed twice a week, producing the following mean values: pH 7.6, dissolved oxygen 8.6 mg/L, temperature 23.1 ^{0}C, alkalinity 75 mg/L, and conductivity 360µS/cm. Water was pumped from a reservoir to mixing head boxes, then to aquaria. Flow to aquaria was set at 25 ml/min using a Masterflex® model 7523-60 12-channel peristaltic pump with a (Masterflex®,Cole-Parmer®, Veron Hills, IL) model 7519-85 cartridge pump head pumping through 2.79 mm Santoprene® tubing.

The required concentration of ibuprofen was dispensed to mixing head boxes via a Harvard Apparatus® (Warner Instruments, Hamden, CT) model PHD 2000 infusion pump at the rate of 5µl/min. The infusion pump delivered ibuprofen through 0.58 mm tubing, fitted to 30-ml B-D® syringes (Watts, N. Andover, MA) producing a dilution rate of 5000. Thus, ibuprofen concentrations were calculated from this factor, such that a 1.0 µg/L target concentration was achieved using a 5.0 mg/L stock, 10.0 µg/L concentration on 50.0 mg/L stock, and 100.0 µg/L concentration on 500.0 mg/L stock. Aquaria were aerated using a commercial air pump and air stones, with zebrafish fed flake food twice daily for 14 days during ibuprofen exposure. The

control zebrafish were maintained under the same conditions, but without exposure to ibuprofen. All experimental fish were allowed to acclimatize for at least a week to the standard laboratory conditions of 23± 2^0 C and 16:8 hour light: dark cycle.

Figure 3. A continuous fish flow-through exposure system used for exposure tests.

3.2.2 Fish Maintenance and Exposure to Naproxen

Male zebrafish were kept in similar aquaria and were maintained in a similar condition as in the exposure of ibuprofen. The flow-through system was also established similarly as in the ibuprofen exposure setup. The infusion pump, tubing, syringe pumps used similarly to produce a dilution rate of 5000. The naproxen exposure concentrations were calculated using this factor, such that a concentration of 10.0µg/L and 100.0µg/L was achieved using 50.0 mg/L and 500.0 mg/L stock. The zebrafish were exposed for 14 days in the aquaria and fed with flake food twice

daily. The control zebrafish were maintained under the same condition, but without exposure to naproxen.

3.2.3 Extract Preparation

In order to examine the effect of ibuprofen and naproxen exposure on the protein composition of zebrafish, proteins were extracted from gill tissues of both the exposed and control groups. For all control and exposed zebrafish for all the treatments, three 2-D gel replicates were prepared from 3 fish (6 gills) per replicate. The collection of gill tissue was done under microscopic lenses aided by small forceps to avoid contamination from other tissues. To minimize protein modification or degradation, dissection of fish was carried out on ice. Gloves were worn to avoid contamination from human tissue. Once the tissues were collected, they were kept in microfuge tubes on ice until homogenized. Dissected gills of 30-40 mg from three zebrafish were homogenized using 2 ml, glass homogenizers (Kontes Vineland, NJ, USA), in 500 µl of homogenization buffer from Bio-Rad Total Protein Extraction Kit, as in Charles *et al. (Charles, et al. 2005)*. The homogenization buffer contains 7 M urea, 2 M thiourea, 1% (w/v) ASB-14 detergent, 40 mM tris base, 0.001% bromophenol blue (BPB), 10 µl of 200mM tributyl phosphine (TBP) and 25 µl of protease inhibitor cocktail. Homogenates were centrifuged at 2500 x g for 30 min at 4 ^{0}C. The insoluble material in the pellets was discarded and the supernatant was stored at -80 ^{0}C until further use. To extract protein in the higher pI range, the composition of the homogenization buffer was adjusted slightly to include only 0.1% of ASB-14 (Dobly, et al. 2004).

3.2.4 Sample Cleanup for 2D Gels

The supernatant was cleaned up using BioRad ReadyPrep 2-D Cleanup Kit. The kit was used to remove chemicals that can interfere with 2-D electrophoresis, such as, detergents, salts,

peptides, nucleic acids, lipids and phenols from the sample. The cleanup was performed using the manufacturer's protocol and was followed by mixing with prechilled acetone at - 20^0C and then briefly vortexing to mix the sample. The mixture was incubated on ice for 30 min and then centrifuged at 2500 x g in a refrigerated microfuge tube for 5 min to form a tight pellet. The pellet was washed with Mili-Q water, air dried and resuspended very thoroughly by vortexing with a volume of 2-D rehydration/sample buffer from the Bio-Rad Ready prep sequential extraction kit (containing 5 M urea, 3 M thiourea, 2% CHAPS, 2% SP310, 40 mM Tris, 0.2% Bio-lyte (w/v), 50 mM DTT and a trace amount of BPB). The total protein concentration of the sample was determined using Bradford assay, and was repeated for all replicate samples of control and each treatment. For pI range 7-10, the sample cleanup procedure was the same as in pI 4-7; however, the washed and dried pellet at the end was resuspended in the same rehydration buffer with higher concentration (7 M) of urea.

3.2.5 Isoelectric Focusing

The 11 cm IPG strips stored at -20^0 C were thawed to room temperature. A 300 µg sub-sample of total protein, in 185 ul volume, from each replicate across all treatments and controls was loaded onto a Protean IEF cell (Bio-Rad). pH 4-7 IPG strips were laid on each well with careful attention to avoid air bubbles. The IPG strips were covered with mineral oil to avoid evaporation of the sample. The frozen IPG strips were thawed at room temperature for 10 min before soaking them in IPG equilibration buffer I (6 M urea, 2%SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT) for 20 min and then in IPG equilibration buffer II (6M urea, 2%SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT and 2.5% iodoacetamide) for 20 min on an orbital shaker. Samples in the 7-10 pI range, were treated identically with the exception that the Iso-electric focusing ramp was performed for 35,000 volt-hours.

3.2.6 Second Dimension Electrophoresis and Gel Staining

Equilibrated IPG strips were laid on top of the gels (Criterion Precast Gels (Tris-[HCl, 8-16% resolving, 4% stacking]) (Bio-Rad, Co., Hercules, CA) and 8 µl of precision standard marker was loaded in the well to co-electrophorese with the samples. They were sealed in place using 0.5% low melting point agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, and a trace of Bromo Phenol Blue (BPB). Second dimension gels were electrophoresed at 175 Volt for 90 min at room temperature. After electrophoresis, the gels were washed in three 10 min changes of 200 ml distilled water, and placed in a fixing solution (40% methanol, 10% acetic acid, and 50% H_20) for 1 hr. The gels were subjected to two, 5 min rinses with distilled water. The gels were washed again in a solution containing 50% ethanol and 50% H_2O overnight at 4 ^{O}C, followed by rinsing with H_2O three times. Gels were placed in 200 ml Colloidal Coomassie Blue G250 (Bio-Rad's Bio-Safe) staining solution until the protein spots were adequately stained. The stained gels were rinsed three times, and washed overnight with H_2O. During all the steps for gel staining, the gels were shaken in a rocking shaker. The gels were stored in 2% acetic acid solution in water and wrapped with an aluminum foil at 4^0C.

3.2.7 Image Analysis

All the gels judged to be acceptable on the basis of uniformity and intensity of staining was used for quantitative image analysis. The 2-D protein profiles were used to construct a 2-D database for the gill tissue. The gels were scanned at a resolution of 200 dpi using a Bio-Rad Quantity One (Chemidoc XRS) densitometer and digitized images stored and analyzed with PDQuest 2-D analysis software (7.4.0). Images were filtered to minimize background, and protein spots were identified using an automated "Spot Detection Wizard", combined with manual editing to remove artifacts. One gel from the replicates was selected as the basis for the

construction of a reference gel against which the remaining gels were matched using standard routines from within the software. Each spot within the reference gel was assigned a spot number, and used in the subsequent descriptions to refer to individual spots. The amount of protein present in a given spot was estimated by the area of the spot multiplied by the pixel density and referred to as the spot volume (Bosworth, et al. 2005). Following correction for background, individual spot volumes were normalized to the total protein amount detected within each gel so the amount of each spot volume expressed as the relative volume. Each protein spot was confirmed using 3D analysis and individual spots were compared between the replicate gels of each treatment and control using spot review tool. Once the 3D and "Spot Review" tools confirmed the presence of the particular spot in all the replicates, these spots were subjected to comparative analysis with their counterparts of each treatment of the experiments. The fold analyses of up-and down-regulated spots were identified by qualitative and quantitative search algorithms in PDQuest. An automated t-test was conducted with a command within the software, and those comparisons with P values of ≤ 0.05 were considered statistically significant. Molecular masses of the over-and-under expressed proteins were estimated by comparing with the co-electrophoresed Precision Plus Protein Kaleidoscope standard marker. Iso-electric points were estimated based on the linearity of the IPG strip. The significantly up- or down-regulated spots were excised (1.0 mm^3 cubes) using an EXQuest Spot Cutter (Bio-Rad) and kept at -20 ^oC in silianized glass inserts.

3.2.8 Pre In-Gel Digestion Step

The excised protein spots were subjected to two, 30 min agitations in 200 µl of deionized H_2O. The washed spots were destained three times, by agitating in 200 µl of destaining solution (25 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) for 10 min at 30 ^0C), and

washed in 100 ul of 90% ACN for 10 min in 200 µl deactivated glass inserts from Agilent Technologies, where the gel pieces were shrunk and turned opaque/white (Sigdel, et al. 2004). These opaque white gel pieces were dried in a speedvac (Savant, Richmond, Virginia) for 30 min and subjected to in-gel digestion. However, for pI range of 7-10 samples, the washing and destaining of the gel pieces were followed by reduction and alkylation in which the dried gel pieces were treated with 25 µl of 25mM dithiothreitol (DTT) in 25 mM ABC, and incubated at 60 ^{o}C for 1 hr. The supernatant was removed and the gel pieces were kept in 25 µl of 55 mM iodoacetamide and incubated at room temperature for 45 min in the dark. The supernatant was discarded, and the gel pieces were dried for 30 min in Speedvac.

3.2.9 In-Gel Trypsin Digestion and Peptide Extraction

The dried gel pieces were treated with 10 µl of 12.5 ng/ µl trypsin in 10 µl of 50 mM ABC and incubated on ice until the gel pieces were fully swollen (approximately 30 min.). The fully swollen gel pieces were further treated with 15 µl each of trypsin (12.5 ng/ µl) and 50 mM ABC, and incubated overnight (16-18 hrs) at 37 ^{o}C. The supernatants from the trypsin-digested mixtures were collected in separate 100 µl deactivated glass inserts (Agilent Technologies, Concord, CA). The gel pieces were gently mixed by slow vortex for 10 min, sonicated for 3 min and centrifuged briefly in 25 µl of 25 mM ABC, twice in 25 µl of 50% ACN/5% formic acid in deionized H_2O, 25 µl of 50% ACN/isopropanol and 2% formic acid, and finally in 15 µl of 95% ACN/5% formic acid. The protein extracts were frozen in liquid N_2 and lyophilized (Labconco, Kansas, MO) (Olsen 2004). The lyophilized protein flakes were resuspended in 8 µl of 50% ACN, 45% water, and 5% formic acid and briefly sonicated. The protein solution was kept at -20 ^{o}C until LC/MS/MS analysis.

3.2.10 Electro Spray Tandem Mass Spectrometry (ESI-MS/MS) and Database Searching

Eight µL volumes of the resuspended samples were automatically injected into a capillary LC-MS (ESI-MS/MS). For the liquid chromatography, an Agilent 1100 binary capillary pump (Agilent technologies, Palo Alto, CA) was used, together with a reversed-phase capillary column, 0.3mm x 150 mm, (3.5 µm particles size). The LC method consisted of, Solvent A [H_2O/0.1% formic acid (FA)] and Solvent B [(ACN/0.1% FA) in a 3-100% gradient (10 min 3 % ACN, 10 min 30%, 20 min 70%, 20 min 100%) with a flow rate of 4 uL/min for 60 minutes. The gradient proceeds from 3% B to 100% B over the course of approximately one hour. Peptides were eluted primarily from 18 to 35 minutes. The peptides eluted were analyzed by ESI-MS/MS. The spectrometer was operated in an automated data-dependent mode. For each scan, the most intense ions were sequentially fragmented in the ion trap by collision-induced dissociation using helium collision gas, and resulting in MS/MS peaks. The most intense MS/MS peaks, in positive ion mode, were automatically selected after defining an intensity threshold. Monoisotopic masses from the tryptic digests were used to identify the corresponding proteins by searching the *D. rerio* databases in SWISS-PROT (http://us.expasy.org) and NCBI using the MASCOT search algorithm (http://www.matrixscience.com). The database searches were done with MS/MS ion search with a mass accuracy of 0.1 Da. Search options included were: only specific tryptic cleavage with two missed cleavage sites permitted. Variable modifications permitted were as follows: for cysteine as S-carbamidomethyl-derivative, oxidation of methionine, deamidated asparagines and glutamine (Blomqvist, et al. 2006). Statistically significant matches were determined from the MASCOT score, by the relationship: Score = -10 x Log_{10} (*P*), where P = 1(1,000 x number of entries in a database). Using the parameters above, MASCOT assigns a significance threshold or confidence level to a match and the score is

displayed as a bar diagram in its peptide summary report (http://www.matrixscience.com) (Bosworth, et al. 2005). The sensitivities of these approaches were determined using a commercial standard protein (BSA) digestate, from Agilent Technology, with an on-column injection of 8 pmol. At least five peptides, representating a minimum of 5% sequence coverage, were considered as the minimum criteria for protein identity.

CHAPTER 4

RESULTS

4.1 Flow- through Exposure System

This dissertation endeavored to establish a standardized system which would permit evaluation of proteomic changes in aquatic organisms exposed to degradable environmental toxicants within the University of North Texas (UNT) aquatic toxicology group. The" pseudopersistent" nature of many emerging contaminants precludes traditional exposure protocols based on static renewal. Therefore, a preliminary requirement for the establishment of a standardized system was confirmation of a reliable flow-through exposure capability. The chosen concentrations for the flow-through system were 1 ppb, 10 ppb and 100 ppb for ibuprofen while only the two higher concentrations 10 ppb & 100 ppb were used for naproxen. The measured concentrations of the ibuprofen and naproxen in the exposure tanks varied from 65% to 61% of the nominal targets and were within the range of environmentally measured concentrations of ibuprofen and naproxen (Bhandari and Venables 2011). In these exposure conditions, the fish appeared healthy and active and fed normally. However, there was a modest trend of increased mortality with exposure which ranged from 3-4% in controls, 5-6% in 1 ppb, 7-8% in 10 ppb and 12-14% in 100 ppb of ibuprofen and naproxen.

4.2 Extraction of Gill Proteins

In comparative proteomics, reproducible sample preparations are crucial. Variability in sample preparation was minimized by optimizing procedures for isolation of gills from liveanimals, storage and extraction of excised tissues. The optimum number of gills found to be appropriate for our analysis was 6 gills per replicate for each treatment. Our optimized extraction buffer for 4-7 pI was 7 M urea, 2 M thiourea, 1% (w/v) ASB-14 detergent, 40 mM tris base,

0.001% bromophenol blue (BPB), 10 µl of 200mM tributyl phosphine (TBP) and 25 µl of protease inhibitor cocktail. However, the optimum buffer composition to extract basic proteins at pH 7-10 was adjusted slightly to include 0.1% of ASB-14. The subsequent clean up steps after the extraction of protein in high or low pI range was found to reliably yield a total protein mass of 400 µg-500 µg as indicated by Bradford assay.

4.3 2-D Gel Electrophoresis and Protein Spot Analysis

Following cleanup, the extracted protein samples were subjected to 2-DGE. Use of 11cm long IPG strips was adequate to separate proteins with minimum horizontal and vertical streaking using the following optimized program performed in four stages: a 12 hour period of passive rehydration was followed by 250 V for 20 min. followed by 8000 V for 2 hrs in a linear voltage slope ramped to 8000 V (30,000 volt-hours) and finally sloped rapidly to a 500 V final hold (at stages of maximum 50 µA to a minimum of 2µA). To ensure optimal performance of protein separation, the second dimension gel electrophoresis conditions were optimized for 175 V to run the experiment for 70 minutes with the gel-runner set up to run only two gels at a time. The gels were stained with SYPRO ruby, silver staining, and Bio-safe Coomassie. However, our ability for the detection and identification of proteins using ESI-MS/MS indicated that use of Bio-safe Coomassie was adequate for detection of proteins that were present in sufficient concentration for final identification.

The 2-D gels from control and each treatment were prepared in triplicates. Hence, there were a total of nine gels comprising three gels from the control and three from each treatment of 1 ppb and 10 ppb of ibuprofen for 4-7 pI. However, for both acidic and basic range pI for all the exposure concentrations of naproxen, and, basic range pI for ibuprofen the total of nine gels were comprised of three gels from control and three gels each from the 10 ppb and 100 ppb exposure,

only. The distribution and intensities of some protein spots were consistent in all the replicates of each experiment and also included clusters of spots having similar M_r and pI values, which were considered as a characteristics of conserved proteins in 2-D gel (Bosworth, et al. 2005). These spots were used as an origin to initiate the optimization process to fix the parameters for the detection of proteins. Once the parameters for spot intensity, size, and purity of spot were fixed, the same parameters were used to detect the spots in the replicate gels. Furthermore, the 100 % matching strategy available in the software was used to prepare a match set for each treatment and control, and ensured the analysis of the same protein on each gel in the experiment. The unmatched proteins spots lying in the gel-borders and in smear regions were excluded from the detection using the manual editing mode. PDQuest software, which requires tedious manual editing options, was used to confirm the authentic spots in all the triplicates of each treatment and the control. To authenticate the spots we optimized the "detection parameters" which should be useful for future work as well. The reproducibility of protein spots in each replicate of each treatment and control was confirmed by spot-to-spot analysis of each identified spot with its counterparts on the 2-DGE control gels using spot review tool within the software. The success of the optimized protocol was indicated by achieving a low relative standard deviation (RSDs) of the protein amounts for each identified proteins within a given treatment group (below 20 %).

The comparative analysis of protein spots of replicate gels of a treatment with the spots of control gels could be performed qualitatively as well as quantitatively to determine the fold changes among treatments and controls. The fold change for up- or down-regulation was also confirmed using an integrated t-test within the software. Proteins showing a significant ($p< 0.05$) fold change in abundance compared to controls were considered as differentially expressed proteins. Following successful detection of proteins and careful qualitative, quantitative and

statistical analysis, these proteins were confirmed through manual spot-to-spot analysis. Once their authenticity was justified, they were considered candidates to excise for MS/MS analysis. The processed, detected and analyzed spots in the digitized image (example gel, Figure. 4) of each replicate gel of each treatment and control were aligned with the real spots of the gel during spot excision process using the PDQuest controlled spot cutter excision robot, which was also optimized to minimize errors.

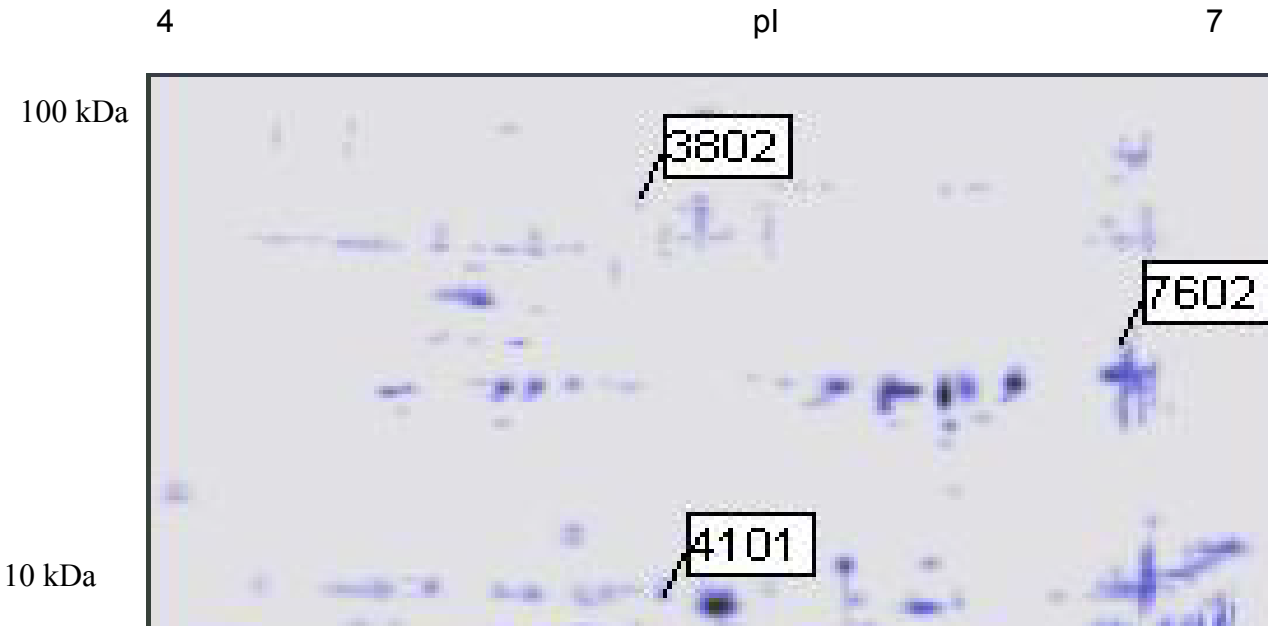


Figure 4. Representative 2-D electrophoresis protein profiles resolved from gill tissue of male zebrafish exposed to 10 ppb of ibuprofen for 14 days. Proteins were extracted from zebrafish gills and separated in the first dimension by IEF using immobilized dry strips, pH 4-7. Separation in the second dimension was performed using gradient gels (8-16%) followed by bio-safe Coomassie staining. Differentially expressed proteins are marked with their SSP number as assigned by PDQuest software and were excised from gels and identified by ESI-MS/MS.

4.3.1 Comparative Study of Ibuprofen Exposed Protein Spots

The level of protein expression was examined by comparing electrophoretic profiles of 1 ppb and 10 ppb in 4-7 pI range and 10 ppb and 100 ppb in 7-10 pI range exposed to ibuprofen, and unexposed gill tissues. Our optimized 2-DGE protocol and manually selected spot detection

parameters resulted in the detection of 300 ± 10 (range of 3 replicate gels) spots in replicates of control samples in the pI range of 4-7 and molecular weight range of approximately 12 kDa-100 kDa. In ibuprofen-exposed gill tissue the number of spots in replicate gels tended to increase to 315 ± 6 spots for the 1 ppb exposure and 335 ± 12 spots for the 10 ppb exposure. The numbers of protein spots detected in replicate samples of gill unexposed to ibuprofen in the pI range of 7-10 tended be fewer than those detected in the 4-7 pI range (270 ± 10). As in the lower pI range, this spot number tended to increase in those gels from exposed gills to 300 ± 9 in gill tissue exposed to 10 ppb and 330 ± 12 in 100 ppb. Table 1 displays the detected spots in the control and 1 ppb, 10 ppb and 100 ppb of ibuprofen exposed samples in pI range of 4-7 and 7-10. The bar diagram corresponding to the number of spots detected as shown in Table 1 is depicted in Figure 5.

Our PDQuest analysis was able to detect up- and down-regulated spots in the ibuprofen exposed samples. The number of up-regulated consensus (seen in all 3 replicates) proteins in 1 ppb and 10 ppb exposure of ibuprofen in pI range of 4-7 was 6 and 23 respectively, while down-regulated proteins were 1 and 3 at the two concentrations. The number of up-regulated proteins in 10 and 100 ppb exposure of ibuprofen in the pI range of 7-10 was 2 and 5 respectively, while down-regulated proteins were 12 and 11 at the two concentrations.. Table 2 shows the number of up- and down- regulated spots, and, Figures 6 and 7 show the bar diagrams corresponding to the up –and down-regulated protein spots in pI 4-7 and 7-10, respectively. Table 3 and 4 shows the up-and down regulated protein spots with their estimated Mr, pI, and fold changes in 1 ppb and 10 ppb of ibuprofen exposed gill proteins in pI range 4-7, respectively, as revealed by PDQuest. Tables 5 and 6 show the up-and down- regulated protein spots, their estimated Mr, pI and fold changes in 10 and 100 ppb ibuprofen exposed gill proteins in pI range of 7-10. In our PDQuest analysis we identified some proteins (4) which appeared in treatment samples but were absent in

controls. These proteins are reported with + sign in the table. Similarly, a – sign represents proteins present in the controls but absent in treatments. All the new proteins detected by PDQuest resulting from exposure were in the acidic pI range while those undetected in the exposed samples were in the basic pI range. From the location of expressed proteins, it was also observed that most of the differentially expressed proteins in the samples of exposed gels occurred in the range of MW under 100kDa and pI range below 9.1. PDQuest analysis was also able to detect proteins beyond 9.1 pI, and above 100 kDa. However, these proteins were less abundant, lacked consensus among the replicates were of poor quality and consistency and were therefore not subjected to PDQuest analysis or excision.

Table 1. Total number of protein spots in control and 1 ppb, 10 ppb and 100 ppb of ibuprofen exposed gill samples in 2D gel as detected by PDQuest, in pI ranges of 4-7 and 7-10. The X signs underneath pH 4-7 and 7-10 indicate that the corresponding exposure concentration was not studied.

	pI 4-7	pI 7-10
Control	300 ± 10	270 ± 10
1 ppb	315 ± 6	x
10 ppb	335 ± 12	300 ± 9
100 ppb	x	330 ± 12

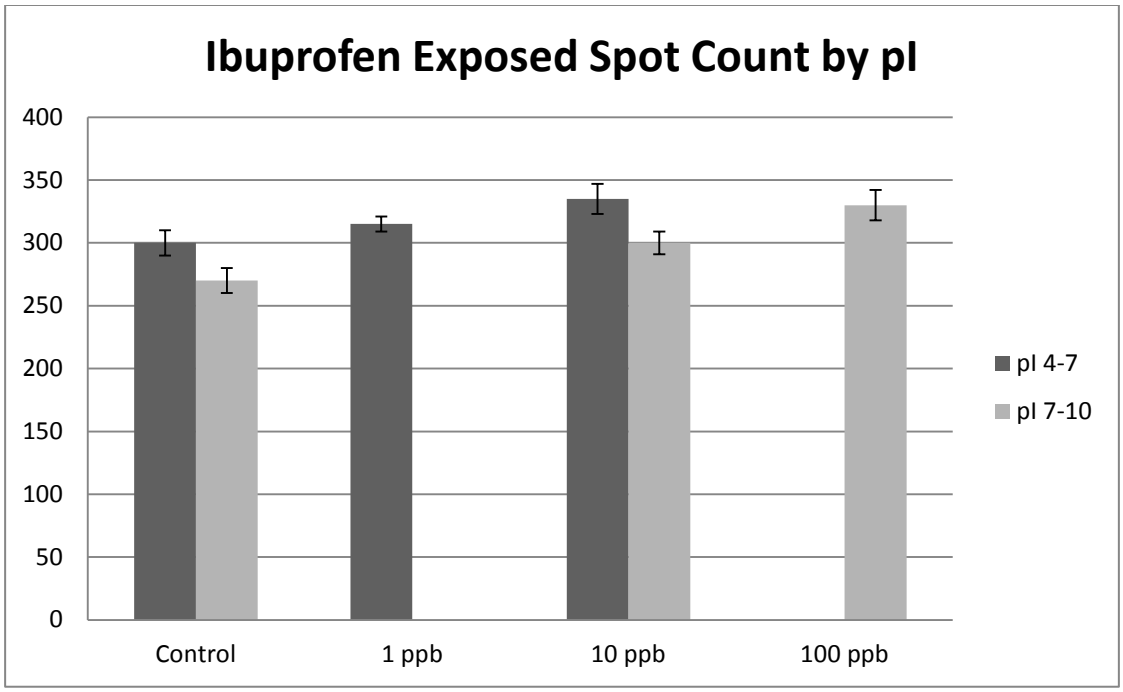


Figure 5. Bar diagram showing the total number of protein spots in 2D gel exposed to 1 ppb, 10 ppb, and 100 ppb of ibuprofen in 4-7 pI and 7-10 pI. The error bar shows the difference in the number of proteins (i.e. range) in three replicate gels.

Table 2. Table showing the total number of up- and down- regulated proteins in the gill tissue samples due to the exposure of 1ppb, 10ppb and 100 ppb of ibuprofen in pI range 4-7 and 7-10.

NSAID	pI Range	Exposure Concentration, (ppb)	Up-Regulated	Down - Regulated	Total
Ibuprofen	4-7pI	1	6	1	7
		10	23	3	26
Ibuprofen	7-10 pI	10	2	12	14
		100	5	11	16

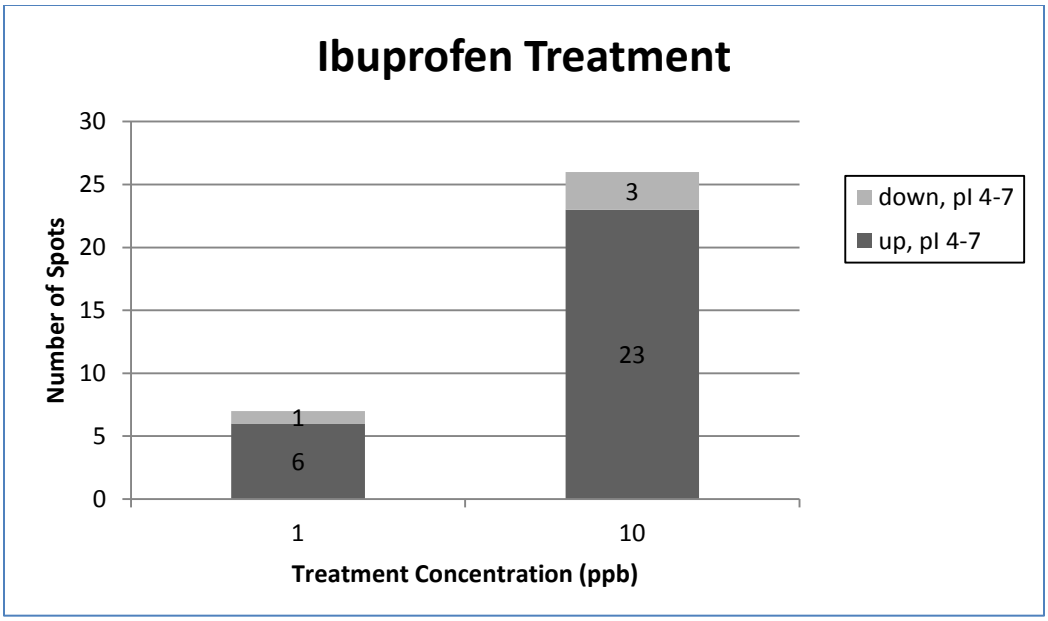


Figure 6. Bar diagram showing the up- and down- regulated protein spots due to the exposure of ibuprofen concentration of 1 ppb, and 10 ppb in range 4-7 pI. The dark color shows the total number of up-regulated proteins, and the light color shows the total number of down-regulated proteins.

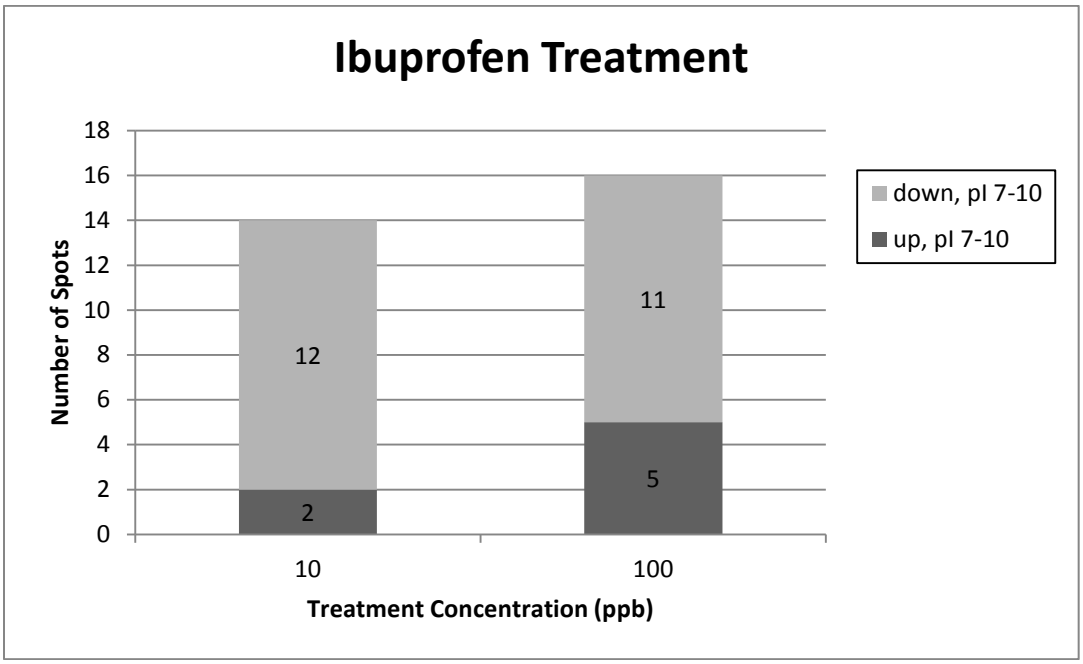


Figure 7. Bar diagram showing the up- and down- regulated protein spots due to the exposure of ibuprofen concentration of 10 ppb, and 100 ppb in range 7-10 pI. The dark color shows the total number of up-regulated proteins, and the light color shows the total number of down-regulated proteins.

Table 3. Up-and down-regulated protein spots and their fold changes in 1 ppb of ibuprofen exposed gill proteins in pI range 4-7, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot.

Up- Regulated			
SSP#	Fold Change	ProteinMass (Mr) kDa	Protein- pI Value
4102	1.5	18.0	6.5
8001	3.5	15.0	6.9
1603	2.3	42.0	5.4
4101	2.0	18.0	5.4
3101	2.0	42.0	4.2
7602	2.0	45.5	4.5
Down-regulated			
1605	0.3	15.0	5.1

Table 4. Up-and down- regulated protein spots with their fold changes in 10 ppb of ibuprofen exposed gill proteins in pI range 4-7, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (+) sign indicates that the spot is absent in its counterpart position in controls.

Up- Regulated			
SSP#	Fold Change	ProteinMass (Mr), kDa	Protein pI value
5104	2.0	16.3	6.7
1608	4.1	15.0	5.0
8401	1.2	12.0	5.0
9001	2.0	100.0	4.8
6102	2.0	80.0	4.9
2502	2.0	48.0	4.5
6402	2.0	90.0	6.1
8201	2.0	100.0	6.3

1102	2.0	15.0	6.3
3802	+	57.0	5.2
4102	1.5	15.5	5.2
8001	3.4	18.0	6.0
4101	+	17.0	4.8
3101	2.0	42.0	4.2
7602	+	75.0	6.8
2504	1.5	50.0	4.7
5104	2.0	16.0	6.5
5506	1.5	65.0	5.4
8201	1.2	15.0	6.5
5503	1.5	60.0	5.3
8803	+	100.0	7.3
3204	+	40.0	5.0
7203	3.0	72.0	6.6
Down- Regulated			
6301	0.5	70.0	6.5
6302	0.5	70.0	6.7
7901	0.5	95.0	5.8

Table 5. Up-and down- regulated protein spots and their fold changes in 10 ppb of ibuprofen exposed gill proteins in pI range 7-10, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 10 ppb exposed ibuprofen in range 7-10 pI but is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Review tools.

Up- Regulated			
SSP#	Fold Change	ProteinMass (Mr), kDa	Protein pI Value
7891	2.0	15.0	8.2
4702	2.0	15.0	8.0
Down -Regulated			
6803	-	22.0	9.0
4001	0.5	90.0	8.5

7801	0.5	17.0	6.7
3802	-	75.0	7.9
6403	-	30.5	7.5
6601	-	35.5	8.9
6603	-	25.0	7.7
6604	-	25.0	8.2
6902	-	20.0	8.5
7104	0.7	17.5	7.5
8901	-	20.0	7.8
9901	-	15.5	8.0

Table 6. Up-and down- regulated protein spots and their fold changes in 100 ppb of ibuprofen exposed gill proteins in pI range 7-10, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 100 ppb exposed ibuprofen in range 7-10 pI. However, it is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Analysis tools.

Up- Regulated			
SSP#	Fold Change	Protein Mass (Mr), kDa	Protein pI Value
7003	2.0	33.0	9.1
5202	2.0	30.0	8.5
2506	5.3	45.0	8.7
3201	3.0	18.0	7.6
1608	2.0	57.0	7.5
Down- Regulated			
6101	0.8	30.0	7.8

8903	-	15.5	8.3
3802	-	55.0	8.6
6403	-	30.5	7.5
6601	-	35.5	8.9
6603	-	25.0	7.7
6604	-	25.0	8.2
6902	-	20.0	8.5
7104	-	17.5	7.5
8901	-	20.0	7.8
9901	0.75	17.5	8.0

4.3.2 Comparative Study of Naproxen Exposed Protein Spots

The protein profiling for naproxen exposed samples was performed with 10 ppb and 100 ppb exposures with gels in both acidic and basic pI ranges, and was compared with control samples using PDQuest analysis techniques. As with ibuprofen exposure, naproxen exposure also resulted in differential expression of several proteins compared to control samples. In pI range 4-7 the total proteins detected in controls was 310 ± 12, in 10 ppb 335 ± 12, and in 100 ppb 350 ± 8. However, in 7-10 pI range, the numbers of spots in the replicate gels decreased to 250 ± 10 in control. The detected spot number in 10 ppb and 100 ppb exposure of naproxen in range 7-10 pI increased to 290 ± 10 and 330 ± 10, respectively. The protein profiling and the corresponding bar diagrams are shown below in Table 7 and Figure 8.

The PDQuest analysis revealed 2 proteins up-regulated in 10 ppb and 3 in 100 ppb, in 4-7 pI range. Similarly, the analysis found 7 and 12 proteins down-regulated in 10 ppb and 100 ppb of naproxen in 4-7 range, respectively. The number of proteins up-and down-regulated in 7-10 pI

range in 10 ppb and 100 ppb of naproxen was 4, 8 (up) and 6, 5 (down), respectively. Table 8 shows the number of up- and down- regulated spots caused by the exposure of 10 ppb and 100 ppb of naproxen, and, Figures 9 and 10 show the bar diagrams corresponding to the up –and down-regulated protein spots in pI 4-7 and 7-10, respectively. Also seen in Tables 9 & 10, are the up-and down- regulated protein spots and their fold changes in 10 ppb and 100 ppb of naproxen exposed gill proteins in pI 4-7, respectively, along with the estimated Mr & pI value from PDQuest analysis. Similarly Tables 11 and 12 show the up-and down- regulated protein spots and their fold changes in 10 ppb and 100 ppb of naproxen exposed gill proteins in pI 7-10, respectively, along with the estimated Mr & pI value from PDQuest analysis. In our PDQuest analysis we identified some proteins present in exposed samples but absent in controls which had consensus among gels. These proteins are reported with + sign in the tables below. Similarly, – sign indicates that their detection is reversed in controls and exposed members of the gel.

The PDQuest analysis detected 7 new consensus proteins in member gels in the basic pI range, all with a high level of confidence. A total of 17 proteins in controls whose counterpart, as confirmed by Mr and pI value of the location were missing and undetectable level in acidic pH range. Most of the differentially expressed proteins in the samples of exposed gels were spread over the MW under 75kDa and pI range below 9.8. As in the ibuprofen analysis, PDQuest analysis was also able to detect proteins beyond 9.1 pI, and 75 kDa. However, these proteins were less abundant, lacked consensus and were not further analyzed.

Table 7. Total number of protein spots in control, 10ppb & 100 ppb of naproxen exposed gill tissue samples in 2D gel as detected by PDQuest, in pI range of 4-7 and 7-10

	pH 4-7	pH 7-10
Control	300 ± 12	250 ± 10
10 ppb	335 ± 10	290 ± 10
100 ppb	350 ± 8	330± 10

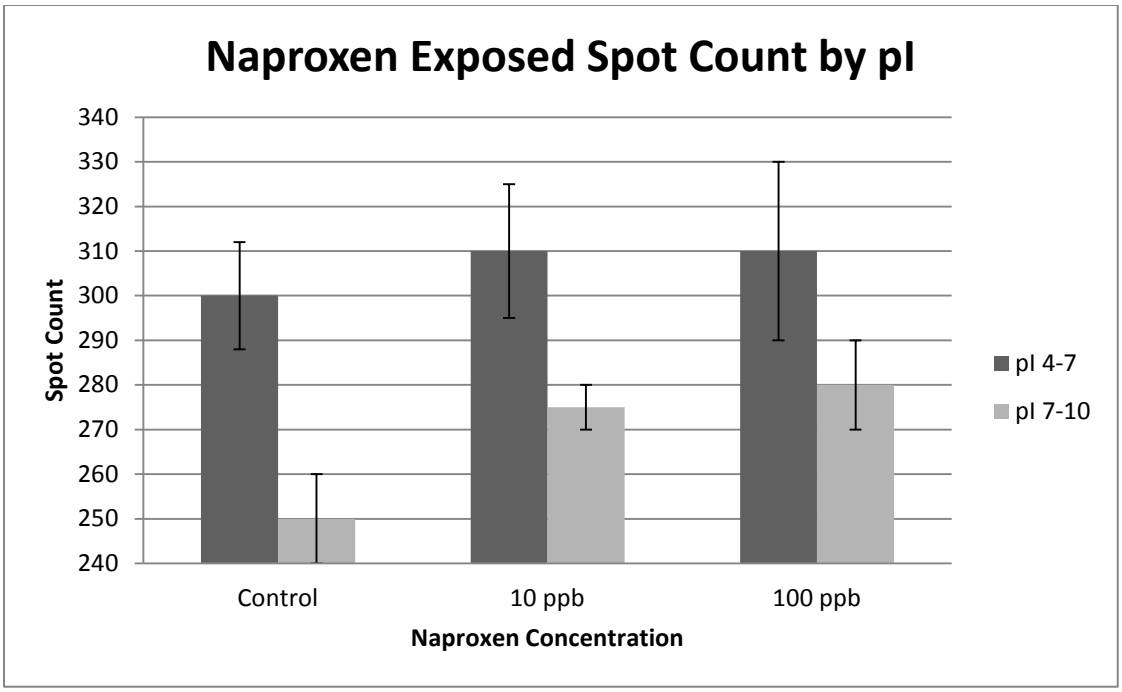


Figure 8. Bar diagram showing the total number of protein spots in 2D gel exposed to 10 ppb and 100 ppb of naproxen in 4-7 pI and 7-10 pI. The error bar shows the difference (range) in the number of proteins in three replicate gels.

Table 8. Total number of up-and down- regulated protein spots in 10 and 100 ppb of naproxen exposed gill proteins in pI ranges 4-7 and 7-10 as depicted in Figure 9 and 10

NSAID	pI Range	Exposure Concentration (ppb)	Up-Regulated	Down-Regulated	Total
Naproxen	4-7 pI	10	2	7	9
		100	3	12	15
Naproxen	7-10 pI	10	4	6	10
		100	8	5	13

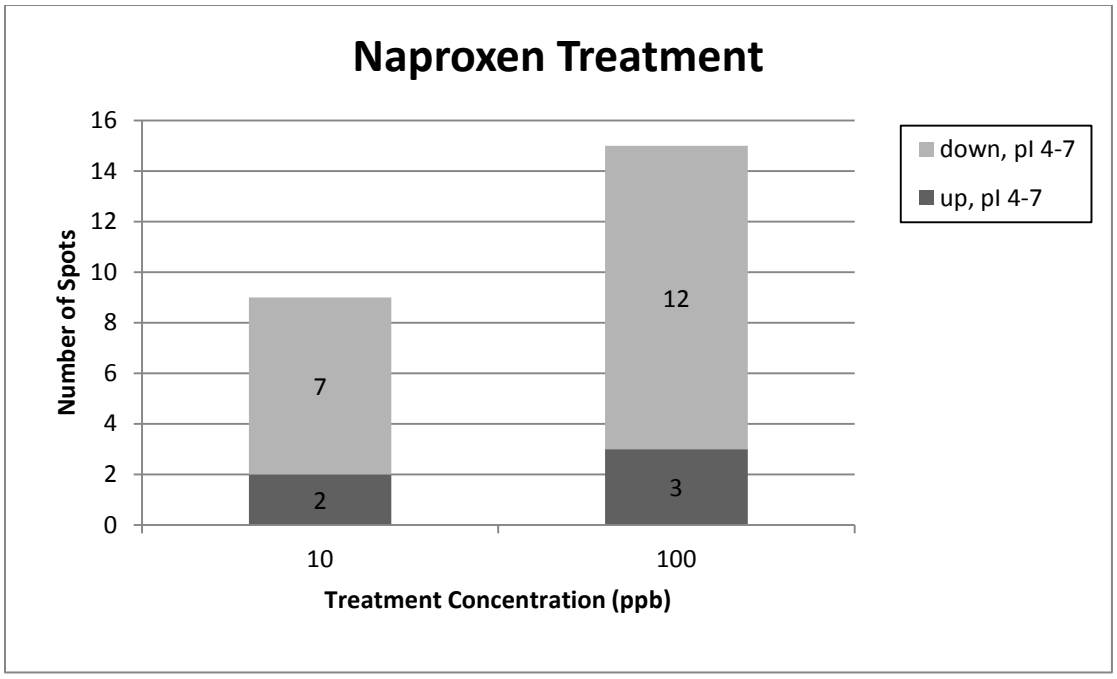


Figure 9. Bar diagram showing the up- and down- regulated protein spots due to the exposure of naproxen concentration of 10 ppb, and 100 ppb in range 4-7 pI. The dark color shows the total number of up-regulated proteins, and the light color shows the total number of down-regulated proteins.

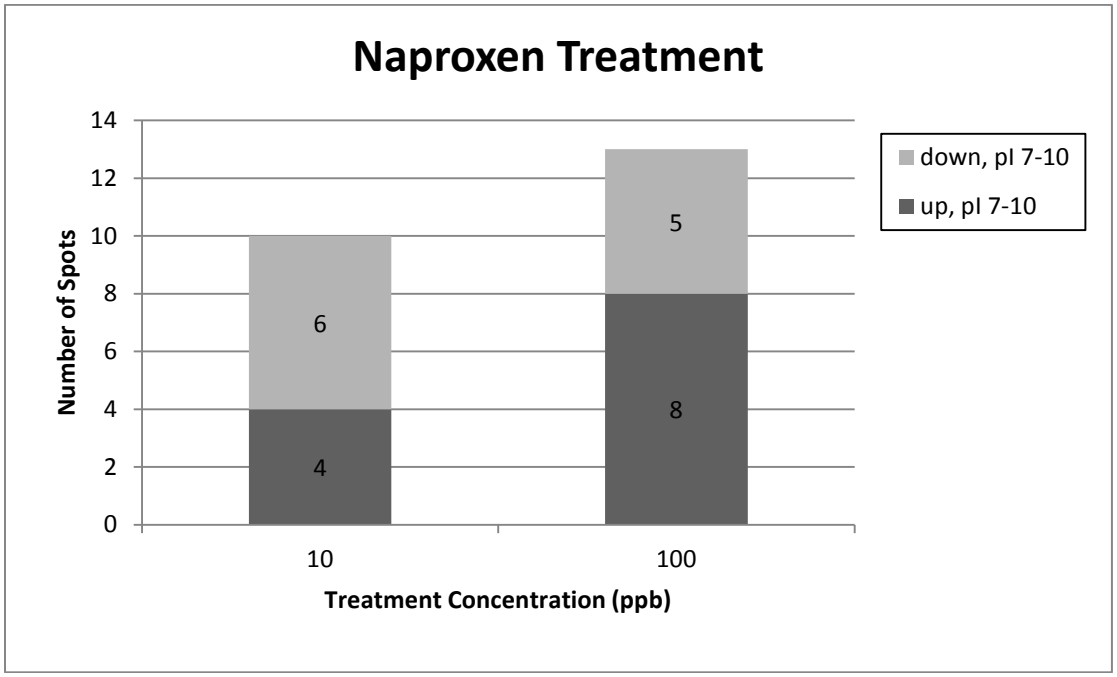


Figure 10. Bar diagram showing the up- and down- regulated protein spots due to the exposure of naproxen concentration of 10 ppb, and 100 ppb in range 7-10 pI. The dark color shows the total number of up-regulated proteins, and the light color shows the total number of down-regulated proteins.

Table 9. Up-and down- regulated protein spots and their fold changes in 10 ppb of naproxen exposed gill proteins in pI 4-7, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 10 ppb exposed naproxen in 4-7 pI. However, it is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Analysis tools.

Up-regulated			
SSP#	Fold Change	Protein Mass (Mr), kDa	Protein pI Value
0103	2.0	75.5	4.7
7602	2.4	18.0	6.4
Down-regulated			
6201	2.0	25.0	5.8
8102	0.5	15.5	4.8
3301	-	52.0	6.8
3302	-	52.0	6.7
4801	-	45.0	6.2
5403	-	35.0	4.4
6802	-	20.0	5.2

Table 10. Up-and down- regulated protein spots and their fold changes in 100 ppb of naproxen exposed gill proteins in pI range 4-7, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 100 ppb exposed naproxen in pI range 4-7 pI but is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Analysis tools.

Up-regulated			
SSP#	Fold Change	ProteinMass (Mr), kDa	Protein pI Value
3101	1.6	18.0	4.6
2303	3.0	32.7	4.7

1001	3.4	17.0	6.2
Down-regulated			
8103	0.5	15.5	4.7
8210	0.5	15.0	6.2
9001	0.5	15.0	6.8
2103	0.5	30.0	5.0
4101	0.5	35.0	4.7
8102	0.5	15.0	4.8
3301	-	52.0	6.8
3302	-	52.0	6.7
4801	-	45.5	6.2
5403	-	35.0	4.4
6201	-	25.5	5.8
6802	-	20.0	5.2

Table 11. Up-and down- regulated protein spots and their fold changes in 10 ppb of naproxen exposed gill proteins in pI 7-10, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 10 ppb exposed naproxen in range7-10 pI but is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Analysis tools.

Up- Regulated			
SSP#	Fold Change	Protein Mass (Mr), kDa	Protein pI Value
4202	2.0	32.5	7.7
4501	2.5	35.0	8.4
7203	1.6	15.5	8.8

8102	4.7	14.7	9.8
Down-Regulated			
5602	0.8	45.5	8.1
1401	0.5	65.0	9.2
1701	-	22.0	8.0
1702	-	55.5	7.6
1903	-	50.0	8.2
6203	0.8	48.0	8.1

Table 12. Up-and down- regulated protein spots and their fold changes in 100 ppb of naproxen exposed gill proteins in pI 7-10, along with the estimated Mr & pI value from PDQuest analysis. The SSP number is automatically generated by software and is used to assign the particular spot. The (-) sign indicates that the spot is absent in all the member gels of 100 ppb exposed naproxen in range 7-10 pI but is present in the counterpart position in control gel, as verified by spot-to-spot analysis using 3D and Spot Analysis tools.

Up-Regulated			
SSP#	Fold Change	Protein Mass (Mr), kDa	Protein pI value
2603	2.0	42.0	7.9
0701	+	70.0	8.2
0702	+	70.5	8.5
0901	+	68.0	7.9
1401	+	65.0	9.2
1701	+	58.0	7.6
1903	+	50.0	8.2
1702	+	55.0	7.6
Down-Regulated			

3302	0.5	38.0	8.5
8102	0.7	17.0	8.0
9102	0.5	15.0	9.0
6203	0.5	48.0	8.1
5602	-	45.5	8.1

4.4 Protein Identification

Our approach to identify up- and down- regulated protein resulting from exposure of ibuprofen and naproxen using 2D-gel electrophoresis followed by mass spectrometry based identification using MASCOT was able to identify a total of 14 differentially expressed proteins. This final number was partially limited by requiring that all protein spots considered to be differentially expressed had to demonstrate consensus among 3 replicate gels. Additionally, inherent sensitivity limitations of capillary ESI LC/MS (as opposed to nanospray) restricted the analysis to relatively abundant proteins. However, the optimized protocol for digestion and post digestion process in a single deactivated glass insert with resulting decreased loss of proteins due to adsorption, helped to overcome the sensitivity limitations of our system (Castellanos-Serra, et al. 2005). All the proteins identified and their change in abundance specific to each treatment of ibuprofen and naproxen are summarized in Table 15.

4.4.1 Protein Identification in Ibuprofen Treatment

Altogether 63 proteins were found to be expressed differentially at higher concentration of ibuprofen compared to their counterpart in the control gel. These proteins were excised in duplicates, one from the control and other from the counterpart from the treated gel, processed for enzymatic digestion, LC/MS/MS analysis and searched on the MASCOT databases for

identification. A total of 11 proteins were identified, 9 of which were up-regulated and 2 were down-regulated. These proteins all had MW values less than 60 kDa. Spot number shown in Table 13 represents the numbers utilized on the gel for PDQuest analysis. All other information shown in the Table 13 was obtained from the MASCOT database system.

4.4.2 Protein Identification in Naproxen Treatment

In naproxen exposures, a total of 41 proteins were excised which showed differential expression compared to their counterpart in the control gels. These proteins were also excised in duplicates one each from control and naproxen treatment. Each of them was treated separately for pre- digestion, digestion, post digestion steps and Mass Spectrometric analysis followed by MASCOT database analysis. A total of 6 proteins were successfully identified, with 5 proteins as up-regulated and one protein as down-regulated. Most of the proteins identified had MW values under 60 kDa. Table 14 shows the accession number, calculated *Mr* & *p*I value, the MOWSE ion score, and number of peptide matches. The spot number was used to represent the spot in the gel for analysis and excision.

Table 13. Up-and down-regulated protein spots and their fold changes in three replicates of ibuprofen exposed gill proteins, along with the estimated Mr & pI value from PDQuest analysis. The corresponding protein name, gi value, and calculated Mr & pI value as identified by ESI-MS. The MS-MS analysis followed by MASCOT shows the number of peptides matched, their score and % coverage of the amino acid sequence of the corresponding protein. (+) Sign represents the presence of the protein spot in all the replicates of 10 ppb ibuprofen exposed but absent in the controls and 1 ppb exposures. All of the proteins were up-regulated in 10 ppb exposure except Apolipoprotein A-I and Nucleotide Diphosphate Kinase (NDKB). Keratin 5, alphaglobin like and Transferrin a were not detected in 1ppb exposure while ba1 globin, aA1 globin isoform 1 and ictacalcin were not altered their abundance in 1 ppb exposure of ibuprofen. Values in parentheses represent result of confirming analysis of same spot from a second replicate. NA = not analyzed.

SSP#	Accession number	Fold Change for Replicates	MASCOT				% Coverage	Identified Proteins
			Nominal Mass (Mr) kDa	Calculated pI value	Mowse Score	No. of Peptide Matches		
4102	gi 68384019	1.2, 1.4, 1.3	15.5 (15.5)	6.6 (6)	182 (181)	15 (8)	55 (44)	Apolipo protein
8001	gi 1477579	1.7, 6.3, 2.6	11.6 (16.3)	7.9 (7.9)	63 (38)	5 (3)	15 (8)	Beta globin
1603	gi 28279111	2.9, 2.2, 1.9	41.7 (41.7)	5.3 (5.3)	78 (76)	9 (10)	31 (28)	Bactin 1
1605	gi 18858281	0.1, 0.2, 0.5	30.2 (30.2)	5.06 (5.06)	75 (73)	11 (11)	38 (34)	Apolipoprotein A-I
8401	gi 47086523	1.1, 1.2, 1.1	10.4 (10.4)	5.03 (5.03)	110 (70)	3 (2)	13 (13)	ictacalcin
3802	gi18858425	+	58.5 (58.5)	5.3 (5.3)	58 (51)	3 (2)	5 (3)	Keratin 5
8001	gi18858329	2.4, 1.9, 2.9	16.4 (16.3)	6.7 (7.9)	126 (38)	8 (3)	37 (8)	Ba1 globin
4101	gi 74315904	+	15.5 (15.5)	5.8 (8.8)	146 (92)	9 (7)	53 (43)	Predicted Similar to aA1 globin isoform 1

7602	gi 47264590	+	73.4 (73.4)	6.81 (6.81)	111 (111)	5 (4)	10 (7)	Transferrin a
7801	gi 41053595	0.5, 0.5, 0.4	17.1 (17)	7.8 (7.8)	53 (106)	5 (6)	36 (23)	Nucleoside diphosphate kinase B
1608	gi125813907	6.5, 2.3, 3.6	15.5 (NA)	5.1 (NA)	141 (NA)	6 (NA)	57 (NA)	aA1 globin isoform 1

Table 14. Up-and down- regulated protein spots and their fold changes in three replicates of naproxen exposed gill proteins in pI 4-7, along with the estimated Mr & pI value from PDQuest analysis. The corresponding protein name, gi value, and calculated Mr & pI value as identified by ESI-MS. The MS-MS analysis followed by MASCOT shows the number of peptides matched, their score and % coverage of the amino acid sequence of the corresponding protein. All the proteins shown in table were up-regulated except NDK. TMP 1 in 10 ppb and UbA-52 and Hemoglobin alpha adult-1 in 100 ppb exposure did not satisfy our criteria (were not consensus in all the replicates) of detection in PDQuest analysis

SSP#	**Accession Number**	**Fold Change**	**MASCOT**				**% Coverage**	**Identified Protein**
			Mass (Mr) kDa	**Calculated pI value**	**Mowse Score**	**No. of Peptide Matches**		
7203	gi 33468640	2.5, 1.1, 1.2	14.7 (14.7)	8.8 (8.8)	50 (47)	3 (3)	5 (3)	Hemoglobin α-adult 1
8102	gi 80751129	2.1, 8.0 4.1	14.7 (NA)	9.8 (NA)	40 (NA)	4 (NA)	31 (NA)	Ubiquitin A-52
6203	gi 41053595	1.1, 0.9, 0.3	17.1 (17.1)	7.8 (6.8)	106 (51)	6 (3)	43 (23)	Nucleoside diphosphate kinase B
3101	gi 74315904	1.8, 1.2, 1.9	15.5 (15.5)	4.75 (4.75)	45 (26)	3 (1)	15 (12)	Alphaglobin Like
2303	gi 28277767	1.5, 2.1, 2.4	32.7 (32.7)	4.7 (4.69)	227 (200)	16 (12)	21 (21)	Tmp 1 protein

1001	gi 18858329	2.9 3.4 3.8	16.3	6.7	66	7	51	Ba 1 globin

Table 15. Summary of identified proteins with their alterations, specific to each treatment of ibuprofen and naproxen, compared to their counterpart in control. ↑ shows up-regulated, ↓ shows down-regulated, ± indicates unaltered status and, X = not detected, in the treatment. * indicates this protein is up-regulated in dose dependent manner in naproxen treatment. # indicates the down-regulation is consistent in all the treatments of ibuprofen and naproxen.

Name of the Protein	1ppb	10ppb		10ppb	100ppb		10ppb	100ppb		10ppb	100ppb
	pI 4-7	pI 4-7		pI 4-7	pI 4-7		pI 7-10	pI 7-10		pI 7-10	pI 7-10
	ibu	ibu		napro	napro		ibu	ibu		napro	napro
Apolipoprotein	↑	↑									
Beta Globin	↑	↑									
Bactin 1	↑	↑									
Apolipo A-I	↓	X									
ba 1 globin *	±	↑		↑	↑						
aA 1 globin isoform 1	±	↑									
Ictacalcin	±	↑									
Keratin 5	X	↑									
"predicted similar to aA1 globin isoform 1" *	X	↑		↑	↑						
Transferrin a	X	↑									
NDK #							↓	↓		↓	↓
UbA-52										↑	X
Hemoglobin alpha adult-1										↑	X
tmp				X	↑						

CHAPTER 5

DISCUSSION

5.1 General Experimental Approach

The overall goal of this work was to establish a standardized system which would permit evaluation of proteomic changes in aquatic organisms subjected to chronic exposure to environmental toxicants within the University of North Texas (UNT) aquatic toxicology group and use this system in an evaluation NSAID exposure to zebrafish. A syringe pump diluter system achieved chronic trace exposure concentrations and successful protocols were established for tissue collection, storage and protein extraction in optimized acidic and basic pH buffer systems and subsequent cleanup. The proteins were then subjected to 2-DGE.

Our choice of gill tissue for profiling of differential expression of proteins after exposure to ibuprofen and naproxen was based on the central role this tissue has for multiple functions making it an especially appropriate indicator of environmental chemical insult (Goldsmith 2004). Towards that end, I was able to find changes in abundances of several proteins after exposure to both ibuprofen and naproxen.

We used 2D-gel electrophoresis to identify the impact of the NSAID exposure on the differential expression of proteins. High quality of 2D-gel preparation is critical to distinguish treatment-specific changed patterns of proteins from natural variability. The total variation of this method is the sum of the biological variability within the samples and technical measurement errors. The variation of 2-DGE technique is due to both qualitative (number ofproteins) and quantitative (amount of proteins). Variations seen in different number of proteins in the replicate gels of the control or the treatment were excluded as we used 100% matching strategy to produce consensus protein patterns valid for all 2-DE gels and ensure analysis of the

same proteins on each gel in the experiment. Furthermore, the quantitative variations can be evaluated by analyzing the RSDs of all detected proteins in replicates which was 20%- 25% in the controls. This variation increased in replicates of exposed gels of varying concentrations. There were highly variable proteins, especially those lying on the border regions, (with pI values above 8.5) and those of low abundance. The use of PDQuest densitometric comparisons after the digitization of gel images allows convenient estimation of relative abundance among replicates and treatments. However, in such comparisons, the reproducibility of the sample preparations was crucial. The poor quality of isoelectric focusing of extremely basic proteins is a special problem for determining relative abundances, and becomes the major problem for establishing consensus among replicates (Encarnación, et al. 2005). I was able to identify only a total of six proteins in the basic pH range from both ibuprofen and naproxen exposures, as compared to acidic pH range in which we identified a total of eleven proteins.

Our criteria for confirming changes in abundances included a requirement for consistency among three replicates of each treatment and three replicates of control. There were several spots which were present only in two replicates, which reduced the number of confirmed protein identifications. Another limiting factor the total number of proteins identified included digests with the presence of multiple proteins which confounded MS identification. This situation arose when proteins were incompletely separated on gels or when gel spots from member gels were combined to increase abundance of trace proteins, which could increase the procedural or human errors.

About 30% to 35% of protein spots were changed in expressions upon exposure of 10 ppb and 100 ppb of NSAID, respectively, indicating substantial shifts in the protein profile including changes in soluble proteins and membrane proteins;

5.2 Biology of Proteins Identified

The 2-D gel analysis of zebrafish gill tissue indicated that the differential expression of dozens of proteins was influenced by exposure to our selected NSAIDs, ibuprofen and naproxen. However, only 14 of the differentially expressed proteins met our criteria for consistency among replicate gels and subsequent successful identification by MASCOT searching of MS/MS spectra from digested peptides. The differential expression of those proteins successfully identified was dominated by various types of proteins including globins, cytoskeleton structure/function (β-actin), motility (tropomyosin), or regulatory pathways (NDK), reflecting the central role of gill in oxygen and ion exchange. Higher order responses related to inflammation, apoptosis or cell death may be inferred from pathway analysis.

5.2.1 Globular Proteins

Globular proteins have a tertiary structure, thus letting the amino acids that make up the proteins to fold up and result in a spherical structure. These types of proteins are soluble in water with the hydrophobic amino acids largely protected within the molecule's interior with polar amino acids found largely on the exterior. These proteins include βA1 globin, βA2 globin or HBB (Hemoglobin beta chain), hemoglobin α adult -1, α globin isoform 1, β globin chain of hemoglobin, and transferrin. These globins are small, globular heme-binding proteins and are involved in efficient transport of oxygen from the respiratory surfaces (lungs, gills, skin) to the inner organs. Their property of solubility makes them multi-functional in the body useful in transport, as enzymes, regulators, messengers, and also to bind with different compounds, thus facilitating their transport from areas of higher concentration to areas of lower concentration. Hemoglobin is a type of globular protein which carries oxygen from respiratory organs in red blood cells. It comprises four hetero tetramers consisting of two α and two β- globin chains

attached head to head in medaka (Oryzias latipes), where each of the chain binds with one hydrophobic pocket in a precise fashion (Maruyama, et al. 2004).

Expression of mutated or non-functional globin polypeptides leads to severe disorders such as sickle cell anemia or thalassemia. In the "classical" thalassemia, the β-thalassemia, the excess of α-chains cannot build heterotetramers with β-chains in the bone marrow thus increasing membrane rigidity. Ba1-globin is found to be 96% identical to βa-globin and differs only in the position of three amino acid residues. Whereas, the αa1-globin isoform differs in one amino acid from α-globin. Hemoglobin alpha adult-1 is considered to be a full length α-globin. The deletion of α-chain gene results in alpha thalassemia (Bertolini, et al. 2001; Flint, et al. 1986). The result of these genetic disorders is decreased efficiency of gas exchange via induced anemia. Up-regulation of the various globins seen in our study may indicate a compensatory response to decreased capacity for oxygen transport associated with NSAID exposure.

Transferrin (Tf) is a globular protein in plasma with a primary transport function. It is responsible for the transport of Fe^{+3} and controls the level of free iron in plasma, as it is a highly abundant iron binding protein. It is a glycoprotein chelator that binds the iron tightly but reversibly. Iron chelation facilitated by transferrin maintains Fe^{3+} in a soluble form under physiological conditions; it also facilitates regulated iron transport and cellular uptake, and maintains Fe^{3+} in a redox-inert state, preventing the generation of toxic free radicals. Tf offers an implicit defense mechanism against systemic infections by depriving the potential pathogens of extracellular iron, which is essential for their growth. Transferrin is generally known to act as a negative acute phase protein (NAPP) during inflammation (i.e. it is downregulated during acute inflammation) (Buetler 1998). However, it has also been reported to act as a positive acute phase protein (PAPP) as well (Neves, et al. 2009). Furthermore, genetic study by Shakor et al.,

confirmed Tf as a biomarker for measuring the efficacy of simvastatin in the treatment of sickle cell anemia, and for prognosis of acute respiratory distress syndrome (Shakor, et al. 2011). Its role in clathrin-mediated endocytosis (also known as "receptor-mediated endocytosis") signaling, cell growth, homeostasis, proliferation, and apoptosis has also been demonstrated (Shakor, et al. 2011).

Few studies have examined the role of Tf in fishes. However, Tf has been demonstrated to be a positive acute phase protein in rabbits and rats (Bayne and Gerwick 2001) and this was confirmed in a rainbow trout study of inflammation triggered by injection of emulsified Aeromonas salmonicida lipopolysaccharide which resulted in Tf up-regulation (Russell, et al. 2006). Toxin exposure (1 ppb Microcystins) has also been found to up-regulate Tf in Japanese medaka (O. latipes) (Sanchez, et al. 2011). Our study found up-regulation of Tf in 10 ppb of exposure of ibuprofen. As the central role of Tf is cell growth, proliferation, delivery of Fe to the cells and as an APP, we can speculate its up-regulation may have been a compensatory response to restore homeostasis or to modulate stress associated with chemical exposure.

A major feature of the differential expression of proteins we observed in response to NSAID exposure was up-regulation of globins involved in the transport of oxygen from gills to other parts of the body and Tf which is a key player in Fe^{3+} transfer and metabolism. It has been shown that the presence of COX-2 inhibitors regulates Tf secretion (Yamaguchi, et al. 2008). Thus, since COX is the primary molecular target for NSAIDs, I can speculate that the combined up-regulation of globins and Tf occurs to maintain homeostasis in the face of NSAID-related challenges to overall regulation of oxygen and iron transport as well as direct effects on the iron containing moiety of the COX enzyme.

5.2.2 Ictacalcin

Ictacalcin is a transporter, regulator, and activator protein. It was originally identified in channel catfish (Ictalurus punctatus), and is a novel calcium-binding protein involved in chemosensory tissues and is highly expressed in barbel and olfactory mucosa and gills of several fish species (catfish, puffer fish, loach, trout, and zebrafish; (Porta, et al. 1996). However, this protein is absent in mammals (Kraemer, et al. 2008). Ictacalcin has been demonstrated to be a dioxin (TCDD) responsive gene in zebrafish and rainbow trout (Cao, et al. 2003)and was modestly up-regulated by exposure to esfenvalerate (a pyrethroid insecticide especially toxic to fish) smelt (Connon, et al. 2009). In this study, the relative abundance of this protein was up-regulated in the 10 ppb exposure but not in the 1 ppb exposure. Little is known about the role of ictacalcin or the significance of its responsiveness to TCDD (but see Cao et al., for a discussion of possible cross-talk between calcium signaling and TCDD AhR/ARNT activity; (Cao, et al. 2003)). Thus we can only speculate that the marginal up-regulation we observed might represent part of an, as yet uncharacterized, generalized response to xenobiotic stress that may be present in fish and absent in mammals.

5.2.3 Ubiquitin A-52

Ub A-52 protein is a 128 amino acid compact globular fusion protein with a 76 amino acid ubiquitin peptide fused to a 52 amino acid tail that is removed from ubiquitin before its maturation (Usami, et al. 2008). This protein has been shown to be highly expressed in inner ear of human where it has a homeostatic functional role (Kitoh, et al. 2007; Usami, et al. 2008). It is a highly conserved protein in all eukaryotes and is involved in selective degradation of intracellular proteins through the ubiquitin proteosome pathway. It is also involved in the maintenance and regulation of gene expression, signal transduction, induction of apoptosis by

extracellular signals, gene expression, stress response, RNA metabolic process, protein trafficking, and many other physiological processes in mammals (Capelson and Corces 2005; Dantuma, et al. 2006; Kinyamu, et al. 2005; Li, et al. 2003). A previous study on rat epithelial tissue revealed the up-regulation of this protein due to the induction of apoptosis caused by the consumption of NSAID (Chiou and Mandayam 2007). Up-regulation of ubiquitin protein will result in the degradation of proteins which in turn will increase the amino acid pool of the cell that can be used for fresh protein synthesis. This suggests the possibility of a typical homeostatic response after the exposure to 10 ppb of naproxen, which is essential to bring back the cell/organ to normal level of function. However, this protein was not detected as differentially expressed in our ibuprofen exposures and, though over-expressed by 10 ppb naproxen exposure, disappeared at the 100 ppb naproxen exposure. No clear conclusion can be reached regarding differential expression of ubiquitin in response to our treatments.

5.2.4 Apolipoproteins

Apolipoproteins are enzymes whose function is to bind lipids (e.g. cholesterol) in order to produce a lipoprotein complex that helps in the transport of water-insoluble lipids in water-based blood and lymphatic system and reduces plasma triglyceride levels (Martín de la Fuente, et al. 2010). A study in rat liver investigated apolipoprotein as a negative acute phase protein whose amount was reduced to 80 to 95 % during inflammation (Buetler 1998). The mutated gene of one of its isoforms in human, ApoE, results in the development of the Alzheimer disease (Monnot, et al. 1999; Roses and Saunders 1994) and another isoform, ApoA, has been shown to cause the development of atherosclerosis (Kawashiri, et al. 2002).

Apolipoprotein A1 is an isoform of apolipoprotein whose function is to solubilize lipoproteins, enzyme co-factors, receptor ligands, and lipid transfer carriers that regulate the

intravascular metabolism of lipoproteins. Furthermore, it is a major apolipoprotein of HDL and its mutant is associated with HDL deficiencies (including Tangier disease) and with systemic non-neuropathic amyloidosis (Li, et al. 2003). It has diverse protective roles in mammals as an anti-oxidant, anticlotting, anti-inflammatory and in anti-thrombic activities and shows APP response (Cabana, et al. 1989; Villarroel, et al. 2007) and has been demonstrated to act as a negative acute phase protein (Cerón, et al. 2005; Martín de la Fuente, et al. 2010). Down-regulation of Apoa1 in human serum is associated with rheumatoid arthritis, schizophrenia, and vascular dementia (Li, et al. 1993). However in a study using rainbow trout (O. mykiss) and Carp (Cyprinus carpio), the up-regulation of this protein was reported as a positive acute phase response (Villarroel, et al. 2007). This study observed down-regulation of apolipoprotein in response to 1 ppb ibuprofen exposure and no detectable apolipoprotein at 10 ppb exposure. Inhibition of COX has been shown to result in reduced expression of Apolipoprotein A1 (Horani, et al. 2004). The up-regulation of apolipoprotein isoforms has also been shown to affect the biosynthesis of cholesterol in Japanese medaka (O. latipes) in which their up-regulation was induced by exposure to the cyanobacterial toxin mcrocystins (Sanchez, et al. 2011). Thus, while generalized chemical stress may be expected to up-regulate expression of this protein, there is evidence that the down-regulation we observed may be attributable to the specific COX-inhibitory mode of action of ibuprofen. This does not, however, explain why no differential expression of apolipoprotein was observed in the naproxen exposures.

5.2.5 Structural Proteins

Structural proteins β-actin 1, keratin 5 and alpha tropomyosin (α-TMP) were found to be differentially expressed. β-actin 1 is involved in the formation of cytoskeleton as well as being a major cytoskeletal protein found in actin filaments (Wang et al. 2010). It is also involved as a

mediator of internal cell motility and in inflammation mediated by chemokine, cytokine and integrin signaling (Etienne-Manneville 2004; Lambrechts, et al. 2004; Olson and Sahai 2009; Tomas, et al. 2006). A study on human and mice intestinal cells, revealed a several fold up-regulation of β-actin 1 in response to stress associated with metals exposure (Calabro, et al. 2011). Thus the up-regulation of β-actin 1 observed in our study might be related to chemical stress and/or some unknown linkage to the inflammation or immune response of the animal.

Keratin 5 is a type II cytokeratin, often paired with keratin 14, and forms the intermediate filament (IF) of the cytoskeleton in basal keratinocytes of stratified epithelia (Huber, et al. 1994; Zatloukal, et al. 2004). A knockout mutation study of the genes of basal cells of epithelium in mice showed its association with a complex disease called epidermolysis bullosa simplex (Porter and Birgitte Lane 2003). Also, this protein has been show to be differentially expressed in papillomas of COX-1-deficient mice when compared with COX-2-deficient mice (Tiano, et al. 2002) indicating some kind of connection to COX metabolism. Mutations in its gene led to a disease that caused progressive and disfiguring reticulate hyper-pigmentation of the flexures, and is called Dowling-Degos disease (Betz, et al. 2006). Certain keratins such as keratin 1, keratin 8, and keratin 14 have physiological functions in addition to their cytoskeletal role. Since keratin 5 was downregulated at 1 ppb ibuprofen, up-regulated 10 ppb ibuprofen and was not found to be differentially expressed in naproxen-exposed treatements, we feel no conclusions can be drawn regarding the physiological significance of its differential expression.

Alpha tropomyosin (α-TMP) is an isoform of muscle tropomyosin found in zebrafish that is involved in a number of cellular events involving the cytoskeleton. It is an abundant cytoskeletal contractile protein and plays an important role in the cell motility and regulation of cardiovascular homeostasis. It has been demonstrated that TMP is essential for both cardiac

myofibril assembly and function and that this protein is an essential component of Alzheimer's disease (Gunning, et al. 2008; Houle, et al. 2007; Salas-Leiton, et al. 2009). It has been suggested to have a role in tumor suppression and has been implicated in hypertension, cancer, nemaline myopathy, and ulcerative colitis (Helfman, et al. 2008). Like Keratin 5, α-TMP differential expression was inconsistent with both up- and down-regulation observed in the naproxen exposures and no differential expression observed in the ibuprofen exposure making any conclusion regarding NSAID exposure difficult.

5.2.6 Nucleoside Diphosphate Kinase (NDK)

Nucleoside diphosphate kinase (NDK) is an enzyme that is required for the synthesis of nucleoside triphosphate (NTP) from nucleoside diphosphate and a phosphate donor. It also functions as a transcription activator in mammals with a net primary function of production of ATP at the expense of GTP conversion to GDP (Dexheimer, et al. 2009; Engel, et al. 1998). Additionally, it plays a negative role in apoptosis, positive regulation of keratinocyte differentiation, cell adhesion, proliferation, colony formation, invasiveness, and growth. It has been described in Japanese medaka, *O. latipes* (Lee and Lee 2000). A proteomic study detected suppression of NDK in zebrafish exposed to 0.5 ppm of perfluorooctane sulfonate (Shi, et al. 2009). It may have an indirect role in the formation of COX products via cyclic adenosine monophosphate (cAMP). It has been shown that the inhibition of NDK causes a substantial increase in cAMP activity catalyzing transformation of adenosine triphosphate (ATP) to cAMP (Kimura, et al. 2003; Lutz, et al. 2001). Increases in the level of cAMP should result in an increased production of prostaglandin E2 (PGE2). This might represent a compensatory response to replenish diminished PGE2 resulting from the direct effects of NSAID exposure. This was the

only protein we identified that was consistently downregulated by both ibuprofen and naproxen at all exposure concentrations.

5.2.7 Physiological Significance of Up and Down-Regulation of Proteins by Exposure to Ibuprofen and Naproxen

It has been widely reported that NSAIDs reduce inflammation and pain by targeting COX, a key enzyme for the synthesis of prostanoids (prostaglandins, prostacyclin, and thromboxane). The COX enzyme has two active sites of which one has a heme group and other one has a peroxidase activity. Inside the cell, to get an active COX enzyme, two molecules, oxygen and iron, are essential. The single most consistent result observed from our examination of the differential expression of proteins resulting from NSAID exposure in zebrafish was an up-regulation of proteins that belong to globin family (beta globin or HbB, alpha globin, hemoglobin alpha adult1, alphaglobin like protein) which are involved in the transport of oxygen from gills and may contribute to heme synthesis required for increased production of COX. Other alterations included up-regulation of transferrin, which is a key player in iron metabolism with an increased production presumably related to increased heme production, and down-regulation of NDK which has been linked to increased cAMP production. After exposure to NSAIDs, the cell has to maintain its homeostatic condition. The up-regulation of hemoglobin protein level will help in increased oxygen transport and up-regulation of transferrin, which is a storehouse for iron, will result in increased heme group production that can be used by hemoglobin or freshly synthesized COX protein which might be expected to have an increased production in compensation to reduced activity of existing COX resulting from NSAID inhibition. On the other hand, NDK which is primarily involved in the synthesis of NTPs, was consistently downregulated in all our NSAID exposures. Although this might be expected to

reduce production of ATP, it may also increase cAMP and PGE2 production to aid in the compensation of reduced COX activity due to NSAID exposure. Up-regulation of ubiquitin protein will result in the degradation of proteins which in turn increases the amino acid pool of cell that can be used for fresh protein synthesis. These results suggest a homeostatic response to NSAID exposure which aids in returning the cell/organ to normal function.

5.2.8 Bioinformatic Summary of Significance of Observed Differential Expression

DAVID: The protein symbol and accession numbers obtained from MASCOT were crosslinked to the zebrafish genomic database ZFIN IDs (http://zfin.or) identifications and were evaluated in a DAVID (Database for Annotation Visualization and Integrated Discovery) Bioinformatics Resources analysis (Huang et al., 2008). The ZFIN IDs were then statistically evaluated for enriched gene ontology (GO) categories including cellular localization, biological process and metabolic functions using the modified Fisher exact test (EASE score) with a statistical significance level of $p \leq 0.05$. Thus, the GO annotation and analysis based on DAVID revealed information regarding metabolic function, cellular localization and biological processes associated with our differentially expressed gene set. The differentially expressed fraction of the gill proteome was found to be primarily associated with oxygen and ion transport and hematopoietic and was primarily localized in the cytoplasm and cytoskeleton followed by non-membrane bound organelles. The DAVID analysis confirms that the dominant phenotype changes observed upon treatment with the NSAIDs involved an increase in hemoglobin synthesis and those additional proteins which were up-regulated, such as Transferrin, are involved in iron transport and hence also functionally related to the same process.

5.2.9 Ingenuity Pathway Analysis (IPA)

Although we had a small set of differentially expressed proteins, we attempted a pathway analysis using Ingenuity Systems, Inc. proprietary software (IPA, Ingenuity Systems Pathway Analysis, Redwood City, California, U.S.A.). The most significant ($p<0.01$) pathways identified, Liver X-Receptor/ Retinoic X Receptor (LXR/RXR) Activation, Acute Phase Response Signaling and Clathrin-mediated Endocytosis Signaling, were all based on only two proteins, transferrin and lipoproteins A-1. Since both of these proteins were unaffected by naproxen exposure and were inconsistently differentially expressed (i.e. both up- and down-regulated depending on exposure concentration) in response to ibuprofen exposure, we feel like our data set represents too few differentially expressed proteins to yield any significant results from a bioinformatics-based pathway analysis.

5.2.10 Summary of Proposed Biological Effects of NSAID Exposure Revealed by Proteomics Analysis

Since the only known direct targets of the two NSAIDs used in our study are the cyclooxygenases (COX-1, COX-2a, COX-2b), the observed increase in the protein components of hemoglobin (and probably also enhanced Hemoglobin synthesis) and Transferrin (that favors increased Heme biogenesis) seems to be indirect. We postulate the following mechanism. Studies have shown that the gills of *D*. rerio strongly express all the three cyclooxygenases (Ishikawa, et al. 2007), and the main product of COX enzymatic action in *D*. rerio is the prostaglandin, PGE2 (Nakatsugi, et al. 1996). PGE2 has been shown to be an important regulator for hematopoiesis during zebrafish development (Grosser, et al. 2002). It is important in angiogenesis, regulation of blood pressure and hematopoiesis in mice (Ben-Av, et al. 1995; Zhang and Riechers 2008). Additionally, PGE2 levels are important in controlling different

stages of hematopoiesis in both embryonic and adult mice. Previous work in our laboratory has demonstrated that NSAID exposure in the ranges used in this study decrease PGE2 levels in bluntnose minnow (Bhandari and Venables 2011). The PGE2 synthesized in the gills, one of the major sites of COX-2a and -2b expression, could have effects on hematopoietic cells and RBCs that are circulating in the gills (paracrine) or in distant tissues such as bone marrow (endocrine). Moreover, PGE2 is synthesized by several other tissues – liver, kidney – and circulating PGE2 levels serve to maintain blood hematocrit levels. Since ibuprofen and naproxen are easily taken up by the *D*. rerio, they are likely to have an inhibitory effect on COX expression both at the gills (the primary uptake site) as well as systemic effects in other organs. The resultant fall in levels of hematopoietic prostaglandins (PGE2) due to inhibition of COX-1, -2, in turn, cause a decrease in generation and maturation of hematopoietic stem cells in the zebrafish. This is likely to reduce oxygen uptake and transport. To counter this potential respiratory stress, the body mounts a homeostatic response in which mature RBCs carry out enhanced biosynthesis of the globin proteins. Increased transferrin levels provide additional iron for heme production. Thus, although ibuprofen and naproxen have probably diminished hematopoiesis, the elevated level of hemoglobin in the already-existing RBCs in circulating blood maintains the circulating oxygen levels above a critical point. Since the gills have extensive vasculature for efficient gas exchange, estimation of protein from gill tissue shows an increase in globin levels. It is probably facilitated by the fact that fish RBCs have a nucleus, and is thus capable of changes in gene expression, unlike mammalian RBCs.

5.2.11 Conclusions

Laboratory exposure of zebrafish to the NSAIDs ibuprofen and naproxen resulted in readily detectable changes in protein expression as revealed by 2-DGE. Alterations occurred at

the lowest exposure concentration tested (1 ppb). Since multiple NSAIDs sharing similar modes of action are routinely reported to co-occur in the environment at concentrations in the low ppb range, it may be expected that exposure to this class of pharmaceuticals results in altered protein expression in wild fish populations, especially those in receiving streams below wastewater treatment plants. The identification of a sub-set of the differentially expressed proteins indicated a potentially wide range of biological effects, but was most indicative of altered heme production. Altered protein expression observed in this laboratory study should be further examined for possible physiological and ecological/fitness consequences that might threaten the long-term ecological health of wild populations.

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