KYNURENINE-TRYPTOPHAN RATIO IN STRESS: CELLS TO SPECIES

FROM MAMMALIAN CELL CULTURES TO AQUATIC SPECIES: DECIPHERING THE ROLE OF THE KYNURENINE-TRYPTOPHAN RATIO UNDER ENVIRONMENTAL STRESS

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TITLE: From Mammalian Cell Culture to Aquatic Species: Deciphering the role of the Kynurenine-Tryptophan Ratio under Environmental Stress

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LAY ABSTRACT

Human activities, especially industrial operations, can significantly impact the environment. To monitor these effects, scientists use various tools and organisms to assess ecosystem health. This research introduces a new approach to measuring environmental stress in wildlife by focusing on two key molecules: tryptophan and kynurenine. These molecules are part of a conserved biological pathway that helps all organisms manage stress, repair cells, adapt to their environment, and maintain overall health. Tryptophan, an essential amino acid, is broken down into kynurenine, and the balance between them—known as the kynurenine-tryptophan ratio (KTR)—can indicate the level of stress an organism is experiencing.

This thesis investigates whether KTR can detect environmental stress caused by industrial activity, particularly from petroleum-derived chemicals in the Athabasca Oil Sands Region (AOSR). In laboratory experiments, mammalian liver cells were exposed to oil sands compounds and complex mixtures from oil sands wastewater. These compounds changed KTR, showing that the liver's stress response was activated, and tryptophan metabolism was disrupted. The study also found that these chemicals affected cellular energy use and the way cells process fats and sugars. Furthermore, we examined fish species in the AOSR: longnose and white suckers. Results showed that KTR varied depending on the species and the location of exposure. In white suckers, KTR increased in response to stress, while in longnose suckers, it decreased, indicating species-specific responses to environmental changes.

Overall, our findings suggest that KTR could serve as a useful tool for measuring environmental stress in different species and ecosystems, especially in areas affected by anthropogenic or industrial activity. Understanding how KTR changes in response to pollution can help scientists better monitor and protect wildlife and ecosystem health.

ABSTRACT

Monitoring the impact of anthropogenic activities, particularly in industrial regions, requires ecological screening tools and frameworks that provide a comprehensive understanding of ecosystem responses to environmental changes. Biological indicators, organisms like algae, insects, fish, and sentinel mammals, are critical for assessing ecosystem health, particularly in areas of high industrial activity. The aim of this thesis was to identify a cross-species biomarker that can assess organismal health and environmental stress across various species, organs, and biological matrices.

A range of biological systems and signaling pathways related to xenobiotic metabolism, energy homeostasis, immune responses, and stress adaptation were explored, leading to the identification of the Tryptophan-Kynurenine Pathway, which consumes 60-90% of tryptophan in vertebrates. Tryptophan and its metabolites play key roles in diverse physiological processes, including cell growth and maintenance, immunity, disease states, and the coordination of adaptive responses to environmental and dietary cues. This adaptive response suggests that kynurenine-tryptophan ratio (KTR) may serve as a marker for exposure to a variety of environmental stress conditions, including toxicants, nutrient scarcity, predatory stress, and habitat loss—stressors that are prevalent in areas of high industrial activity. In recent years, the KTR is increasingly recognized as a sensitive biomarker in human diseases induced or exacerbated by stress; however, its role in environmental exposure and wildlife health remains unexplored. This thesis explores the question of whether KTR can be utilized as a cross-species biomarker for environmental stress or environmental exposure to toxicants, particularly focusing on the Athabasca Oil Sands Region (AOSR).

In vitro studies with mammalian hepatocytes exposed to polycyclic aromatic compounds (PACs): benzo[a]pyrene (BaP), and a Bitumen Water Accommodated Fraction (BitWAF) demonstrated that KTR increases were driven by elevated kynurenine levels, indicating disruption of tryptophan metabolism via the aryl hydrocarbon receptor (AhR). Further studies using acid extractable organics from Oil Sands Process-Affected Water (OSPW), Naphthenic Acid Fraction Components (NAFCs) showed metabolic reprogramming, including altered glucose and fatty acid uptake and mitochondrial dysfunction, mediated through PPARα activation and upregulation of *Tdo2*, the enzyme responsible for kynurenine production.

In vivo studies of longnose and white suckers from the AOSR were conducted to assess the relationship between KTR and CYP1 enzyme activity (EROD). These studies revealed species-specific responses, with an inverse correlation between KTR and EROD in longnose suckers and a direct correlation in white suckers. These findings validate KTR as a biomarker for environmental exposure in wildlife, with significant implications for monitoring ecosystem health. Collectively, this work demonstrates the potential of KTR as a novel biomarker for environmental toxicology, offering a valuable tool for assessing organismal stress across species in response to environmental contaminants.

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Finally, I would like to submit this thesis in the memory of my grandparents, Aurangzeb Khan, Paresha Begum, Begum Parijaan, and Muhammad Yusuf Khan, who are probably walking hand-in-hand with God right now thanking him that I'm finally done.

كُنْ فَيَكون

PREFACE

This thesis is prepared in the "sandwich" format as outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through the School of Graduate Studies at McMaster University.

Chapter 1 consists of a general introduction for the entire body of work. Sections of this chapter have been published in a review article.

Chapters 2, 3, 4 and 5 consist of submitted manuscripts of original research articles, all in peer-review at the time of thesis submission.

Chapter 6 includes a general discussion and future directions of this research project.

The author of this thesis is also the first author of all five submitted and/or published articles included.

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LIST OF ABBREVIATIONS AND SYMBOLS

3-HAA	3-hydroxyanthranilic acid
3-HAAO	3-hydroxyanthranilate 3,4-dioxygenase
3-HK	3-hydroxykynurenine
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AA	Anthranilic acid
ACCα	Acetyl-CoA Carboxylase Alpha
ACMS	2-amino-3-carboxymuconic semialdehyde
ACMSD	2-amino-3-carboxymuconate semialdehyde decarboxylase
AhR	Aryl hydrocarbon receptor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANGPTL4	Angiopoietin-Like 4
ATP	Adenosine triphosphate
BaP	Benzo[a]pyrene
BitWAF	Bitumen Water Accommodated Fraction
CAT	Catalase
CD36	Cluster of Differentiation 36 (also known as FAT, Fatty Acid Translocase)
CO2	Carbon dioxide
CPT1α	Carnitine Palmitoyltransferase 1A
CRH	Corticotropin releasing hormone
CYP	Cytochrome P450
CYP1A1	Cytochrome P450 Family 1 Subfamily A Member 1
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
DDT	Dichlorodiphenyltrichloroethane
EROD	Ethoxyresorufin-O-Deethylase
FASN	Fatty Acid Synthase
FICZ	6-formylindolo[3,2-b]carbazole
G6Pase	Glucose-6-Phosphatase
GC	Glucocorticoid
GPx	Glutathione peroxide
GR	Glucocorticoid receptor
GSI	Gonado Somatic Index
HPA	Hypothalamic-pituitary-adrenocortical
IAA	Indole-3-acetic Acid
IDO	Indoleamine 2,3-dioxygenase

IFN-γ	Proinflammatory interferon-gamma
IL	Interleukin
IPA	Indole-3-pyruvic acid
ITE	2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester
KAT	Kynurenine aminotransferase
KMO	Kynurenine monooxygenase
KTR	Kynurenine-Tryptophan Ratio
KYN	Kynurenine
KYNA	Kynurenic acid
KYNU	Kynureninase
LSI	Liver Somatic Index
MAOA	Monoamine oxidase A
NAD+	Nicotinamide adenine dinucleotide
NAFC	Naphthenic Acid Fraction Components
NIC	Nicotinic acid
NMDA	N-methyl-D-aspartate
OS	Oxidative stress
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzofuran
PGC1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
PGE3	Prostaglandin
PIC	Picolinic acid
PPAR	Peroxisome Proliferator-Activated Receptor
QPRT	Quinolinate phosphoribosyltransferase
QUIN	Quinolinic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDO	Tryptophan 2,3-dioxygenase
TGF-β	Tumor growth factor-beta
Th	T-helper
TIPARP	TCDD-Inducible Poly(ADP-Ribose) Polymerase
TNFα	Tumor necrosis factor-alpha

TPH	Tryptophan Hydroxylase
TRP	Tryptophan
TRP-KYN	Tryptophan-kynurenine
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UV	Ultraviolet
XA	Xanthurenic acid

DECLARATION OF ACADEMIC ACHIEVEMENT

Chapter I. Introduction. An Emerging Cross-species Marker for Organismal Health: Tryptophan-kynurenine pathway
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Chapter V: Assessing the Kynurenine-Tryptophan Ratio and CYP1 Activity in Longnose (*Catostomus catostomus*) and White Suckers (*Catostomus commersonii*) exposed to Petroleum-Derived Contaminants from the Athabasca Oil Sands Region.
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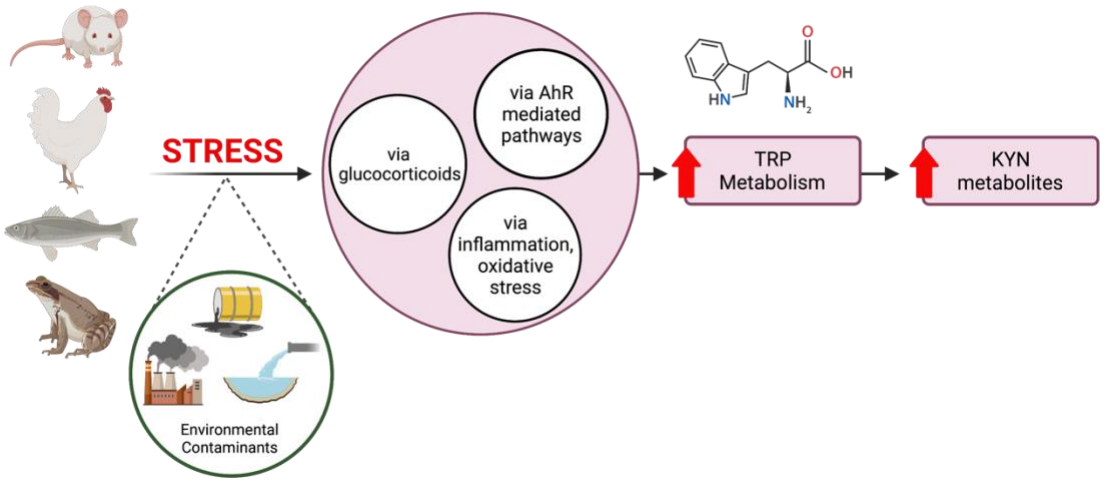
CHAPTER I: AN EMERGING CROSS-SPECIES MARKER FOR ORGANISMAL HEALTH: TRYPTOPHAN-KYNURENINE PATHWAY

This chapter is a reprint of the original article with the same title, published in the *International Journal of Molecular Sciences*, 2022 June (doi: 10.3390/ ijms23116300).

1.1 Abstract



Graphical Abstract.

Tryptophan (TRP) is an essential dietary amino acid that, unless otherwise committed to protein synthesis, undergoes metabolism via the Tryptophan-Kynurenine (TRP-KYN) pathway in vertebrate organisms. TRP and its metabolites have key roles in diverse physiological processes including cell growth and maintenance, immunity, disease states and the coordination of adaptive responses to environmental and dietary cues. Changes in TRP metabolism can alter the availability of TRP for protein and serotonin biosynthesis as well as alter levels of the immune-active KYN pathway metabolites. There is now considerable evidence which has shown that the TRP-KYN pathway can be influenced by various stressors including glucocorticoids (marker of chronic stress), infection, inflammation and oxidative stress, and environmental toxicants. While there is little known regarding the role of TRP metabolism following exposure to environmental contaminants, there is evidence of linkages between chemically induced metabolic perturbations and altered TRP enzymes and KYN metabolites. Moreover, the TRP-KYN pathway is conserved across vertebrate species and can be influenced by exposure to xenobiotics, therefore, understanding how this pathway is regulated may have broader implications for environmental and wildlife toxicology. The goal of this narrative review is to (1) identify key pathways affecting TRP-KYN metabolism in vertebrates and (2) highlight consequences of altered tryptophan metabolism in mammals, birds, amphibians, and fish. We discuss current literature available across species, highlight gaps in the current state of

knowledge, and further postulate that the kynurenine to tryptophan ratio can be used as a novel biomarker for assessing organismal and, more broadly, ecosystem health.

1.2 Introduction

The aromatic amino acid L-tryptophan (TRP) is one of nine essential amino acids required by all living organisms for protein synthesis. Although bacteria, fungi and plants can synthesize TRP from phosphoenolpyruvic acid via the shikimate pathway [1], vertebrates must obtain TRP from dietary sources [2,3], such as plants and plant products (i.e., fruit, nuts, oats, chocolate) and other animals and animal products (i.e., fish, turkey, dairy). In addition to its role in protein synthesis, TRP is the precursor of the monoaminergic neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT) [2–5]. 5-HT is known to regulate behaviour and adaptive responses towards environmental stress. These responses include mood (anxiety) [6–8], cognition [9,10], nociception, aggressiveness, appetite [11,12], and temperature homeostasis [13]. Furthermore, there is evidence that 5-HT and its derivative melatonin regulate reproductive processes in vertebrates through the hypothalamo-hypophyseal system and by direct effects on reproductive organs [14–18]. Although the role of TRP and its metabolites may differ among mammals, birds, and fish, all vertebrates produce 5-HT in the central nervous system [4,5,19]. More recently, peripheral 5-HT synthesis and signaling has been identified in mammals [20,21] and fish [15,22]. Peripheral 5-HT has been reported to modulate gonadal hormone secretion, immune and inflammatory responses, and metabolic homeostasis in the gut [4,23]. Although TRP is a critical precursor for serotonin synthesis, only 1-2% of dietary TRP is converted to 5-HT; approximately 95% of dietary TRP is metabolized through the tryptophan-kynurenine (TRP-KYN) pathway [3].

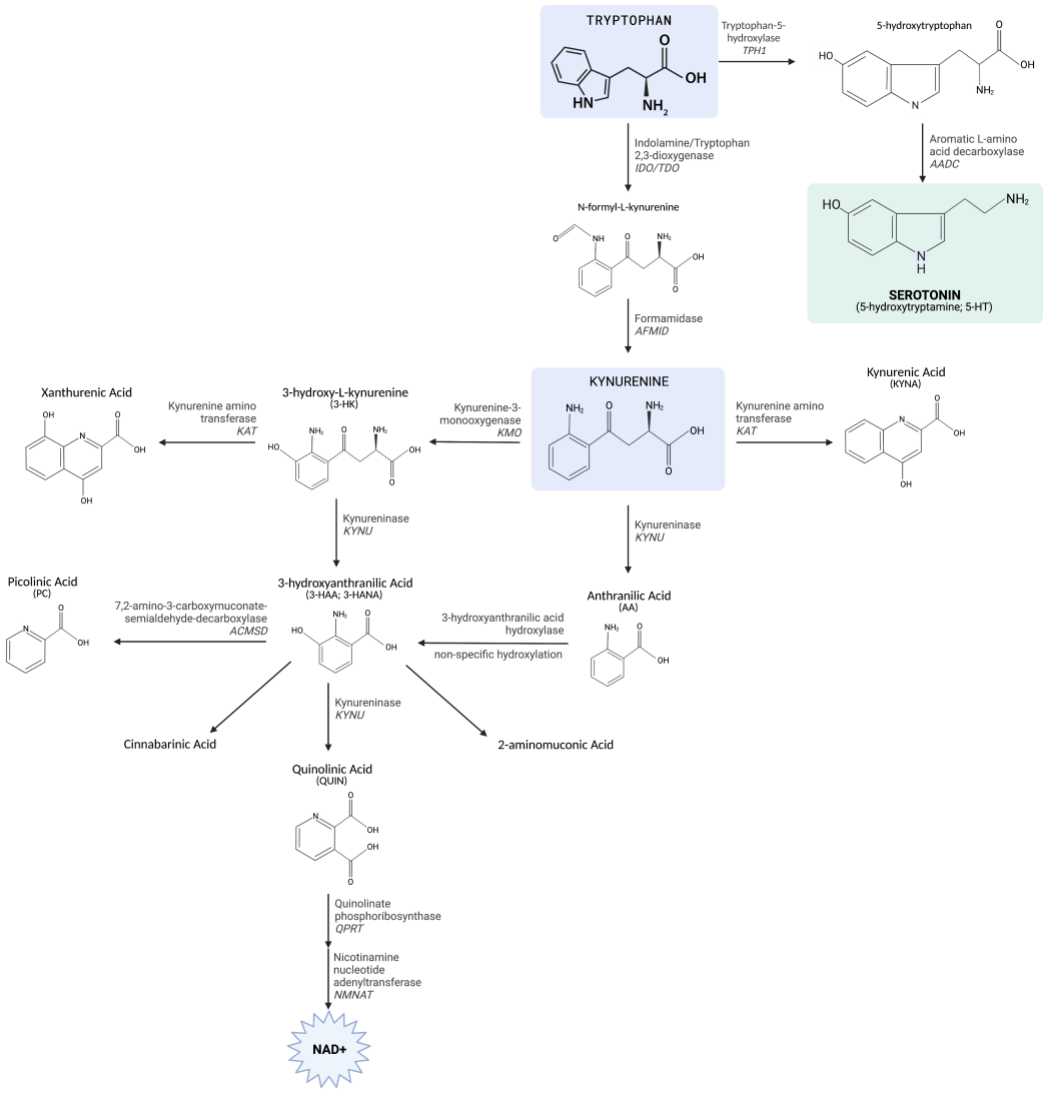


Figure 1. Key enzymes regulating Tryptophan (TRP) metabolism. The pathway depicting key regulators and targets that play a role on the TRP metabolism with a connection to Kynurenine (KYN) and Serotonin (5-HT). Figure created on biorender.com.

1.2.1 Tryptophan Metabolites

The kynurenine (KYN) pathway of TRP metabolism produces biologically active metabolites involved in inflammation, immune responses, and excitatory neurotransmission [3,24,25]. The first step involves oxidation of the indole ring and is catalyzed by two enzymes that are differentially distributed: tryptophan 2,3-dioxygenase (TDO) (predominantly expressed in the liver) and indoleamine 2,3-dioxygenease (IDO) (expressed in most peripheral tissues) [3,26,27] (Figure 1). These heme-containing enzymes are structurally distinct proteins that have evolved to catalyze the conversion of TRP to N-formylkynurenine. N-formylkynurenine is rapidly converted to KYN by N-formylkynurenine formamidase. At this point, KYN metabolism can follow one of two

branches: (1) metabolized to kynurenic acid (KYNA) and anthranilic acid (AA) via kynurenine aminotransferase (KAT) and kynureninase (KYNU), respectively, or (2) converted to the neurotoxic and free-radical generator 3-hydroxykynurenine (3-HK), which is further transaminated to xanthurenic acid (XA). KYNU catalyzes the hydrolysis of 3-HK to form the immunomodulatory and free-radical generator 3-hydroxyanthranilic acid (3-HAA), which can spontaneously transform to nicotinic acid (NIC) via 3-hydroxyanthranilate 3,4-dioxygenase (3-HAAO) or be completely oxidized to carbon dioxide (CO2). Initially, 3-HAAO converts 3-HAA to the unstable intermediate 2-amino-3-carboxymuconic semialdehyde (ACMS), which spontaneously rearranges to the excitotoxin and N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (QUIN) or is further converted to the neuroprotective picolinic acid (PIC) after enzymatic decarboxylation via 2-amino-3-carboxymuconate semialdehyde decarboxylase (ACMSD). QUIN is decarboxylated to form NIC, the NAD+ coenzyme precursor. QUIN is also the substrate for quinolinate phosphoribosyltransferase (QPRT), which initiates several metabolic steps to ultimately produce the essential cofactor, NAD+ [3,4,25].

1.2.2 Kynurenine Metabolites as Biomarkers of Human Disease

TRP and its metabolites have key roles in diverse physiological processes such as cell growth and maintenance (where TRP serves as a building block for proteins) and the coordination of adaptive responses to environmental and dietary cues (where TRP metabolites serve as neurotransmitters and signaling molecules). For example, many of KYN metabolites are neuroactive and are considered cytoprotective (such as KYNA) or cytotoxic (such as 3-HK, 3-HAA, QUIN, and NIC) [3,28]. These effects are highly dependent on accumulation of metabolites within specific tissues [Reviewed In: [29]]. KYNA acts as an antagonist to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA and kainate glutamate receptors, while QUIN acts as an NMDA receptor agonist [30,31]. NAD+ is an important coenzyme in many energy metabolism pathways including glycolysis, β-oxidation, and oxidative phosphorylation, [32] and picolinic acid modulates immune function and antimicrobial activity [33].

Over the past 15 years, there has been increasing interest in the role of KYN metabolites in human disease models (Table S1). Indeed, altered levels of TRP-KYN pathway metabolites have been reported in aging and sleep disorders [34,35], metabolic syndrome [23,24,36], cardiovascular disease [37,38], cancer [39], autoimmune disease [40–42], anxiety and depression [6,7], neurodegenerative diseases [30], as well as obesity, anorexia and bulimia nervosa, and other diseases presenting peripheral symptoms [19,43]. Changes in TRP metabolism can alter the availability of TRP for protein and 5-HT biosynthesis and dysregulate the levels of immuno- and neuro-active KYN metabolites. Recently, research has begun to assess KYN metabolites as biomarkers to many neuro- and immune-associated illnesses, including Alzheimer's Disease [44,45], ALS [46], and Major Depressive Disorder [47].

1.2.3 Objective of Study

However, to assess KYN enzymes and metabolites as markers of altered homeostasis, it is critical to understand and appreciate the integration of TRP catabolism. KYN pathway activation, enzymatic activity, and metabolite formation and function is dependent on exogenous stimuli and endogenous ligand binding. There is now considerable evidence showing that the KYN pathway of TRP metabolism can be influenced by various stressors including glucocorticoids (GCs) [24,48–52], infection [53–56], inflammation and oxidative stress [57–62], and environmental contaminants [3,63–66].

Importantly, the TRP-KYN pathway is conserved across vertebrate species, understanding how this pathway is regulated may have broader implications for environmental and wildlife toxicology. In mammalian species and birds with monogastric systems including duck (*Anatidae*) and chicken (*Gallus gallus*), the KYN pathway is the central catabolic route for TRP's indole ring [2]. As reviewed by Hall and colleagues [27,67], the genes of the major TRP catabolizing enzymes: IDO and TDO have undergone gene duplications leading to multiple isoforms of IDO. For example, all mammals have two IDO genes (IDO1 and IDO2) via gene duplication, whereas fish and amphibians have IDO2-like genes. Interestingly, while IDO and TDO enzymes show functional convergence, TDO has a higher catalytic efficiency for TRP catabolism and as such, its conservation across metazoans and vertebrates is clear [27]. More importantly, the expression of these enzymes differ across species by tissue/organ/cellular localization, by enzymatic characteristics, signaling properties, and by biological function following induction by distinct stimuli [27,67].

The goal of this narrative review is to identify (1) key pathways affecting TRP-KYN metabolism in vertebrates and (2) consequences of altered TRP metabolism in mammals, birds, amphibians, and fish. We provide evidence of studies linking exposure to environmental contaminants and altered TRP-KYN metabolites, which contribute to a range of adverse health outcomes. Further, we postulate that alterations in TRP metabolism, KYN metabolites, and KYN:TRP ratio may be an indication of exposure to environmental contaminants. Thus, we suggest that levels of KYN metabolites and the KYN:TRP ratio can be used as a novel integrative biomarker for assessing organismal exposure to environmental contaminants, and more broadly, ecosystem health.

1.3 Methods

A literature search was conducted in NCBI using MeSH on "Tryptophan" OR "Kynurenine" AND the terms: "Glucocorticoids", "Stress, Physiological", "Infections", "Inflammation", "Oxidative Stress", "Environmental Pollution/adverse effects", "Environmental Pollution/genetics", "Environmental Pollution/immunology", "Environmental Pollution/metabolism", "Environmental Pollution/pathogenicity", "Environmental Pollution/pathology", "Environmental Pollution/physiology", "Environmental Pollution/physiopathology", and "Environmental Pollution/toxicity". Collectively, between the years 1970–2021, a total of 1959 publications were identified. From these, we selected publications that specifically identified changes in key

cellular/physiological pathways that led to alterations in TRP-KYN metabolites in vertebrates (i.e., mammals, birds, fish, amphibians), and publications that identified altered tryptophan metabolites and their physiological consequences. A total of 265 papers were selected, and of those 185 were used in this review to focus on (1) glucocorticoids and stress, (2) infection, inflammation, and oxidative stress and (3) environmental contaminants. The break down by species and by physiological processes/pathways is depicted in Figure 2.

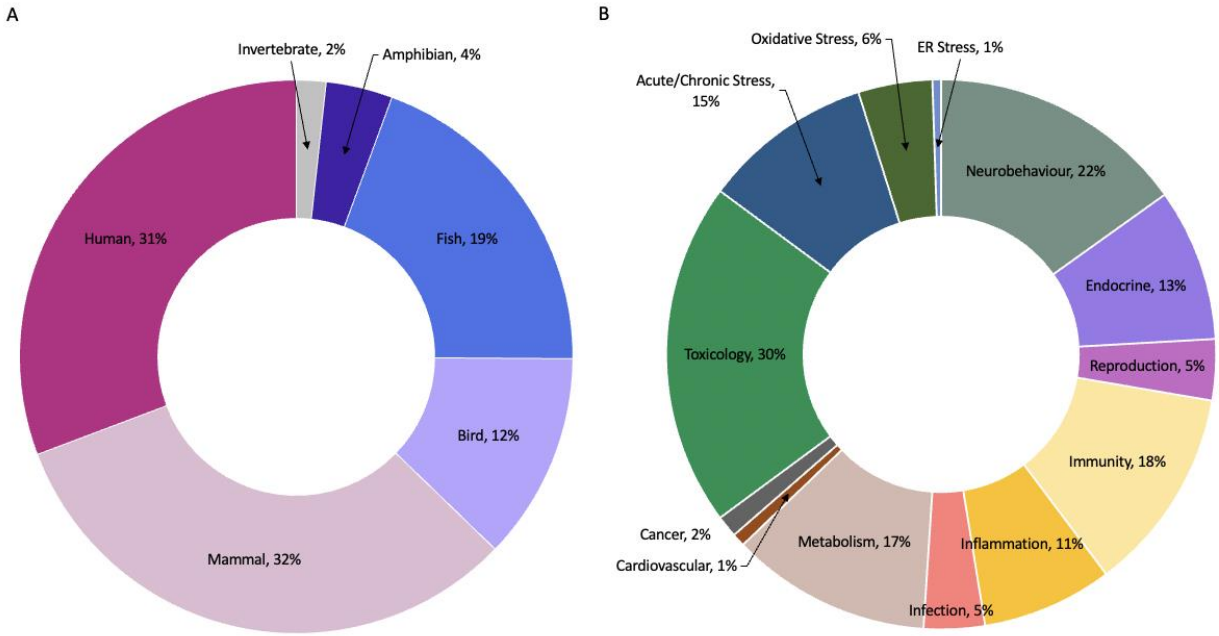


*Figure 2. A review of the literature by (**A**) species and (**B**) physiological processes / pathways.*

An additional screening was completed to identify enzyme and metabolite presence across vertebrate animal kingdoms (Figure 3). Both gene detection (i.e., PCR of mRNA, RNA, and DNA) and protein levels (i.e., HPLC, LC, MS, Western Blots) were methodologically screened using literature searches and databases. Gene data was collected through PubMed and NCBI searches specified for "gene name" (i.e., IDO, TDO, TPH, KYN, KYNA etc.) AND "homo sapiens" OR for mammals ["mammal", "rat", "mouse", "rabbit"] OR for fish ["fish", "medaka", "salmon", "trout", "carp"] OR for birds ["bird", "pigeon", "chicken"], OR for amphibians ["amphibian", "frog"]. It is important to note that when screening for enzyme and gene names, all isoforms of IDO and KAT were identified and used as "gene name". If genomic information was present, it was considered "detected". For protein levels, the UNIPROT database was screened with "kingdom" (i.e., mammal, bird, fish, frog, amphibian) and "Enzyme Name". Entries were then confirmed by identifying protein name, organism, and whether it was reviewed (Swiss-Prot) or unreviewed (TrEMBL, computational curation). If it was reviewed, it was considered "detected". If it was unreviewed, a PubMed and NCBI check was conducted, using MeSH terms for "Enzyme

Name", selecting "Other Animals" and a species name if needed (e.g., *Gallus gallus*). If an enzyme was unreviewed, and PubMed and NCBI identified primary articles that showed protein levels of the enzyme, it was considered "detected". If an enzyme was unreviewed and no additional information could be found, it was considered "orthologs may be present but have not been confirmed". If no information was found, it was considered "unknown". Metabolites could not be screened using UNIPROT, so literature data was used primarily to identify the presence across animal kingdoms.

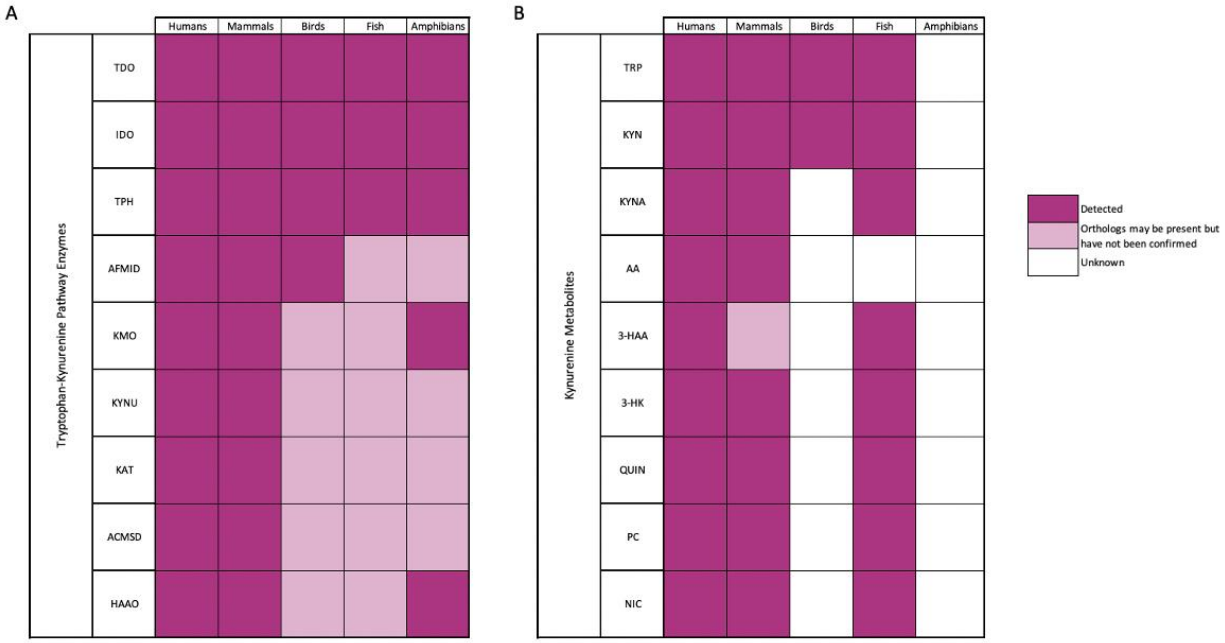


*Figure 3. A review of kynurenine pathway (**A**) enzymes and (**B**) metabolites detected across animal kingdoms. If both gene and protein data was available, it was considered detected and labelled dark pink. If enzymatic and metabolite orthologs may be present (via. computational curation of databases) but have not yet been confirmed in gene or protein levels, it was labelled light pink. If no information could be found yet, it was considered unknown and labelled white.*

1.4 Discussion

In the following sections, we discuss critical stress-inducing pathways that may play a role in driving TRP metabolism down the KYN pathway. As the TRP-KYN pathway is altered in response to glucocorticoids and chronic stress, infection, inflammation, and oxidative stress, and environmental toxicants, and these stressors are ubiquitous phenomenon across animal kingdoms, we explore the current literature available with regard to mammals, birds, fish, and amphibians.

Glucocorticoids and Chronic Stress

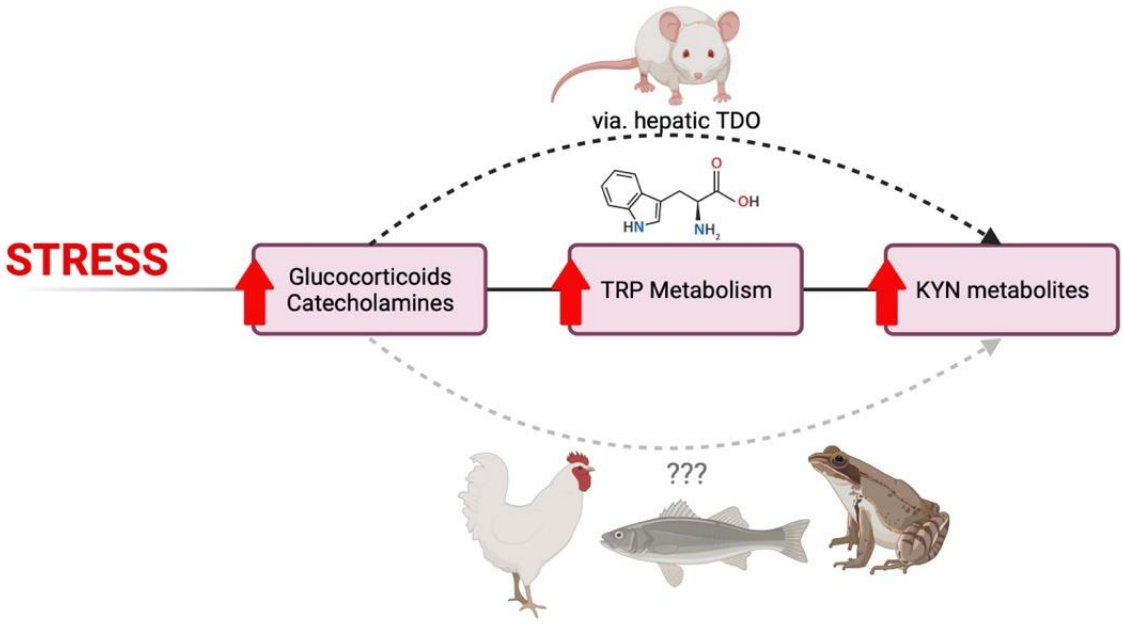


Figure 4. Across species, mammals, birds, and fish have similar responses to stress induced changes in Tryptophan-Kynurenine (TRP-KYN) metabolism. Stress responses include increased glucocorticoid (GC) and catecholamine signaling, which elevate TRP metabolism through the KYN pathway, as indicated by the levels of downstream metabolites. While it is generally accepted that this occurs through hepatic Tryptophan 2,3 dioxygenase (TDO) induction in mammals, the molecular mechanism is not yet clear in other vertebrate species. Figure created on biorender.com.

Glucocorticoids (GCs) are hormones of the endocrine system responsible for regulating stress responses. GCs are modulated by the hypothalamic-pituitary-adrenal (HPA) axis through the release of tropic hormones and negative feedback regulation. Although GCs exhibit circadian variation, they can be induced in response to physical, chemical, and psychological stressors. The molecular mechanism begins with the binding of GCs to the nuclear glucocorticoid receptor (GR), which functions as a transcription factor and modulates downstream gene expression. In general, increased GC signaling has anti-inflammatory and immunosuppressive activity. When responding to stress, the body mobilizes energy through sympathetic activation, which governs the "fight-or-flight" response. For this reason, the stress response sits at the interface of neuroendocrine, immune, and behavioural systems.

The degree of the stress response often depends on the duration and intensity of the stressor. Acute, immediate stress responses tend to be protective and allow an organism to adapt to

a changing environment [68,69]. Conversely, chronic, prolonged stress activation can lead to the accumulation of wear-and-tear on physiological systems and result in impaired health outcomes. For this reason, it is important to manage the balance between protective and damaging stress responses. In environmental toxicology, biomarkers such as metabolic cytochrome P450 (CYP) enzymes and heat shock proteins are often used as indicators for exposure to acute stressors and environmental insults [70]. Not only are these markers sensitive to contaminants and inexpensive for use in field monitoring, but they are also reliable in a wide variety of organisms. While acute stress-induced GC increase in plasma is associated with increased 5-HT production through TRP hydroxylase activity, chronic stress reduces 5-HT turnover and release and favours the KYN pathway [7]. Given the role of the TRP-KYN pathway in stress responses, its metabolites and enzymes may be useful biomarkers for stress across species.

It is generally accepted that GCs increase expression and activity of hepatic TDO in rats and mice [29,71–74]. GC signaling involves *de novo* synthesis of TDO at the transcriptional level, which is mediated by GC-responsive elements on the promoter region of TDO [73,74]. TDO activity can be increased through substrate activation by TRP and cofactor activation by heme, neither of which involve *de novo* synthesis. Chronic stress responses, which involve both HPA signaling and sympathetic activation, increase metabolism of TRP to KYN by TDO, which reduces the availability and transport of TRP to the brain [3] for 5-HT production. While IDO expression is not directly induced by GCs, HPA tropic hormones and/or stress can increase the expression and activity of IDO. This has been shown in the hippocampus of mice treated with adrenocorticotropic hormone [75], and in the serum and frontal cortex of rats exposed to chronic mild stress [76,77]. Some studies have suggested that TRP uptake into the brain is increased with acute stress and HPA activation. For example, in rats, foot shock stress increased levels of TRP, KYN, KYNA, and 3-HK in the brain. Similarly, acute stress induced by physical restraint in mice increased TRP metabolism to KYN in the brain and plasma, along with an increase in IDO1, IDO2, and TDO2 [78]. Moreover, following exposure to a novel stressor, brain TRP and KYN levels were raised in mice with no change in the KYN:TRP ratio and a reduction in the 5-HT:TRP ratio [79]. This suggests that while brain TRP levels increase with stress, brain IDO activity may also be induced, which increases KYN levels and shifts metabolism away from the 5-HT pathway [5].

Stress-induced alterations in TRP metabolism in the brain and periphery in mammals can also be due to an interaction between the immune system and the HPA axis [79]. Exposure to stressors induces the release of proinflammatory cytokines, which further activate the HPA axis. For example, proinflammatory interleukin-1 (IL-1) is a potent stimulant for corticotropin releasing hormone (CRH) synthesis. By increasing CRH levels, GC production can be increased. Additionally, the proinflammatory cytokine interferon-gamma (IFN-γ) induces IDO expression in hypothalamic and pituitary neurons, which produce 3-HK and QUIN [79]. Induction of IDO by IFN-γ can be potentiated by dexamethasone, which has no effect when administered alone in human monocytes [80]. In mice, stimulation of the HPA axis through immune activation by IL-1 and lipopolysaccharide has

been shown to increase brain TRP levels. However, since stress-induced changes in brain TRP levels occur in adrenalectomized rats and mice, this is not necessarily dependent on adrenocortical activation [79]. Similarly, Rose *et al*. (2020) reported increased KYN and decreased TRP levels in plasma, and decreased expression of monoamine oxidase A (MAOA) following exposure to ozone; the addition of metyrapone, a GC inhibitor, was able to reverse the effects of ozone on MAOA and kynurenine monooxygenase (KMO), but did not change the effects on TRP and KYN levels [81]. Interestingly, it has been shown that GC release is inhibited with increased KYNA levels in some areas of the central nervous system [82]. Further, the enol tautomer of indole-3-pyruvic acid (IPA), another metabolite in the TRP-KYN pathway, has been reported to reduce plasma GC levels and GR activity in the hippocampus following repeated stress [82]. Taken together, these data suggest that stress plays a key role in mammalian TRP-KYN metabolism through both GC-dependent and -independent mechanisms.

There is little known regarding the molecular effects of stress on the regulation of the TRP-KYN pathway in birds. However, in avian species, the TRP-KYN metabolic pathway is associated with undesirable behavioural patterns. For example, when chickens were under socially disruptive stress due to housing changes and manual restraint, it was found that increased destructive feather-pecking behaviour was associated with decreased plasma KYN:TRP ratios. In the same study, the socially disrupted group had gained more weight. Since plasma metabolite levels can vary with food intake, and stress can either increase or decrease feeding behaviours, it is unclear whether the observed results were due to a stress response or increased food intake [83]. Similar to rats, it was found that chickens with a TRP-supplemented diet show reduced aggressive behaviour such as feather pecking; however, it is not clear if this is due to alterations in TRP-KYN pathway metabolites or increased 5-HT production [83].

In fish, stress responses involve release of catecholamines from chromaffin tissue in the head kidney and adrenergic nerves, as well as cortisol from the interrenal tissue into the circulation. Chronic, prolonged stress responses can lead to metabolic dysfunction and immunosuppression, which are characteristic of HPA hyperactivity [84]. The majority of work in fish has focused on the impact of TRP-supplemented diets on stress response reduction. [85–90]. For example, studies in meagre (*Argyosomus regius*), rainbow trout (*Oncorhynchus mykiss*), Sengalese soles (*Solea sengalensis kaup*), Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) species have investigated the effects of a TRP-supplemented diet and chronic stress on TRP-KYN metabolism. Chronic stress in fish fed a control diet did not affect brain levels of 5-HT or TRP concentrations, but liver TRP, KYN, and QUIN were increased; in fish given a TRP-supplemented diet, brain 5-HT and liver TRP were reduced, and hepatic KYN and QUIN were increased [78]. Similarly, Wish *et al*. (2022) have found increased KYN levels in the brain and liver of rainbow trout following exposure to an acute stressor [91]. Taken together, this suggests that the KYN pathway is enhanced in stressed fish, regardless of diet.

Across species, it appears that mammals, birds, and fish have similar responses to stress with respect to changes in TRP-KYN metabolism (Figure 4). Increased GC and catecholamine signaling, which are characteristic of stress responses, elevate TRP metabolism through the KYN pathway as indicated by the levels of downstream metabolites. While it is generally accepted that this occurs through hepatic TDO induction in mammals, the molecular mechanism is not yet clear in other vertebrate species. In bird and fish species, TRP-enhanced diets have been shown to reduce stress-induced plasma GC levels and aggressive behaviours.

Infection, Inflammation and Oxidative Stress

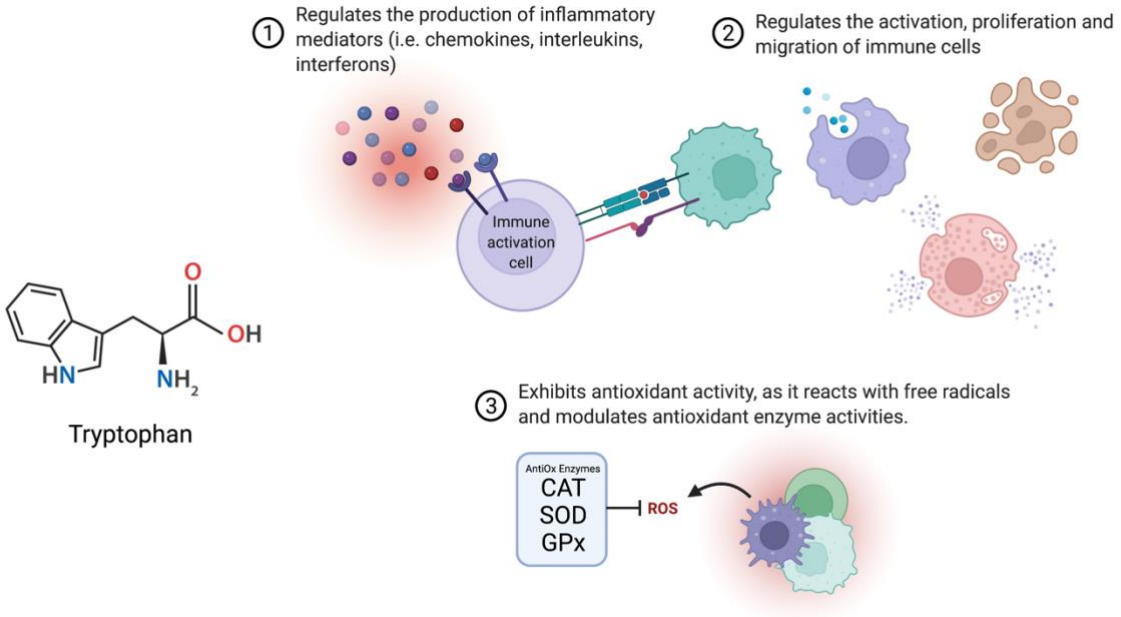


Figure 5. TRP functions as a regulator of inflammatory signaling molecules that control the immune response and the antioxidant response to cell stress and tissue damage. Figure created on biorender.com.

In all species, there is an interaction between the neuroendocrine and immune systems. Both immune and endocrine cells share common receptors, and different hormones and cytokines are involved in many of the same physiological processes. TRP functions similarly in all higher vertebrates to regulate (1) the activation, proliferation, and migration of immune-surveillant cells (i.e., T- and B-lymphocytes, macrophages, and natural killer cells) and (2) the production of inflammatory signaling molecules, cytokines, nitric oxides and superoxides (Figure 5). In mammals, KYN metabolites are reported to be involved in inflammation, immune response, and excitatory neurotransmission [24]. In recent years, metabolites including KYN, KYNA, and QUIN are emerging as key targets in diseases such as diabetes, HIV [92], atherosclerosis [93], neurodegenerative diseases including schizophrenia, Alzheimer's and Huntington's [94–96], and cancer [24,97]. Primarily, these

pathologies converge on inflammatory events which include target organ infiltration of circulating immune cells, activation of pro-inflammatory signaling pathways, expression of cytokines [24]/chemokines [98], and production of reactive oxygen species (ROS) [93]. Recent studies have reported that TRP deficiencies result in immunosuppression and a significant increase in the susceptibility of humans [60], pigs, and teleost fish [99] to disease, infection, morbidity, and mortality. As such, the TRP-KYN pathway is recognized as an important player in inflammation and immune response [95,100].

Of the rate-limiting enzymes in the TRP-KYN pathway, the IDO enzymes are known to play a role in modulating infection and autoimmunity [101]. In fact, the biological role of IDO on the infiltration of circulating immune cells has been coined the TRP-depletion hypothesis; IDO suppresses microbial infection by reducing TRP availability in infected tissues [102,103]. Furthermore, IDO is highly regulated by superoxides and inflammatory mediators, where its expression is increased by IFN-γ [103–106], tumor necrosis factor-alpha (TNFα) [106,107], tumour growth factor-beta (TGF-β) [97], interleukins 1-beta, 2, 12, 18 (IL1β, IL2, IL12, and IL18) [97], and prostaglandins (PGE2) [106,108,109], as well as pathogenic infections including parasites, viruses, and bacteria. Downstream of IDO activation, the metabolite KYNA promotes monocyte extravasation and IL6 production, and controls cytokine release [24,110], while the metabolite 3-HAA can induce apoptosis in T-cells through glutathione depletion [111]. In line with this, high KYN levels can increase the proliferation and migratory capacity of cancer cells and aid tumours to evade immune surveillance. In addition, KYN metabolites can then act via the aryl hydrocarbon receptor (AhR) to mediate T-cell anergy and apoptosis, proliferation of T-regulatory and T-helper(Th)17 cells, and the Th1/Th2 response [101]. Similarly, KYN metabolite KYNA has also been found to activate human, mouse, and rat GPR35 – a G protein-couple receptor with orthologs found in a variety of mammals and one amphibian species (*Xenopus tropicalis*). While KYNA has been found to be an endogenous activator of GPR35 in humans and rats altering immune responses in inflammation, pain, cancer, cardiovascular disease and energy homeostasis [Reviewed In: [112]], its role as an activator of GPR35 in other species remain unknown.

There is increasing evidence that IDO's role in immune function may begin in gestation. Histochemical studies of the human decidua have reported alterations in the expression of IDO and KMO throughout pregnancy. In the first trimester, IDO and KMO expression is present in stromal and glandular epithelial cells of the decidua. In both the first and second trimester placenta, they become localized to the synctiotrophoblast, stroma, and macrophages before shifting to fetal endothelial cells and macrophages in terminal villi of term placenta [113–116]. This shift in expression suggests that the function of these enzymes may change from a role of immunosuppression at the maternal-fetal interface in early pregnancy to one associated with the regulation of fetoplacental blood flow or placental metabolism in late gestation [113]. Work done by Williams and colleagues found that 24 hours following inflammation induced by intrauterine endotoxin administration, there was a significant upregulation of IDO in the placenta and fetal brain, which was associated with increased IFN-γ expression and increased in KYN, KYNA and QUIN

levels. These increases occurred in parallel with decreased levels of 5-hydroxyindole acetic acid, a precursor for 5-HT [106]. Taken together, these results indicate that maternal inflammation can shunt TRP metabolism away from 5-HT and down the KYN pathway.

IFN-γ also upregulates other enzymes in the KYN pathway including KMO, KYNU, and 3-HAAO activities [117]. For example, in adipose tissues, which have resident macrophages with high levels of KMO, the catabolism of TRP via KMO induction following immune system activation leads to increased production of QUIN rather than KYNA, which is reported to have anti-inflammatory properties [118]. Interestingly, plasma neopterin levels, an indicator of IFN-γ activity, have been paralleled by increased levels of the KYN:TRP ratio, which is reflective of IDO activity in patients with metabolic disorders such as type 2 diabetes [119] and neurodegenerative disorders such as Huntington's Disease [96]. In fact, in Huntington's patients, the KYN:TRP ratio was higher and associated with elevated levels of C-reactive protein, neopterin and lipid peroxidation products. Increased plasma KYN levels and KYN:TRP ratios have been found in patients with systemic inflammatory response syndrome, sepsis and septic shock, but the biological significance and prognostic value of these findings have remained uncertain [120]. Together, KYN:TRP and plasma neopterin levels are considered systemic markers of inflammation and oxidative stress [93,96].

Similar to mammalian IFN-γ, chicken IFN-γ is a known regulator of the immune response through the regulation of proinflammatory cytokines that correlate to immune activation and antiviral status. In fact, studies have previously shown that immunocompromised chickens have downregulated IFN-γ [121], which resulted in higher mortality following viral infection than normal chickens. Yuk and colleagues (2016) showed that siRNA suppression of IFN-γ in chicken embryo fibroblasts results in increased replication of viral genes during infection [122]. Taken together, these studies suggest that the immune state of chickens, and other avian species are mediated by interferons [123,124]. Interestingly, Emadi and colleagues (2010) have shown that supplementing broiler chicken feed with double the National Research Council's levels of TRP enhanced the IFN-α, IFN-γ, and immunoglobulin G response to infection [125]. Taken together, these studies show that TRP may also be playing a vital role in the immunity of avian species.

In fish, TRP plays a critical role in macrophage and lymphocyte function. When faced with infection, TRP and IDO levels are related to the induction of anti-inflammatory signaling molecules, the main effectors being T-cells (Reviewed in: [126,127]). Similar to the other vertebrates discussed, interferons that regulate IDO play analogous antiviral roles in teleost fish (Reviewed in: [128,129]). Notably, fish interferons possess the same exon/intron structure as mammalian IFN-γ [128], also playing a similar critical role in adaptive cell-mediated immune responses produced by Th1 and cytotoxic T-lymphocytes [128,130]. Interestingly, TRP supplementation has not shown improvement in immune status in European seabass (*Dicentrarchus labrax*) [131] or Persian sturgeon (*Acipenser persicus*) [132]. Furthermore, Machado *et al*. (2015) have shown that following TRP

supplementation and subsequent increases in cortisol, European seabass had decreased monocyte/macrophage activation in response to infection. The decreased levels of lymphocytes and monocytes/macrophages were accompanied with decreased mRNA expression of proinflammatory cytokines (IL1β, IL8 and TGFβ), diminished IFN-γ, and lowered levels of superoxide dismutase (SOD), an enzyme involved in antioxidant defence [131]. Conversely, in mice [133] and broiler chickens [125], dietary TRP supplementation was able to alleviate inflammatory responses by attenuating the migration of inflammatory cells. While an interesting difference amongst vertebrates, it has been suggested that these difference may be a result of teleost fish requiring an optimal amount of dietary TRP for growth—in fact, alterations to dietary TRP has resulted in osmotic-based acute stress [99,131,134], where TRP supplementation in non-stressed fish has resulted in increased plasma cortisol level, while the opposite effect has been observed in stressed groups [13,86]. Moreover, exogenous TRP supplementation varies depending on fish species and size, as well as exposure to stressful environments (i.e., crowding, high stocking density, pollutants) [99,135–137].

Oxidative stress (OS) is another mechanism by which cell and tissue damage can occur. OS is an increase between the production and accumulation of ROS, which are generated as by-products of oxygen metabolism in processes including immune activation, apoptosis, cell differentiation, and protein phosphorylation, as well as through exposure to environmental stressors and xenobiotics [138]. The consequences of increased cell exposure to ROS include reduced levels of adenosine triphosphate (ATP), lipid peroxidation, cell membrane depolarization, morphological changes in cell surfaces, and DNA damage [139]. Cellular damage marked by OS includes lipid peroxidation, increased levels of protein carbonyl, and decreased levels of antioxidant enzymes (SOD, catalase [CAT] and glutathione peroxidase [GPx]) [138]. The TRP-KYN pathway plays a role in the onset of OS (Reviewed in: [140]) and it has been proposed that TRP exhibits antioxidant activity, as it reacts with free radicals and modulates antioxidant enzyme activities. In fact, there is accumulating evidence to support the use of TRP and its metabolites as antioxidants [141]. In rabbits, dietary TRP supplementation was effective in protecting against free radical generation and lipid oxidative damage produced by hypoxic myocardial injury [142]. Similarly, in weaned piglets, increasing dietary TRP levels resulted in enhanced antioxidant capacity (SOD and GPx), which attenuated the OS response induced by diquat injection [143]. In line with the work done in mammals, dietary TRP supplementation in ducks alleviated stress, and improved growth performance and antioxidant activity (GPx and CAT). Similarly in fish (Reviewed In: [144,145]), it has been shown that while increased supplementation of dietary TRP increases total SOD, CAT, glutathione activity, and total antioxidant capacity, an optimal range of TRP supplementation is dependent on species [146].

Some TRP metabolites including 5-hydroxytryptophan (5-HTP), indole-3-acetic acid (IAA), 3-HAA, 3-HK and XA can act as ROS scavengers and modulate antioxidant enzymes. Interestingly, other KYN metabolites including QUIN, 3-HK and 3-HAA can generate oxygen radicals and ROS, thereby modulating OS [147–149]. Accordingly, the

pharmacological inhibition of the KYN pathway can decrease levels of OS. For example, in a rat model of schizophrenia, inhibitors of TDO, IDO and KMO were able to prevent lipid peroxidation, decrease protein carbonyl levels, and increase SOD levels and catalase activity [150]. There is also considerable evidence linking aging with OS; in many aging-associated disorders, there are consistent reports of upregulated IDO (Reviewed in: [151,152]). Currently, reliable markers of OS include carbonylated proteins, malondialdehyde, 4-hydroxy-2-nonenal, and F2-isoprostanes analyzed by LC-MS/MS [153]. However, given that TRP and KYN metabolite levels have been repeatedly shown to be sensitive and specific to antioxidant capacity and associated gene changes, TRP-KYN levels, or varying ratios of the metabolites (i.e., 3-HAA/AA) may be surrogate markers for the organism's response to insults causing increased ROS.

Environmental Contaminants

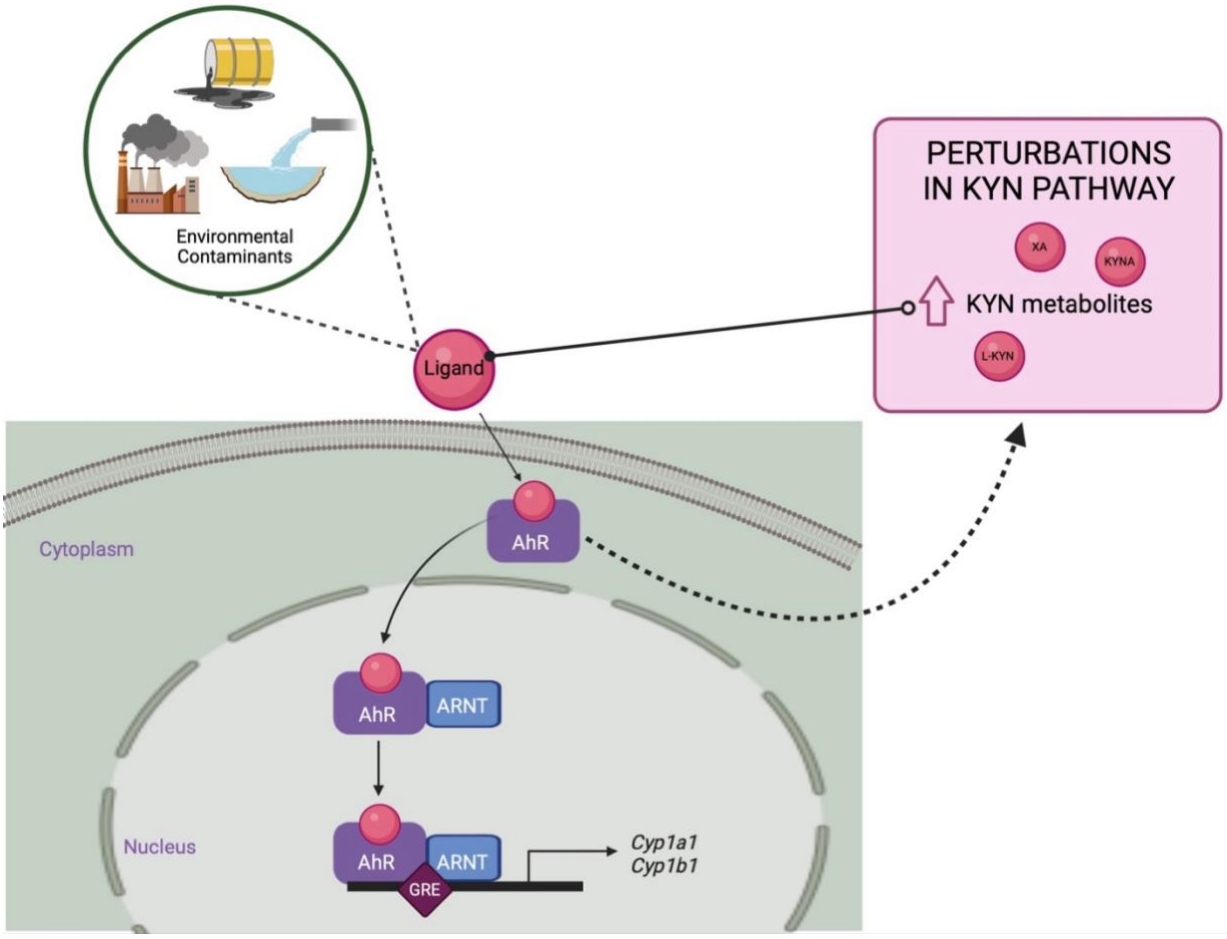


Figure 6. Alterations in TRP metabolism and KYN metabolites may be an indication of exposure to environmental contaminants. Many environmental contaminants (i.e., PACs) are known to act as AhR ligands and are also known to regulate IDO and TDO expression, thereby affecting the production of immunomodulatory KYN metabolites. Moreover, many

of these metabolites can also act as AhR ligands and induce AhR target genes. Figure created on biorender.com.

Environmental stressors are factors that constrain the productivity, survival, and reproductive success of organisms. These stressors can range from biological or social stress (i.e., predation, competition, and disease); physical disturbances to the landscape (i.e., weather events like tsunamis, hurricanes, volcanic eruptions, and wildfires, or anthropogenic deforestation, machinery trampling, and hikers); and chemical pollution (i.e., pesticides, flame retardants, personal care products, waste chemicals from industrial development, and air pollution). Increasingly, stressors due to anthropogenic activities have become the most critical influence on species and ecosystems [154]. Rapid industrialization and urbanization can dramatically change the composition and diversity of biotic communities.

Alongside urbanization comes the increased distribution of environmental toxins through human activity. Some of these contaminants include heavy metals; polycyclic aromatic compounds (PACs) which include benzo[a]pyrene (BaP), polycyclic aromatic hydrocarbons (PAHs) and their heterocyclic, alkylated, halogenated, oxygenated, sulphated and nitrated analogs (Reviewed In: [155]); dioxins, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzo-p-dioxins (PCDDs); organochlorine compounds including polychlorinated biphenyls (PCBs), hexachlorocyclohexane isomers, dichlorodiphenyltrichloroethane (DDT) compounds, and hexachlorobenzenes (Reviewed In: [156]). Many of these contaminants can be released into the environment from natural and anthropogenic sources but have become ubiquitous in recent years. For example, PACs can be found in sea ice in the Arctic, industrialized harbors, the Oil Sands Region in Canada, and major oil spills (e.g., Exxon Valdez Oil Spill, Alaska; Prestige Oil Spill, Spain; Deepwater Horizon Oil Spill, Gulf of Mexico) [155,157]. Similarly, exposure to dioxins and furans can occur naturally through wildfires, and through industrial processes such as waste incineration, burning fuels (i.e., wood, coal, and oil), and accidental/residential/structural fires.

Other contaminants such as pesticides have an even greater public health concern; while they are designed to kill specific plants and insects, they often have harmful effects on non-target species. In mammals, exposure to pesticides has been linked to cancer; neurotoxicity and immunotoxicity [158]; endocrine disruption [159], reproductive effects, and birth and developmental defects (Reviewed In: [160]); and changes in energy homeostatic organs such as the liver and adipose tissue [161]. Similarly, in birds, organophosphates, neonicotinoids [162], carbamates and second-generation insecticides can result in decreased food consumption, weight loss, delayed migration, and decreases in the production, fertility, and hatchability of eggs (Reviewed In: [158,163]). Fish and other aquatic organisms are the most exposed in their environments due to runoff of pesticides [164–166], oil spills [167–170], wastewaters from sewage containing personal care products [171–174], and chemical plant disposals; and have been extensively reviewed.

Animal and human exposure to these chemicals can occur directly through pollution, or indirectly through food chain effects [175–177].

Many physiological homeostatic responses to environmental contaminants can be attributed to perturbations in pathways associated with GC signaling, inflammation, OS and ultimately, alterations in amino acid metabolism. For example, rats exposed to pyrethroid pesticides (deltamethrin and fenpropathrin) had significantly lowered KYNA production in cortical brain slices [178]. In chicken embryos, exposure to organophosphate and methylcarbamate insecticides was associated with lowered embryo NAD+ levels [179]; this effect was attributed to the inhibition of KYN formamidase, which impairs conversion of TRP to essential pyridine nucleotide cofactors. Metabolomics studies have demonstrated that exposure to various environmental pollutants, such as particulate matter (PM2.5), fires, and other sources of complex mixtures containing substance including heavy metals, PAHs, and persistent organic pollutants, disrupt key metabolic pathways, notably amino acid metabolism [180–182]. Among these pathways, tryptophan metabolism has demonstrated notable sensitivity to environmental stressors ranging from habitat loss, ecosystem disturbances, and contamination from industrial and anthropogenic activities. For example, untargeted metabolomics profiling of urinary metabolites in occupational firefighters has revealed significant alterations in the TRP-KYN pathway, marked by significant changes in KYN metabolites: kynurenic acid and indole-3-acetic acid, both of which are well-established indole ligands of the AhR [182]. Indeed, the majority of research in this area has focused on the link between environmental contaminants that act as AhR ligands and perturbations in the TRP-KYN pathway.

Many environmental contaminants, particularly PACs, are known to act as AhR ligands; these compounds are also known to regulate IDO and TDO expression, thereby affecting the production of immunomodulatory TRP metabolites. For example, in mammals, it is well established that IDO expression and activity can be induced by AhR ligands including TCDD, BaP, and several PAHs [183,184]. Interestingly, KYN metabolites, including L-KYN, KYNA, XA, cinnabarinic acid, indigo, indirubin, and ultraviolet (UV) photoproducts of TRP such as 6-formylindolo[3,2-b]carbazole (FICZ) can act as AhR ligands and induce AhR target genes (CYP1A1, CYP1A2, and CYP1B1) (Reviewed In: [185]). Novikov *et al*. (2016) investigated the role of TRP-derived metabolites within malignant and non-malignant breast cancer cell lines and showed that (1) cell lines that expressed TDO produced sufficient intracellular KYN and XA concentrations to activate the AhR, (2) TDO overexpression led to excess KYN and XA which accelerated the migration of tumour cells in an AhR-dependent manner, and (3) environmental ligands TCDD and BaP, as well as endogenous TRP-derivative FICZ mimic this effect [186]. Furthermore, AhR knockdown or inhibition significantly reduced TDO2 expression. KYN has also been shown to activate AhR in an autocrine/paracrine fashion, resulting in the suppression of antitumour immune responses and promotion of tumour cell survival and motility [183]. Furthermore, FICZ—the UV photoproduct of TRP—binds to AhR with the highest affinity known to date of endogenous ligands; at high concentrations, FICZ behaves similarly to TCDD, exhibiting toxicity in fish and bird embryos, and playing a role in immunosuppression [187]. In birds,

including the chicken (*Gallus gallus domesticus*), ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Coturnix japonica*), and common tern (*Sterna hirundo*), FICZ has been identified as an avian AhR ligand [188], However, with exception to changes in the avian CYP1A gene, other pathways mediated by AhR remain to be studied. 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)—a dietary TRP derivative—was discovered in porcine lung tissues and has been shown to be a high-affinity AhR ligand in humans, mice, and fish [189]. Taken together, these data suggest that across vertebrates, environmental exposure to certain contaminants can result in altered TRP-KYN metabolites that can contribute to a range of adverse health outcomes.

1.5 Conclusions

Many physiological responses to environmental contaminants can be attributed to perturbations in pathways associated with GC signaling, inflammation, oxidative stress, and ultimately, alterations in TRP metabolism. Notably, environmental contaminants and their responses in vertebrate species are strikingly similar. Given that AhR and TRP metabolism are evolutionarily conserved across vertebrates (i.e., mammals, birds, fish), the crosstalk between xenobiotic receptors such as AhR, IDO/TDO immunoregulatory pathways, and altered stress indicators (i.e., antioxidant levels, ROS levels, and cortisol) suggest that alterations in TRP-KYN metabolism, metabolite levels, and ratio can be a cross-species marker of environmental exposure to chemical contaminants. Moreover, as ecotoxicological assessments are slowly moving away from evaluating the effects of a single compound to complex environmental mixtures, the TRP-KYN pathway provides a promising avenue to model the impacts of exposure to complex mixtures. Given its role in other biological processes, the TRP-KYN pathway provides many integrative biomarkers, both enzymatic ratios and metabolite levels can link environmental exposures to animal health, and broadly, ecosystem health. These integrative markers can be used as part of environmental effects monitoring (EEM) programs, where a few biomarkers could be monitored over a larger spatial area, across multiple species, trigger “investigation of cause” (IOC) studies, or focused studies to aid in the identification of mixtures and environmental factors that cause sublethal effects over acute and chronic time points.

1.6 References

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1.7 Supplemental

Table 1. Clinical Implications of Kynurenine Metabolites in Humans

Organ	Clinical Implication	Disease Phenotype	Reference(s)
Brain	Cancer	Cancer	[190]
	Neurobehaviour	Addiction	[191]
		Bipolar	[192], [193],[194], [195]
		Depression	[194], [196], [197], [198]
		Schizophrenia	[195], [194], [199]
	Neurodegenerative	ALS/MS	[46], [200]
		Alzheimer's Disease	[201], [45]
		Dementia	[202]
		Parkinson's Disease	[203], [204]
		REVIEW	[205], [206]
	Neurological Illness	CNS and PNS disorders	[207]
		Migraine	[208], [209]
		Neurotransmission	[210]
		REVIEW	[211]
		Somatic Symptoms	[212]
	Neuropathic Pain	Neuropathic Pain	[213], [214], [215]
Breast	Cancer	Cancer	[216]
Heart	Cardiometabolic	Cardiometabolic	[217]
		Cardiovascular Disease	[218],[219], [220], [221]
Endocrine	Metabolism	Obesity	[222], [223]
Gastrointestinal	Gastrointestinal Regulation	Gastrointestinal Disorders	[224]
		Gut Homeostasis	[225], [226]
		Inflammatory Bowel Disease	[227]
	Immunity	Endocrinopathies	[228]

Immune System		Inflammation	[229], [118]
		SARS-CoV-2	[230], [231]
Kidney	Kidney Disease		[232], [233]
Liver	Cancer	Cancer	[234], [235]
	Metabolism	Energy Homeostasis	[236], [237]
		Inflammation	[238]
		Liver Cirrhosis	[239]
		Liver Fibrosis	[240]
Muscle/Skeletal	MuscoSkeletal	Inflammation	[241]
		SARS-CoV-2	[242]
Placenta	Reproduction	Pregnancy	[243], [244], [245], [246]
	Reproductive Disorders	Preeclampsia	[247], [248]
REVIEW	Cancer		[249]
	Gerontology		[250]
	All disease phenotypes		[251], [252], [253]

RATIONALE

Tryptophan is an essential amino acid that plays a critical role in multiple metabolic pathways. In monogastric organisms—including humans, rats, dogs, and birds—more than 95% of free tryptophan is metabolized via the tryptophan-kynurenine (TRP-KYN) pathway. The first and rate-limiting step in this pathway is the conversion of tryptophan to N-formylkynurenine, a process mediated by the enzymes tryptophan 2,3-dioxygenase (TDO2) and indoleamine 2,3-dioxygenase (IDO1/IDO2). Changes in tryptophan metabolism can alter the availability of tryptophan for protein synthesis and serotonin production, as well as influence the levels of immunomodulatory and neuroactive metabolites along the TRP-KYN pathway.

There is growing evidence that the key enzymes in this pathway (TDO2 and IDO1/IDO2) are sensitive to various stressors, including glucocorticoids, inflammation, oxidative stress, and exposure to environmental toxicants. Moreover, studies, including work from our lab, have demonstrated that certain environmental contaminants known to affect glucose and lipid homeostasis—such as dioxins, ozone, and bisphenol A—can also disrupt components of the TRP-KYN pathway. This disruption suggests that altered tryptophan metabolism may be closely tied to the metabolic disturbances associated with exposure to environmental pollutants.

This study investigates the effects of bitumen-derived compounds, complex mixtures primarily composed of carbon and hydrogen with smaller amounts of nitrogen, oxygen, and sulfur. Among these, naphthenic acid fraction compounds (NAFCs) and polycyclic aromatic compounds (PACs) are abundant in oil sands environments and are key environmental concerns in the Athabasca Oil Sands Region (AOSR). Elevated oil sands activity has increased the levels of these contaminants, which are linked to metabolic disturbances such as disrupted glucose and lipid homeostasis. However, the relationship between NAFC/PAC exposure, tryptophan metabolism, and metabolic dysfunction remains poorly understood.

Given that the TRP-KYN pathway is highly conserved across species, this study aims to explore whether altered tryptophan metabolism can serve as a cross-species biomarker of environmental stress. By focusing on how exposure to these complex mixtures disrupts the TRP-KYN pathway in both *in vitro* and *in vivo* models, this research seeks to determine whether the kynurenine-tryptophan ratio (KTR) can be used to measure stress in various species. This would provide a valuable tool for monitoring ecosystem health, particularly in regions affected by industrial pollution, and for understanding the broader metabolic consequences of environmental contaminants.

HYPOTHESIS

The kynurenine-tryptophan ratio (KTR) can serve as a sensitive and reliable cross-species biomarker for environmental stress and exposure to environmental toxicants. Specifically, it is hypothesized that exposure to petroleum-derived compounds from the Athabasca Oil Sands Region (AOSR), including polycyclic aromatic compounds (PACs) and Naphthenic Acid Fraction Components (NAFCs), will disrupt tryptophan metabolism, as indicated by alterations in KTR, and that these alterations can be correlated with markers of environmental stress in both *in vitro* mammalian hepatocyte models and *in vivo* wildlife species.

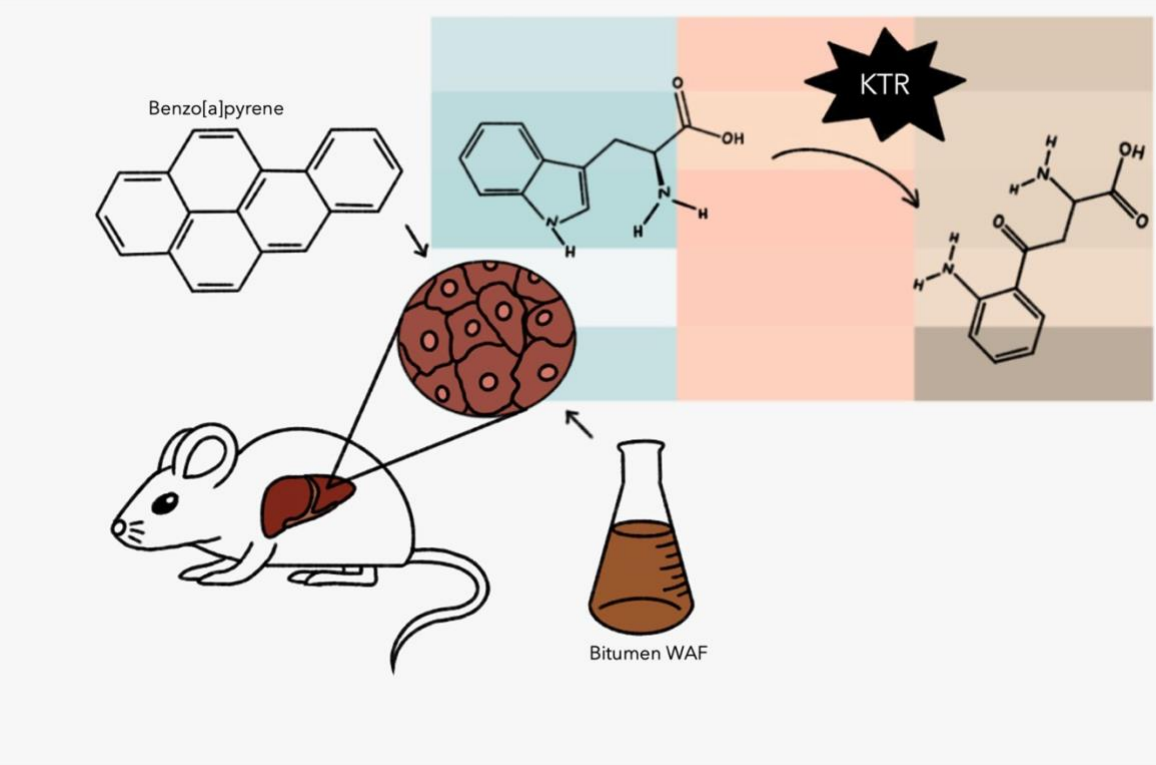
OBJECTIVES

1. To assess the impact of polycyclic aromatic compounds (PACs), specifically benzo[a]pyrene (BaP) and Bitumen Water Accommodated Fraction (BitWAF), on the kynurenine-tryptophan ratio (KTR) in mammalian hepatocytes, and to elucidate the metabolic pathways involved in KTR alterations, particularly the role of the aryl hydrocarbon receptor (AhR) pathway.
2. To investigate the effects of Naphthenic Acid Fraction Components (NAFCs) from Oil Sands Process-Affected Water (OSPW) on cellular metabolism in rat hepatocytes, with a focus on how NAFC-induced metabolic reprogramming influences KTR as a marker of environmental stress.
3. To explore the role of peroxisome proliferator-activated receptor alpha (PPARα) in regulating the tryptophan-kynurenine pathway in response to NAFC exposure, specifically investigating whether KTR modulation is mediated through PPARα activation and its transcriptional control over the rate-limiting enzyme *Tdo2*.
4. To validate the use of KTR as a biomarker for environmental exposure by examining its correlation with established markers of environmental stress, such as CYP1 enzyme activity (EROD), in wildlife species from the AOSR, thereby assessing the ecological relevance of KTR alterations observed *in vitro*.

CHAPTER II: RAT HEPATOCYTES EXHIBIT ALTERED KYNURENINE-TRYPTOPHAN RATIO IN RESPONSE TO POLYCYCLIC AROMATIC COMPOUND EXPOSURE

This chapter has been submitted as an original article with the same title and is currently in peer-review, 2024 September.

2.1 Abstract



Graphical Abstract. This study is the first to demonstrate an increase in the kynurenine-tryptophan ratio (KTR) in rat hepatocytes following environmental exposure to PACs, specifically benzo[a]pyrene and a Bitumen Water Accommodated Fraction.

Polycyclic aromatic compounds (PACs) are ubiquitous environmental contaminants and potent aryl hydrocarbon receptor (AhR) ligands, formed during the incomplete combustion or pyrolysis of organic material, or diagenetic processes. These compounds are widely distributed in air, water, soils, and sediments, including near unconventional oil and gas extraction activities and petroleum refineries. Bitumen, a heavy crude oil containing a complex mixture of PACs, is often diluted with lighter hydrocarbons for transport via pipelines or rail. Accidental spills or pipeline breaches can release this diluted bitumen into the environment resulting in wildlife exposure. Although there are many studies which have examined the toxicity of bitumen spills in aquatic species, toxicity in mammals remains largely unexplored. In this study, we propose the kynurenine-tryptophan ratio (KTR) as a potential biomarker for xenobiotic exposure to AhR ligands. We investigated whether AhR ligands, such as benzo[a]pyrene (BaP) and a Bitumen Water Accommodated

Fraction (BitWAF), alter the KTR in a mammalian hepatocyte model. BitWAF was generated using the standardized CROSERF method. McA-RH7777 cells were exposed to 10 μM BaP and 0.01-10% BitWAF for 8, 24, and 48 hours, followed by measurement of kynurenine and tryptophan output and their ratio (KTR) by HPLC/MS/MS. Both BaP and BitWAF increased KTR, with BitWAF exhibiting this effect at lower concentrations (0.01% and 0.1%). The increase in KTR is primarily driven by elevated kynurenine levels, indicating that both BaP and BitWAF influence KTR through similar metabolic pathways. These results suggest that KTR is a sensitive marker for detecting exposure to PACs.

2.2 Introduction

Tryptophan (TRP) is an essential dietary amino acid that is critical in various physiological processes, including cell growth, communication, and the coordination of adaptive responses to environmental stressors and dietary cues. In vertebrates, TRP, when not used for protein synthesis, is predominantly metabolized via the Tryptophan-Kynurenine (TRP-KYN) pathway. This pathway produces several metabolites, including kynurenine, kynurenic acid, anthranilic acid, quinolinic acid, and NAD+ which are involved in critical functions such as immune modulation, neurotransmission and the maintenance of cellular homeostasis. The TRP-KYN pathway is increasingly being recognized for its sensitivity to various stressors, including psychological stress, inflammation, and environmental toxicants.[1]

Various environmental contaminants have been shown to alter enzymes in the TRP-KYN pathway leading to increased levels of kynurenine metabolites, including kynurenic acid and indole compounds.[2–7] Although the role of TRP metabolism following exposure to complex mixtures of environmental contaminants is not yet fully understood, emerging evidence suggests a link between chemically induced metabolic perturbations and alterations in TRP-converting enzymes and metabolites.[8] Notably, the majority of observed changes in TRP-KYN metabolism have been linked to exposure to well-studied aryl hydrocarbon receptor (AhR) agonists, such as benzo[a]pyrene (BaP) and tetrachlorodibenzo-p-dioxin (TCDD). BaP exposure has been shown to alter tryptophan metabolism by activating AhR signaling and regulating the tryptophan metabolizing enzyme tryptophan 2,3-dioxygenase (TDO).[8] Similarly, TCDD was shown to induce expression of the other major tryptophan metabolizing enzyme, indolamine 2,3-dioxygenase (IDO).[9] While these findings are well-documented in human disease models, particularly cancer research, there is a significant gap in our understanding of TRP-KYN pathway alterations in wildlife exposed to environmental toxicants.[1]

In the Athabasca Oil Sands Region (AOSR) of Northern Alberta, Canada, there are considerable concerns about the health impacts of exposure to polycyclic aromatic compounds (PACs). Among these PACs are polycyclic aromatic hydrocarbons (PAHs), a class of AhR ligands that are known to regulate the expression and activity of key enzymes in the TRP-KYN pathway and influence downstream metabolites. The oil sands deposits contain bitumen, a viscous form of crude oil, which contains a complex mixture of PACs and their alkylated derivatives (Supplemental Figure 1). To facilitate transportation through

pipelines, bitumen is typically diluted with natural gas condensate, rich in lower molecular weight volatile organic compounds, a practice that exacerbates existing environmental concerns, particularly in the event of spills or leaks, which have the potential to negatively impact the health of wildlife living in affected areas.[10,11] Given that the TRP-KYN pathway is sensitive to environmental toxicants, including AhR ligands like PAHs, this study aims to investigate whether exposure to a prototypical AhR ligand (BaP) and a bitumen water-accommodated fraction—a complex mixture containing multiple AhR ligands—alters the KTR in a mammalian system.

2.3 Methods

2.3.1 Preparation of Bitumen Water-Accommodated Fractions (BitWAF)

We used a bitumen water-accommodated fraction (BitWAF) as a model of a bitumen spill. Based on the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) method[12,13], bitumen (reference material obtained from Environment and Climate Change Canada, Emergency spill response and restoration, Oil and fuel forensics and monitoring, Ottawa, ON, 1g) was dispersed into 1L of distilled H_2O and stirred in the dark at room temperature for 18 hours, such that the vortex created by the stir bar was 1/3 the height of the distilled H_2O. The mixture was allowed to settle for 6 hours to ensure the WAF was separated from any submerged oil. The bottom 10% of the WAF was collected and considered to be 100% WAF. For the control, 1g of commercial Mineral Oil (Life Brand$^{TM/MC}$) underwent the same process to obtain 100% Mineral Oil WAF. Cell exposure and treatments were conducted immediately after generation and dilution of the WAF.

2.3.2 Cell Culture Maintenance and Treatment

McA-RH7777 cells, an immortalized rat hepatoma cell line (CRL-1601; ATCC, Manassas, VA) were cultured at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM 1X with 4.5 g/L glucose, L-glutamine, and sodium pyruvate; DMEM; Corning, Manassas, VA), supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone^{TM}, Marlborough, MA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Unless otherwise noted, experimental protocols were carried out in DMEM media supplemented as described.

McA-RH7777 cells were grown in 10mm dishes (Corning). Fresh medium was provided every 48h and cells were sub-cultured when ~80% of the cells were fully adhered and cells showed a spindle-like morphology. Cells were considered confluent when 80% of cells were attached to the plate after a minimum of two passages. Confluent cells from passages 11-13 were used for experiments. For experiments, confluent cells from passage 11 were seeded in 6-well plates (Corning) at a density of 250,000 cells/mL, 200,000 cells/mL and 150,000 cells/mL for 8, 24 and 48h exposures, respectively, and grown to 85% confluency prior to treatment.

McA-RH7777 cells were treated for 8, 24 and 48h with either (1) Vehicle control (DMSO) and 10 µM BaP (Sigma-Aldrich; Burlington, MA), or (2) a water-accommodated fraction of Bitumen at concentrations of 0.01, 0.1, 1, 10%. The control for BitWAF exposure was

Mineral Oil WAF. The concentrations of BitWAF [0.01, 0.1, 1, 10%] were selected to cover a broad range of potential exposure similar to what has been reported in other studies assessing dilbit toxicity. [14,15] Each treatment was performed in supplemented-DMEM as described above (N=6 independent experiments), and spent media was collected for HPLC/MS/MS analysis to assess tryptophan and kynurenine levels.

2.3.3 qPCR

Real-time quantitative polymerase chain reaction (qPCR) was performed to determine changes in gene expression of the canonical AhR pathway. Downstream mRNA expression of genes transcriptionally regulated following activation of AhR [*Cyp1a1, Cyp1b1,* and *Tiparp*] were assessed. In the AhR pathway, *Cyp1a1* (cytochrome p450 family 1 subfamily A member 1) and *Cyp1b1* (cytochrome p450 family 1 subfamily B member 1) play a pivotal roles in xenobiotic metabolism and serve as primary responders to environmental toxins.[16] *Tiparp* (TCDD inducible polyADP-ribose polymerase), another AhR target is crucial for modulating the transcriptional activity of AhR and subsequent detoxification processes.[17] Following cell treatment, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were measured using the NanoDrop One^{TM} Microvolume UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. RT-qPCR was performed using PerfeCta® SYBR® green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the CFX384 Touch^{TM} Real-Time PCR Detection System (Bio-Rad). The cycling conditions included polymerase activation (95°C for 10 min), followed by 40 cycles of denaturing (95°C for 10 sec), annealing (60°C for 10 sec) and extension (72°C for 15 sec, slow-ramp rate 2.5°C per sec). Levels of gene expression were calculated using the ΔΔCt method[18] and normalized using the geometric means of reference gene expression: beta-2-microglobulin (*B2m*) and peptidylprolyl isomerase A (*Ppia*). Primer sequences are supplied in Table 1.

Table 1. Table of Primer Sequences

Accession number	Gene name	Symbol	Forward Sequence (5'-3')	Reverse sequence (5'-3')
NM_017101.1	Peptidylprolyl isomerase A	Ppia	CCGCTGTCTCTTTTCGCC	GCTGTCTTTGGAACTTTGTCTGC
NM_012512.2	Beta-2-microglobulin	B2m	AATTCACACCCACCGAGACC	GCTCCTTCAGAGTGACGTGT
NM_012540.3	Cytochrome P450 Family 1 Subfamily	Cyp1a1	TTGGGGAGGTTACTGGTTCTG	GAGTTAGGGAGGTAACGGAGG

	A Member 1			
NM_012940.2	Cytochrome P450 Family 1 Subfamily B Member 1	Cyp1b1	CAACCCAACTTACCATACGTCA	AAACAAAGGTGTTGGCAG
XM_039102285.1	TCDD Inducible Poly(ADP-Ribose) Polymerase	Tiparp	GGTGGGATTGGGTTAAGCTCT	CAGTCCATCTATACACCTGCCC

2.3.4 High Performance Liquid Chromatography Tandem Mass Spectrometry screening of the Kynurenine:Tryptophan Ratio

Materials and reagents: Unlabeled analytical standards of L-tryptophan (TRP, ≥ 98% HPLC) and L-kynurenine (KYN, ≥ 98%) were purchased from Sigma- Aldrich Canada Co. (Oakville, ON, CA). Mass labeled L-tryptophan- 2′,4′,5′,6′,7′-d5 (indole-d5) (98%, TRP-d5), L-kynurenine-d4 [4-(2-aminophenyl-3,5-d2)] (99%, KYN-d4) were purchased from CDN Isotopes (Pointe-Claire, QB, CA). All solutions were stored at 4◦C. L-Acetonitrile (ACN) and water were purchased from Fisher-Scientific and of HPLC-grade. Ultrapure Milli-Q was obtained from a Synergy^{TM} Milli-Q purification system from Millipore (Billerica, MA).

Preparation of samples for chromatographic analysis: To a 2 mL glass vial, 60 µL of cell supernatant (*in vitro* media) and TRP-d5 and KYN-d4 (20 µL of a mixture containing 7.5 ng/µL each) were added and adjusted to a final volume of 1.5 mL with ACN (1420 µL). Samples were vortexed for 30 secs prior to chromatographic analyses. Method blanks consisting only of the control media were prepared in an identical manner to our samples. Reagent blanks of ACN were injected periodically to assess the extent of contamination from the solvent and between injections. Matrix effects were assessed by fortifying control media (60 µL) with our native and mass labelled compounds (20 µL each and diluting to a final volume of 1.5 mL) and comparing their signals to that of an external standard prepared in ACN. Limits of detection and quantitation were determined according to Eurachem guidelines by fortifying control media with native TRP and KYN (n=6).

High Performance Liquid Chromatography Tandem Mass Spectrometry: The detection and quantitation of TRP and KYN in biological matrices was based on a previously published validated method.[19] Separations of mass labeled and native KYN and TRP were performed using a SEQUANT ZICR HILIC PEEK coated HPLC column (4.6 mm x 100mm, 3.5 µM particle size, 200 A pore size) (Merck, Darnstadt, Germany). The HPLC was coupled to a Sciex 365 triple quadrupole mass spectrometer retrofitted with an HSID Ionics EP+ orthogonal ionization source. Ionization was performed in the positive ion

mode and the multiple reaction monitoring ion transitions used for detection and quantitation were: TRP: m/z 205.1 → 188.2; KYN: m/z 209.4 → 94.1, TRP-d5: m/z 201.2 → 192.4 and KYN-d4: m/z 213.3 → 196.2. No statistical differences ($p<0.05$) were observed between the response of the target analytes in media relative to the standards prepared in solvent implying that matrix effects were negligible. Limits of detection and quantitation for TRP were 2.8 and 9.5 pg/μL and for KYN were 4.3 and 14.3 pg/μL, respectively.

2.3.5 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism (v.10.3.0, GraphPad Software, San Diego, CA). Data were assessed for outliers using Grubb's Test, tested for normality with the Shapiro-Wilk test, and checked for equal variance. For comparisons between control and benzo[a]pyrene treatments, a Student's t-test was performed. For comparisons between control and the multiple treatment groups of BitWAF, a one-way ANOVA was conducted, followed by Dunnett's test for post hoc comparisons when significant differences were detected ($p \leq 0.05$). Data that did not pass tests for normality or equal variance were analyzed by Kruskal-Wallis one-way ANOVA on ranks. If significance was found, Dunn's post-hoc test was used for comparisons against control. All results are presented as mean ± SEM and were considered significant when $p \leq 0.05$.

2.4 Results

2.4.1 Kynurenine:Tryptophan Ratio in response to BaP exposure

To assess whether environmental contaminants classified as AhR ligands can alter KTR, we tested the effects of benzo[a]pyrene, a known AhR ligand, on McA-RH7777 cells. BaP exposure resulted in an increase in kynurenine production at all three time points (8, 24, and 48 hours) when compared to controls (Figures 1B, 1E, 1H). This increase in kynurenine was accompanied by a corresponding increase in the kynurenine-tryptophan ratio across all time points (Figures 1C, 1F, 1I).

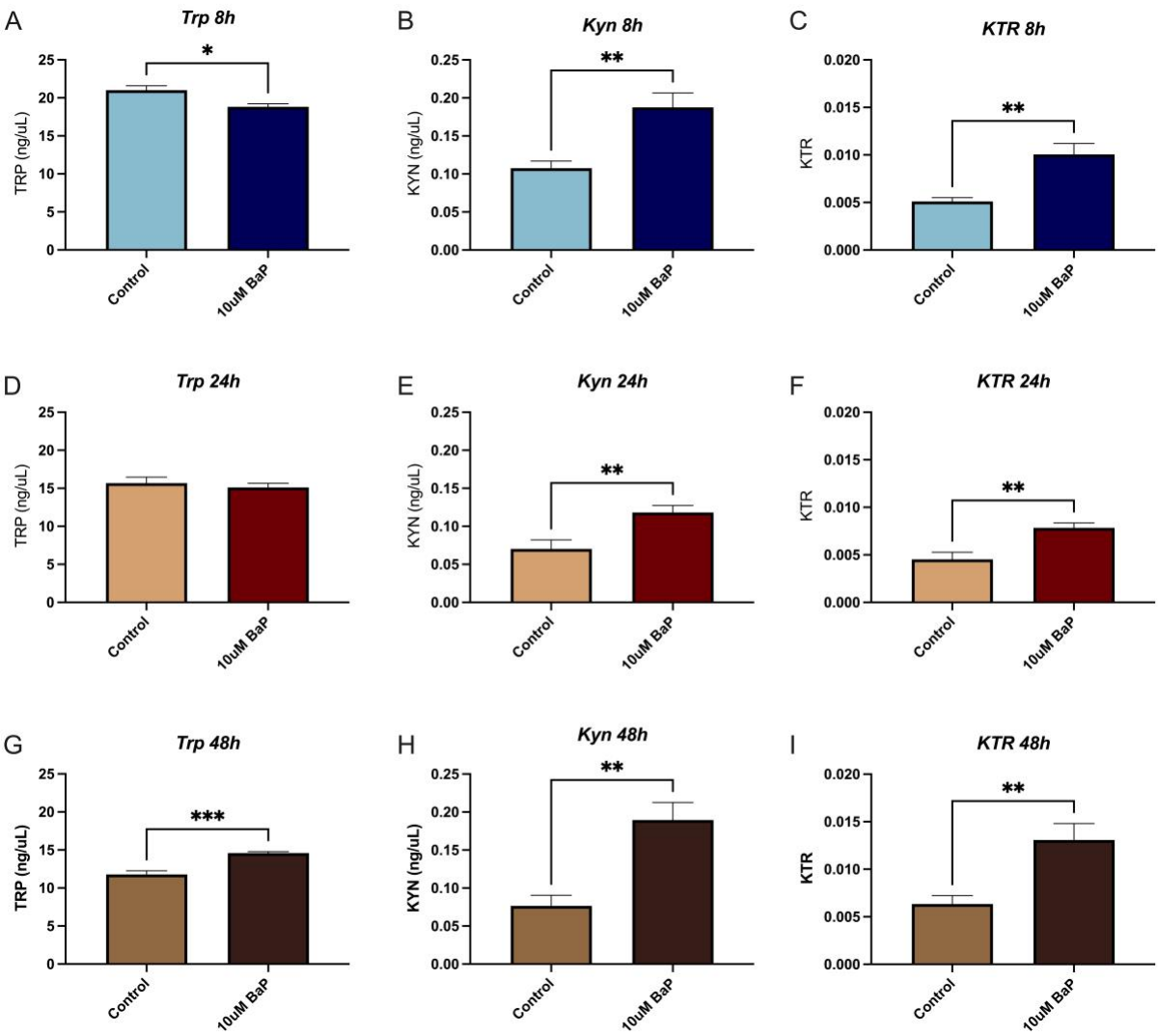


*Figure 1. Concentrations of Tryptophan (A, D, G) and Kynurenine (B, E, H) [ng/uL] in spent media from McA-RH7777 cells following 8 (A, B, C), 24 (D, E, F), and 48h (G, H, I) exposure to 10μM Benzo[a]pyrene (n=6). Calculated Kynurenine-Tryptophan Ratio (KTR) (C, F, I) values are shown. Values are presented as mean ± SEM. *, **, *** indicates significance p ≤ 0.05, 0.01, 0.005 versus control, respectively.*

2.4.2 Kynurenine:Tryptophan Ratio in response to Bitumen WAF exposure

Exposure to the lowest concentration of BitWAF [0.01%] resulted in a significant increase in kynurenine levels and KTR at all three time points (Figures 2C, 2F, 2I). Additionally, 0.1% WAF increased KTR at the 8-hour time point, and 1% WAF increased KTR at the 48-hour time point (Figures 2C and 2I).

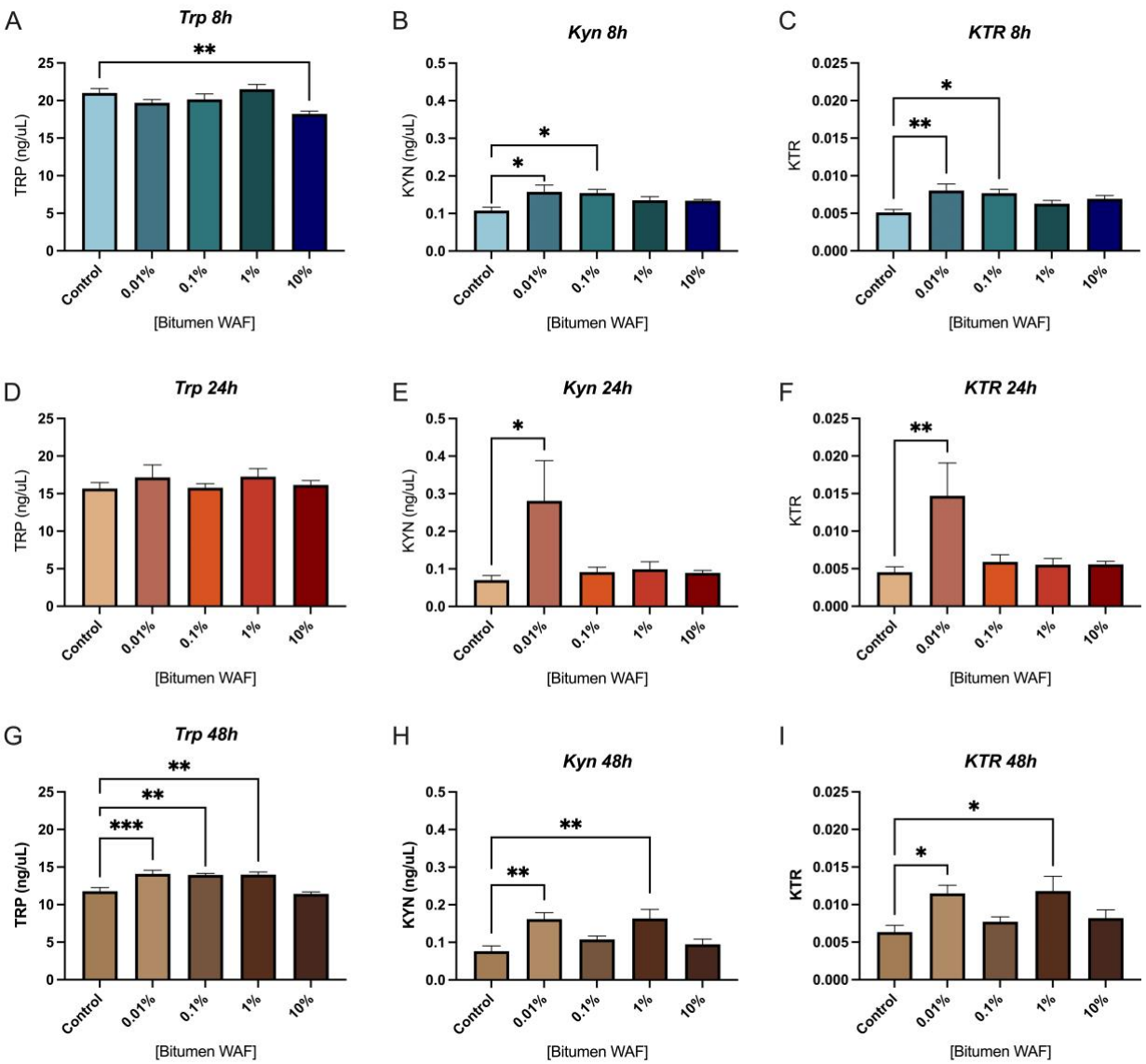


*Figure 2. Concentrations of Tryptophan (A, D, G) and Kynurenine (B, E, H) [ng/uL] in spent media from McA-RH7777 cells following 8 (A, B, C), 24 (D, E, F), and 48h (G, H, I) exposure to a Bitumen WAF dose response [0.01, 0.1, 1, 10%] (n=6). Calculated Kynurenine-Tryptophan Ratio (KTR) (C, F, I) values are shown. Values are presented as mean ± SEM. *, **, *** indicates significance $p \leq 0.05, 0.01, 0.005$ versus control, respectively.*

2.4.3 Correlation between KTR and canonical AhR Gene Expression

To further explore the relationship between KTR and the expression of key genes known to be induced by AhR ligand binding, we performed Pearson correlation analyses comparing KTR with the mRNA expression levels of *Cyp1a1, Cyp1b1, and Tiparp* (Figure 3; Supplemental Figure 2). In the BaP exposure groups (Figures 3A, 3B, 3C), we observed

statistically significant, positive correlations between KTR and the expression of these genes across all time points. Conversely, in the BitWAF exposure groups (Figures 3D, 3E, 3F), no significant correlations were observed between KTR and the expression of these genes, with the data showing greater variability and no consistent pattern across time points.

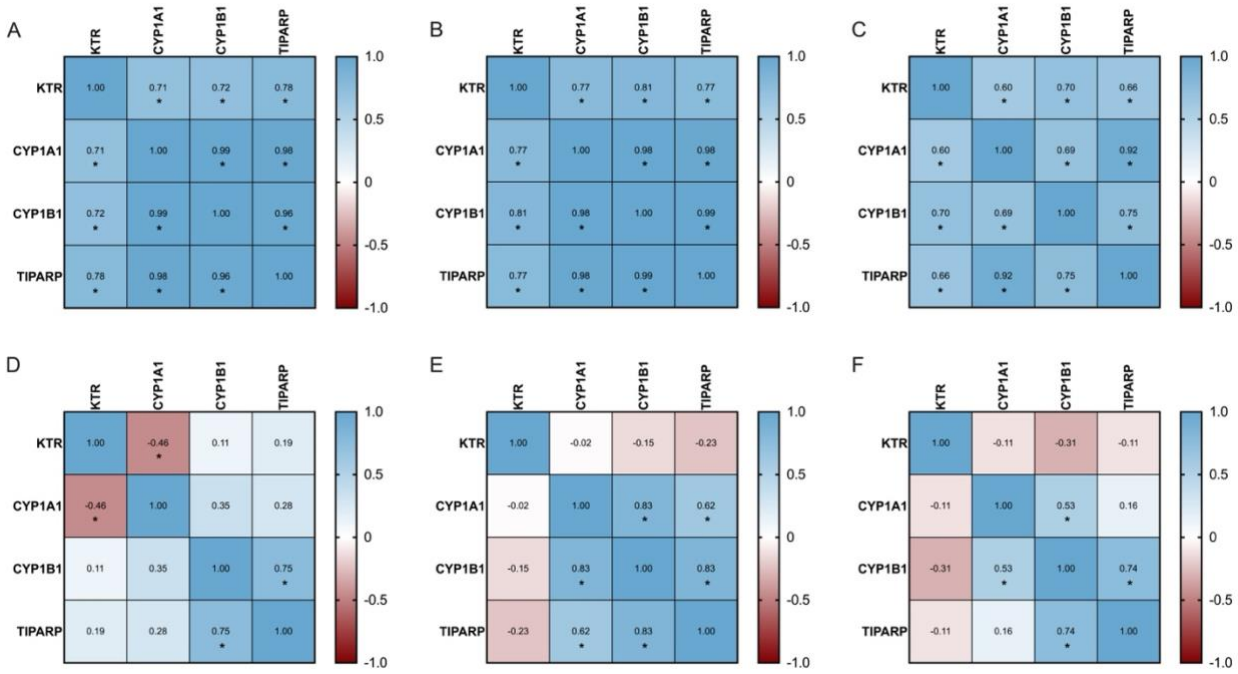


*Figure 3. Heat map of Pearson's correlations comparing the kynurenine-tryptophan ratio (KTR) to CYP1A1, CYP1B1, and TIPARP mRNA expression. Panels A, B, and C represent BaP exposure at 8, 24, and 48 hours, respectively. Panels D, E, and F represent Bitumen Water Accommodated Fraction (BitWAF) exposure at 8, 24, and 48 hours, respectively. Each heat map depicts the strength and direction of the correlation, with color intensity indicating the correlation coefficient value. * indicate a significant ($p \leq 0.05$) correlation.*

2.5 Discussion

In this study, we investigated the effects of benzo[a]pyrene and a BitWAF extract on the kynurenine-tryptophan ratio in mammalian hepatocytes. Our findings demonstrate a proof-of-concept that both BaP and BitWAF can significantly alter the TRP-KYN pathway, with BitWAF showing increased KTR at concentrations as low at 0.01%. These results suggest that the KTR is a sensitive marker for detecting exposure to PACs, consistent with other studies that have explored the impacts of AhR ligands on amino acid metabolism.[8,20] Despite their ability to influence downstream kynurenine metabolites and alter the activity of rate-limiting enzymes within the pathway, no studies have yet demonstrated that the kynurenine-tryptophan ratio (KTR) itself is altered by chemical exposures. Together, this evidence suggests that exposure to these environmental PACs may also result in alterations in the KTR, which could serve as a potential marker for AhR-ligand exposure.

The aryl hydrocarbon receptor is a nuclear protein that, upon binding to endogenous and exogenous ligands, translocates into the nucleus to regulate gene expression. The AhR plays a pivotal role in mediating the toxicological responses to various environmental xenobiotics through the activation of xenobiotic-metabolizing phase I and phase II enzymes, particularly cytochrome p450 enzymes (CYPs). These enzymes are crucial to the metabolic processing of both xenobiotics and endogenous compounds, facilitating the detoxification and excretion of harmful substances. Our study provides proof-of-concept that BaP, a well-studied, prototypical PAH and AhR ligand, consistently increases KTR—a response primarily driven by elevated kynurenine levels while tryptophan levels remained relatively unchanged. This finding is consistent with existing literature that highlight AhR activation by well-characterized ligands like BaP and TCDD in regulating the expression and/or activity of the TRP-metabolizing enzymes TDO and IDO.[21,22] Moreover, the strong correlation between BaP-induced KTR changes and the mRNA expression of *Cyp1a1, Cyp1b1* and *Tiparp* across all three time points (Figure 3) further supports the hypothesis that the BaP-induced changes in KTR are likely due to its effect as an AhR agonist. The correlation between BaP-induced KTR changes and these AhR-responsive genes suggests that the observed metabolic shift in the TRP-KYN pathway can be affected by AhR ligands.

However, while BaP serves as a well-characterized example of a single compound that influences tryptophan metabolism via AhR activation, most exposures to PACs occur in mixtures. Interestingly, some of these environmentally relevant mixtures containing PACs have been shown to alter tryptophan metabolism. For example, metabolomics studies have demonstrated that exposure to particulate matter (PM2.5), which contains a complex of harmful substances including heavy metals, PACs, and persistent organic pollutants, caused dysregulated tryptophan metabolism.[4,23] Furthermore, an untargeted metabolomics analysis in urine samples from firefighters collected at baseline and 2-4 hours post-fire (i.e., following exposure to PACs from the fire) revealed a significant induction of the tryptophan metabolites kynurenic acid and indole-3-acetic acid.[3] These findings suggest that the impact of environmental pollutants on the TRP-KYN pathway is not limited to individual PAHs like BaP but extends to complex mixtures containing compounds that that can act through the AhR to influence tryptophan metabolism.

Our findings with BitWAF, a complex mixture containing multiple PACs, showed significant inductions of KTR at the lower [0.01, 0.1%] but not higher concentrations. Interestingly, with the BitWAF exposure the increase in KTR may not be solely driven by AhR activation, as no consistent correlation was observed between KTR and *Cyp1a1, Cyp1b1* or *Tiparp* mRNA expression (Figure 3). This discrepancy suggests that while both BaP and BitWAF influence the TRP-KYN pathway, the mechanisms underlying these effects may differ. In fact, the diverse composition of BitWAF, is particularly relevant, as bitumen is a highly variable mixture of PACs, including PAHs and their alkylated derivatives, sulfur-containing compounds, and other complex organic molecules. Additionally, bitumen composition varies based on the source of oil, geological location, extraction methods, technological processing, climate conditions, and even slight variations

introduced during the creation of WAF, despite standardized methods. This finding is consistent with existing literature that shows that PAH mixtures do not always uniformly induce CYP enzymes.[24] In fact, it has been hypothesized that depending on their composition, mixtures of PAHs may activate AhR through both canonical pathways (i.e., Cyp1a1), and non-canonical pathways involving transcription factor pathways like NFkB, or hormone signalling pathways (i.e., androgen and estrogen receptors).[25] Alternatively, this variability suggests that additional molecular pathways, such as those involving glucocorticoids (GR) or Peroxisome Proliferator-Activated Receptors (PPARs), may contribute to the altered KTR observed in BitWAF-exposed cells.

Glucocorticoids are steroid hormones that play a critical role in regulating various physiological processes, including stress responses, immune function, and metabolism. Evidence suggests that PAHs, such as benzo[a]pyrene, can interact with glucocorticoid receptors, potentially altering stress-related gene expression. Recently, evidence from our group show that in rainbow trout, acute stress, leading to increased hepatic and brain cortisol levels, is associated with a concurrent increase in KTR.[19] Additionally, exposure to PAHs like naphthalene, β-naphthoflavone, and benzo[a]pyrene in rainbow trout altered plasma cortisol levels and 5HIAA/5HT ratios in several brain regions, with 5-HT being a key component of TRP metabolism.[6] Together, these interactions suggest a link between PAH exposure, glucocorticoid signaling, and TRP-KYN pathway regulation. In addition to GRs, PPARs also play a crucial role in mediating the effects of PAHs on cellular metabolism.[26,27] PPARs, particularly PPARα and PPARγ, are nuclear receptors that regulate lipid metabolism, glucose homeostasis, and inflammation. PAHs, including those present in BitWAF, have been shown to activate PPARs,[28–30] leading to changes in the expression of genes involved in these metabolic processes. Together, these data suggest that the interplay between PPAR signaling, and PAH exposure could be a significant factor in the regulation of KTR and related metabolic pathways in environmentally exposed organisms.

2.6 Conclusion

This study is the first to demonstrate altered kynurenine-tryptophan ratio in non-human mammalian model following environmental exposure to PAHs, specifically benzo[a]pyrene and a Bitumen Water Accommodated Fraction. Importantly, we found that the KTR can be affected even in the context of complex environmental mixtures like BitWAF. Our findings show that despite the potential complexity of interactions between environmental pollutants and cellular pathways, the outcome—altered KTR—remains consistent. This supports the utility of KTR as a marker for detecting xenobiotics exposure, whether they be independent compound and complex mixture. Since tryptophan and its metabolites play key roles in growth, immune function, and cellular homeostasis, changes in the KTR have the potential to be used as a biomarker for organism health following exposure to a broad range of contaminants. Research is ongoing to further explore the utility of KTR as a biomarker of health across diverse scenarios in environmental toxicology.

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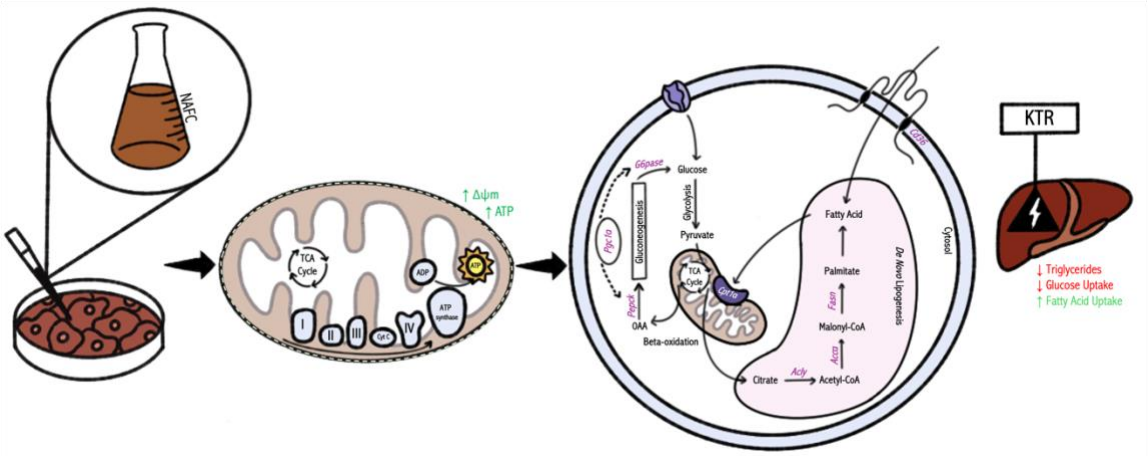
CHAPTER III: NAPHTHENIC ACID FRACTION COMPONENT–INDUCED METABOLIC AND MITOCHONDRIAL ALTERATIONS IN RAT HEPATOMA CELLS: MONITORING METABOLIC REPROGRAMMING WITH THE KYNURENINE-TRYPTOPHAN RATIO

This chapter has been submitted as an original article with the same title and is currently in peer-review, 2024 October.

3.1 Abstract



Graphical Abstract. These findings provide insights into how bitumen-derived organic compounds may be affecting wildlife health through impacts on cellular metabolism and mitochondrial function. Exposure to higher concentrations of naphthenic acid fraction components (NAFC; 14.7 and 73.4 mg/L) resulted in decreased triglyceride levels and glucose uptake, with an increase in fatty acid uptake and expression of beta-oxidation genes, indicating a switch from glucose to fatty acid oxidation in McA-RH7777 cells. The switch in substrate availability due to NAFC exposure suggests altered mitochondrial function, confirmed by ATP, JC-1 and TUNEL assays. The KTR was effectively used to mark the shifts in substrate utilization and altered metabolic state following McA-RH7777 exposure to 73.4 mg/L NAFC.

Altered body condition and diminished growth in wildlife within the Alberta Oil Sands Region (AOSR) have raised concerns regarding the impact of oil sands industrial activity on wildlife in the region. Chemical constituents from bitumen-influenced waters, including oil sands process-affected water (OSPW), can disrupt endocrine signaling, leading to aberrant lipid accumulation, and altered glycemic control in mammals. This study explored the effects of naphthenic acid fraction components (NAFC), derived from OSPW, on energy homeostasis using the McA-RH7777 rat hepatocyte model. Cells were exposed to NAFC at nominal concentrations of 0, 0.73, 14.7, and 73.4 mg/L for 24 and 48 hours. We assessed gene expression related to lipid and glucose metabolism, and measured triglyceride accumulation, glucose, and fatty acid uptake. NAFC exposure (14.7, and 73.4 mg/L) reduced triglyceride levels and glucose uptake, coupled with an increase in fatty acid uptake and expression of beta-oxidation genes suggesting a metabolic switch from glucose to fatty acid oxidation. This switch in substrate availability signifies a shift in bioenergetics,

potentially linked to altered mitochondrial function. To investigate this, we conducted ATP, JC-1, and TUNEL assays to measure cellular ATP levels, mitochondrial membrane potential, and apoptosis, respectively. At both time points, 73.4 mg/L NAFC exposure resulted in increased ATP levels, induced mitochondrial membrane hyperpolarization, and increased apoptosis. These results suggest that mitochondrial efficiency is compromised, necessitating metabolic adaptations to maintain energy homeostasis. Given that cells exhibit metabolic flexibility that allows them to dynamically respond to changes in substrate availability, we further demonstrated that the tryptophan-kynurenine ratio (KTR) serves as a marker for a shift in bioenergetics under these stress conditions. Together, these findings provide insights into the metabolic state of mammalian hepatocytes and a potential mechanism by which bitumen-derived organic compounds may be affecting wildlife health in the AOSR.

3.2 Introduction

The oil sands in Alberta are the third largest oil reserve in the world and are a major source of revenue, energy, and economic success for both the province and Canada.[1] Comprised of three regions – Athabasca, Cold Lake, and Peace River – these deposits consist of a mixture of sand, clay, water and bitumen. The extraction and processing of bitumen from these reserves have provided numerous jobs, spurred infrastructure development, and bolstered Canada's position in the global energy market. However, alongside these economic benefits, continued industrial development in the Alberta Oil Sands Region (AOSR) has sparked significant environmental and health concerns.

A by-product of the extraction process of bitumen results in the production and storage of oil sands process-affected water (OSPW) in tailings ponds.[2–5] The tailings ponds in northern Alberta, store ~1.4 trillion litres of OSPW. A “zero discharge” policy is in place to prevent the release of tailings water into the environment,[6] however, reports of numerous leaks and spills[7] leading to localized contamination have resulted in increased concerns about the effectiveness of tailings management. In recent years, discussions and proposals have emerged considering the eventual release of treated water from tailings ponds into northern Alberta's Athabasca River system. The Athabasca River watershed is a critical habitat for numerous species of wildlife including fish, birds, and mammals. Moreover, it supports the livelihoods and cultural practices of Indigenous communities in the region.[8] As OSPW and its constituents have been shown to be toxic to various species, the efficacy of treatment strategies employed prior to the potential release of OSPW into the environment is a significant concern for scientists, policy makers and peoples living in the region.

While the composition of OSPW varies based on a variety of factors including: i) host ore spatial differences, ii) the method of the extraction (i.e., open-pit mining, *in-situ* recovery, solvent extraction), iii) the geological location of the mine, and iv) the length of time the OSPW has been stored in the tailings ponds, it is evident that certain contaminants are consistently present across all OSPW samples.[4] These include elevated levels of salts, specific metals, polycyclic aromatic compounds (PACs), BTEX (benzene, toluene,

ethylbenzene, and xylenes), and dissolved organic compounds, including naphthenic acid fraction components (NAFCs)[6–8]. NAFCs are naturally occurring in bitumen and are therefore present in the Athabasca River watershed flowing through the oil sands deposits. However, they become highly concentrated in OSPW due to the extraction processes[12,13] and can enter the environment through leachates from tailings ponds and be transported via groundwater flow paths in combination with naturally leached bitumen.[2,4,14–17]

Many studies have identified NAFCs as a key driver of OSPW toxicity. Chronic exposure to NAFCs has been found to cause reproductive, developmental, and behavioural abnormalities in various aquatic species, including fish, amphibians, and invertebrates.[5,8,18] More recently, NAFCs have been found to impact endocrine signaling and metabolic health, resulting in aberrant lipid accumulation and altered glycemic control.[3,19] Importantly, changes in glucose and lipid availability are often linked to altered energy homeostasis including mitochondrial dysfunction and amino acid metabolism.[20,21] While NAFCs have been shown to affect glucose and lipid homeostasis in non-mammalian vertebrates, their effects on mammalian metabolic homeostasis including mitochondrial function and amino acid metabolism are unknown and are the focus of this study.

Cellular resource allocation is central to how cells manage nutrient availability, bioenergetic capacity, and macromolecule production, all of which are crucial to maintaining metabolic homeostasis.[22] Hepatocytes, the liver cells responsible for regulating glucose, lipid and protein metabolism, are functional indicators of this balance and are key to understanding how organisms adapt to their microenvironments. In this study, we investigated the effects of NAFC exposure on metabolic homeostasis using McA-RH7777 cells, a rat hepatocyte model of dyslipidemia. Cells were exposed to environmentally relevant concentrations of NAFCs[23–28] [0, 0.73, 14.7, 73.4 mg/L] for 24 and 48 h to determine how these bitumen-derived dissolved organics influence energy homeostasis in a mammalian system.

3.3 Methods

3.3.1 Preparation of NAFC

Preparation of the NAFC stock solution has been previously described (Frank *et al*., 2006).[11] Briefly, approximately 2000 L of OSPW were collected from an active tailings pond at Industry A in 2009. The OSPW was stored at 4°C in the dark until extraction commenced within 6 months of sample collection. NAFC were extracted, purified[11], quantified following the method of Brunswick *et al*. (2015)[29], and were chemically characterized in Marentette (2015).[23] NAFC extracts were stored in amber bottles at 4°C.

3.3.2 Cell Culture Maintenance and Treatment

McA-RH7777 cells, an immortalized rat hepatoma cell line (CRL-1601; ATCC, Manassas, VA) were cultured at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM 1X with 4.5 g/L glucose, L-glutamine, and

sodium pyruvate; Corning, Manassas, VA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Unless otherwise noted, experimental protocols were carried out in DMEM media supplemented as described.

McA-RH7777 cells were grown in 10mm dishes (Corning). Fresh medium was provided every 48h and cells were sub-cultured when ~80% of the cells were fully adhered and cells showed a spindle-like morphology. Cells were considered confluent when 80% of cells were attached to the plate after a minimum of two passages. Confluent cells from passages 11-13 were used for all experiments. For all assays, cells were seeded in a 96-well flat-bottom tissue-culture treated microplate (Corning) at a density of 150,000 cells/well for 24 h exposures and 100,000 cells/mL for 48 h exposures. Cells were allowed to adhere for 24 h, after which cells were exposed to treatment-supplemented media for 24 or 48 h. All assays were completed between passages 12-13 and are described below.

For dose-response exposure experiments to investigate gene expression, confluent cells from passage 11 were seeded in 6-well plates (Corning) at a density of 200,000 cells/mL for 24 h exposures and 150,000 cells/mL for 48 h exposures and grown to 85% confluency prior to treatment. McA-RH7777 cells were treated for 24 and 48 h with vehicle (DMEM, control), or NAFC [0.73, 14.7, and 73.4 mg/L] (N=6 independent experiments)[11] in supplemented-DMEM as described above. The concentrations of NAFC [0.73, 14.7, and 73.4 mg/L] were chosen to be within the range of naphthenic acid concentrations reported in surface waters, wetlands, tailings ponds, and OSPW from the Athabasca Oil Sands Region.[23–28]

3.3.3 qPCR

Quantitative Polymerase Chain Reaction (qPCR) was used to assess changes in mRNA expression of genes involved in cellular metabolic responses to environmental stresses. These included genes associated with lipid and glucose metabolism, cellular stress response, and tryptophan metabolism. The genes involved in lipid metabolism include *Fasn* (fatty-acid synthase) and *Acca* (Acetyl-CoA carboxylase alpha), which are crucial for fatty acid synthesis, *Cpt1α* (carnitine palmitoyltransferase 1 alpha), which facilitates the transport of fatty acids into the mitochondria for beta-oxidation, and *Cd36* (fatty acid translocase), which is involved in fatty acid uptake. For glucose metabolism, *G6pase* (glucose-6-phosphatase), the enzyme essential for the final steps of gluconeogenesis and glycogenolysis was assessed. *Pgc1a* (peroxisome proliferator-activated receptor-γ coactivator), a key regulator of genes involved in energy metabolism and mitochondrial biogenesis was also assessed.

Following cell treatment, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were measured using the NanoDrop One™ Microvolume UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's

instructions. RT-qPCR was performed using PerfeCta® SYBR® green FastMix® (Quantabio, Beverly, MD) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The cycling conditions included polymerase activation (95°C for 10 min), followed by 40 cycles of denaturing (95°C for 10 sec), annealing (60°C for 10 sec) and extension (72°C for 15 sec, slow-ramp rate 2.5°C per sec). Levels of gene expression were calculated using the ΔΔCt method[30] and normalized using the geometric means of reference gene expression levels: beta-2-microglobulin (*B2m*) and peptidylprolyl isomerase A (*Ppia*). Primer sequences are supplied in Table 1.

Table 1. Table of Primer Sequences.

Accession number	Gene name	Symbol	Forward Sequence (5'-3')	Reverse sequence (5'-3')
NM_017101.1	Peptidylprolyl isomerase A	Ppia	CCGCTGTCTCTTTTCGCC	GCTGTCTTTGGAACTTTGTCTGC
NM_012512.2	Beta-2-microglobulin	B2m	AATTCACACCCACCGAGACC	GCTCCTTCAGAGTGACGTGT
NM_017332.1	Fatty-acid synthase	Fasn	GGACATGGTCACAGACGATGAC	CGTCGAACTTGGACAGATCCTT
NM_022193.1	Acetyl-CoA carboxylase alpha	Acca	ATTGGGCACCCCAGAGCTA	CCCGCTCCTTCAACTTGCT
NM_031559.2	Carnitine Palmitoyl transferase 1A	Cpt1*a*	CACCCCAACCCATATCCAGG	TCCTCACGGTCTAATGTGCG
NM_031561.2	Fatty acid translocase	Cd36	TCTCACACAACTCAGATACTGCT	GCACTTGCTTCTTGCCAACT
NM_013098.2	Glucose-6-phosphatase	G6pase	AACTCCAGCATGTACCGCAA	AAACGGAATGGGAGCGAC TT
NM_031347.1	Peroxisome proliferator-activated receptor-γ coactivator	Pgc1*a*	ATGGAGTGACATAGAGTGTGCT	CACCACTTCAATCCACCCAGA

3.3.4 Metabolic Function

To determine whether transcriptomic changes in key molecular pathways of hepatocyte metabolism following NAFC treatment would translate to functional differences, we quantified triglyceride levels, glucose uptake, and fatty acid uptake.

Glucose Uptake Assay: Glucose uptake was measured using a Glucose Uptake-Glo™ Assay (Promega) according to the manufacturer's protocol (N=10). Briefly, McA-RH7777

cells were plated in 96-well plates and treated with control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 and 48 h, as previously described (N=10 independent experiments). Luminescence was measured using a Synergy H1 Hybrid Reader (Agilent Technologies, Inc.).

Triglyceride Assay: To determine the effects of NAFC on triglyceride content, a Triglyceride-Glo™ Assay (Promega, Madison, WI) was performed according to manufacturer's protocol (N=9 independent experiments). Briefly, McA-RH7777 cells were plated in 96-well plates and treated with control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 and 48 h, as previously described. Luminescence was measured using a Synergy H1 Hybrid Reader (Agilent Technologies, Inc., Santa Clara, CA). Triglyceride concentrations were quantified against an 8-point standard curve.

Fatty Acid Uptake Assay: To determine the effects of NAFC on fatty acid uptake, a Fatty Acid Uptake Assay Kit (Abcam, Cambridge, UK) was performed according to the manufacturer's protocol (N=5 independent experiments). Briefly, McA-RH7777 cells were plated in 96-well plates and treated with control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 h and 48 h, in serum-free, phenol red-free DMEM. Fluorescence measurements (Ex/Em = 488/523 nm) were conducted in kinetic mode at 37°C for 60 min, followed by endpoint florescence in bottom-read mode, using a Synergy H1 Hybrid Reader (Agilent Technologies, Inc.).

3.3.5 Mitochondrial Function

Mitochondrial function is crucial for energy production and overall cellular health. Disruptions in mitochondrial activity can lead to significant metabolic and bioenergetic challenges, impacting cell survival and function. In this study, we employed two assays to assess mitochondrial health in McA-RH7777 cells following exposure to NAFC for 24 and 48 h.

ATP Assay: To determine the effects of NAFC on ATP production, as a direct indicator of mitochondrial function and cellular energy status, we performed a Luminescent ATP Detection Assay Kit (Abcam) according to manufacturer's protocol (N=6 independent experiments). Briefly, McA-RH7777 cells were plated in 96-well plates and treated with control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 and 48 h, as previously described. Luminescence was measured using a Synergy H1 Hybrid Reader (Agilent Technologies, Inc).

JC-1 Assay: To determine the effects of NAFC on mitochondrial membrane potential ($\Delta\psi m$), a JC-1 Assay (Abcam) was performed according to manufacturer's protocol (N=8 independent experiments). Briefly, cells were seeded in 96-well plates and allowed to adhere for 24 h after which cells were exposed to control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 and 48 h, as previously described. Fluorescence measurements were taken for JC-1 aggregate and monomer (Ex/Em = 475/590, 530 nm) on a Synergy H1 Hybrid Reader (Agilent Technologies, Inc.).

3.3.6 Apoptosis

To determine if NAFC exposure caused increased cell death via apoptosis, TUNEL staining was performed with a DeadEnd fluorometric TUNEL system (Promega) following the manufacturer's protocol as previously described[30]. Briefly, cells were seeded in chamber slides (N=4 independent experiments) and treated with control, or NAFC [0.73, 14.7, and 73.4 mg/L] for 24 and 48 h. Positive (DNase I) and negative controls were performed according to the kit recommendations. Stained slides were imaged, and TUNEL-positive cells were quantified using Image J software. The percentage of TUNEL-positive cells per section was calculated and averaged across groups.

3.3.7 Amino acid metabolism

NAFCs have been found to cause oxidative stress, mitochondrial dysfunction, and cell cycle dysregulation; all pathways that have been linked to metabolic perturbations and changes in amino acid pathways.[32] Tryptophan is an essential amino acid involved in numerous physiological processes, including protein accretion (growth), neurotransmitter synthesis, regulation of mood and sleep, immune function and gastrointestinal health. Tryptophan is primarily catabolized through the kynurenine pathway (TRP-KYN), but it is also the precursor for serotonin and melatonin. There are now a number of studies which have shown that tryptophan catabolism is altered in association with perturbations in glucose and lipid homeostasis.[33–35] Moreover, non-lethal end point toxicity studies across species have shown that NAFC exposure can affect aryl hydrocarbon (AhR) signaling, glucocorticoid (GR) signaling, oxidative stress and inflammatory pathways,[36–43] all of which have been reported to regulate the TRP-KYN pathway. Therefore, we assessed changes in TRP, KYN and the kynurenine-tryptophan (KTR) ratio following NAFC exposure.[44]

Materials and reagents: Unlabeled analytical standards of L-tryptophan (TRP, ≥ 98% HPLC) and L-kynurenine (KYN, ≥ 98%) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON, CA). Mass labeled L-tryptophan- 2′,4′,5′,6′,7′-d5 (indole-d5) (98%, TRP-d5), L-kynurenine-d4 [4-(2-aminophenyl-3,5-d2)] (99%, KYN-d4) were purchased from CDN Isotopes (Pointe-Claire, QB, CA). All solutions were stored at 4◦C. L-Acetonitrile (ACN) and water were purchased from Fisher-Scientific and of HPLC-grade. Ultrapure Milli-Q was obtained from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA).

Preparation of samples for chromatographic analysis: To a 2 mL glass vial, 60 μL of cell supernatant (*in vitro* media) and TRP-d5 and KYN-d4 (20 μL of a mixture containing 7.5 ng/μL each) were added and adjusted to a final volume of 1.5 mL with ACN (1420 μL). Samples were vortexed for 30 secs prior to chromatographic analyses. Method blanks consisting only of the control media were prepared in an identical manner to our samples. Reagent blanks of ACN were injected periodically to assess the extent of contamination from the solvent and between injections. Matrix effects were assessed by fortifying control media (60 μL) with our native and mass labelled compounds (20 μL each and diluting to a final volume of 1.5 mL) and comparing their signals to that of an external standard prepared

in ACN. Limits of detection and quantitation were determined according to Eurachem guidelines by fortifying control media with native TRP and KYN (n=6).

High Performance Liquid Chromatography Tandem Mass Spectrometry: The detection and quantitation of TRP and KYN in biological matrices was based on a previously published validated method[45]. Separations of mass labeled and native KYN and TRP were performed using a SEQUANT ZICR HILIC PEEK coated HPLC column (4.6 mm x 100mm, 3.5 μM particle size, 200 A pore size) (Merck, Darnstadt, Germany). The HPLC was coupled to a Sciex 365 triple quadrupole mass spectrometer retrofitted with an HSID Ionics EP+ orthogonal ionization source. Ionization was performed in the positive ion mode and the multiple reaction monitoring ion transitions used for detection and quantitation were: TRP: m/z 205.1 → 188.2; KYN: m/z 209.4 → 94.1, TRP-d5: m/z 201.2 → 192.4 and KYN-d4: m/z 213.3 → 196.2. No statistical differences ($p<0.05$) were observed between the response of the target analytes in media relative to the standards prepared in solvent implying that matrix effects were negligible. Limits of detection and quantitation for TRP were 2.8 and 9.5 pg/μL and for KYN were 4.3 and 14.3 pg/μL, respectively.

Total naphthenic acids were quantified following the method of Brunswick *et al.* (2015).[29] Samples were adjusted to pH 10 with direct injection into an Agilent Infinity 1290 HPLC system fitted to an Agilent 6550 iFunnel AJS Series Liquid Chromatograph Mass Spectrometer with Time of Flight detector (LC/MS-ToF). Analytes were detected by total ion scan (TIC) from *m/z* 110 - 990 and utilized a library of over 400 C_9-C_{30} isomers. Instrument calibrations utilized a Merichem technical naphthenic acid mixture.

3.3.8 Statistical Analysis

All statistical analyses were conducted using GraphPad Prism (v.9.5.1, GraphPad Software, San Diego, CA). Data were assessed for outliers using Grubb's Test, tested for normality with the Shapiro-Wilk test, and checked for equal variance. For outcome measures requiring comparisons between control and multiple treatment groups, a one-way ANOVA was performed, followed by Dunnett's test for post hoc comparisons when significant differences were detected ($p \leq 0.05$). Data that did not pass tests for normality or equal variance were analyzed by Kruskal-Wallis one-way ANOVA on ranks. If significance was found, Dunn's post-hoc test was used for comparisons against control. All results are presented as mean ± SEM and were considered significant when $p \leq 0.05$.

3.4 Results

3.4.1 Exposure to 14.7 and 73.4 mg/L NAFC induces significant metabolic shifts in McA-RH7777 cells.

Glucose

NAFC exposure led to time- and dose-dependent alterations in the expression of gluconeogenesis-related genes and glucose uptake in McA-RH7777 cells. At 24 h, treatment with 73.4 mg/L NAFC resulted in a significant increase in *G6pase* mRNA

expression (Figure 1A), while *Pgc1a* expression was notably decreased (Figures 1B). By 48 h, the increase in G6pase expression at 73.4 mg/L was even more pronounced (Figure 1D), and *Pgc1a* expression was significantly reduced at both 14.7 mg/L and 73.4 mg/L (Figure 1E). In parallel, the glucose uptake assay revealed that at 24 h, there was no significant change in glucose uptake at 73.4 mg/L NAFC, but a small and significant reduction was observed at the lower dose of 0.73 mg/L (Figure 1C). However, by 48 h, glucose uptake was significantly decreased at the 73.4 mg/L concentration (Figure 1F).

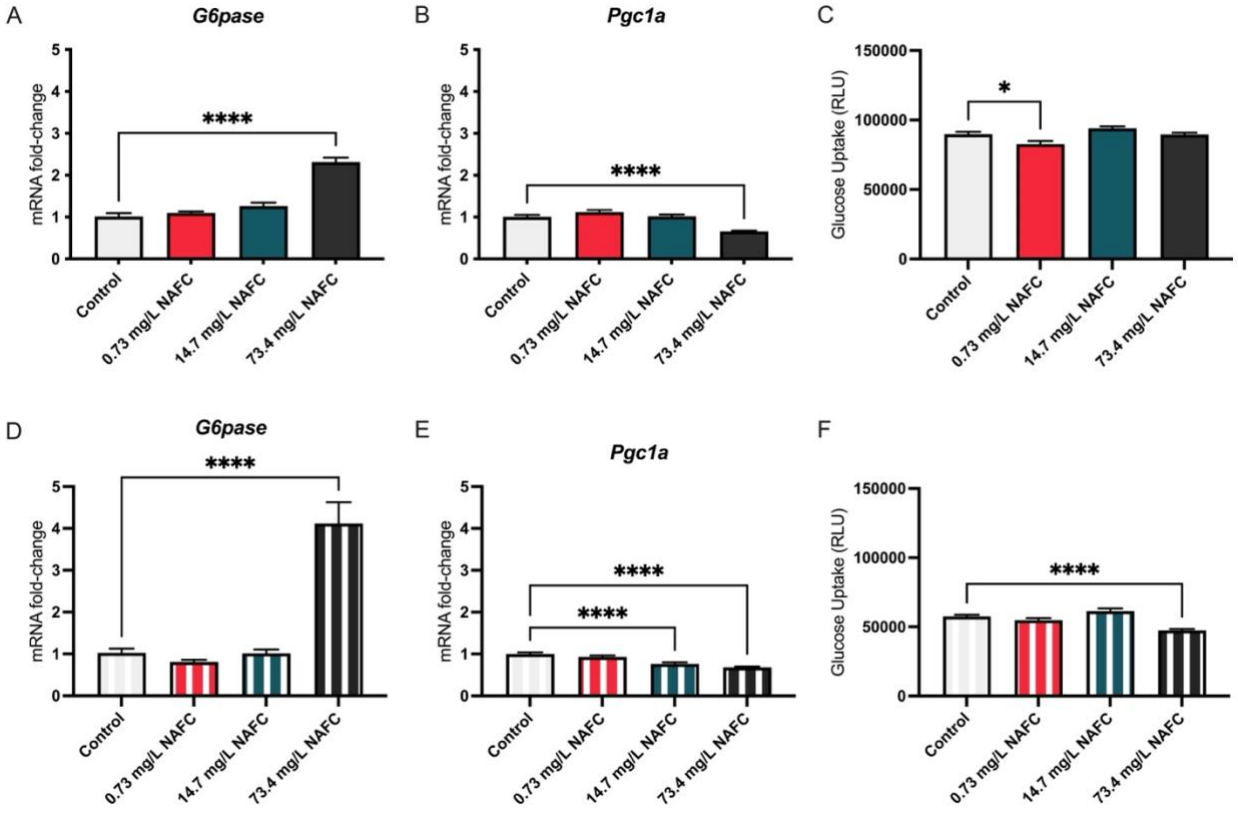


*Figure 4. mRNA expression of gluconeogenesis pathway genes G6pase (A, D) and Pgc1a (B, E), and glucose uptake (C, F) in McA-RH7777 cells following 24 h (colored bars) and 48 h (striped bars) exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. Data are presented as mean ± SEM (N=6 gene; N=10 assay). Statistical significance compared to control is denoted by asterisks: *p < 0.05, ****p < 0.001.*

<u>*Lipids*</u>

NAFC exposure induced time- and dose-dependent changes in both gene expression and triglyceride content in McA-RH7777 cells. At 24 h, a significant decrease in *Fasn* mRNA expression was observed with 73.4 mg/L NAFC (Figure 2A), which corresponded with a reduction in triglyceride levels at the same concentration (Figure 2C). Interestingly, at 48 h, while 73.4 mg/L NAFC reduced triglyceride levels (Figure 2F), it also significantly increased *Acca* mRNA expression (Figure 2E). Notably, at 48 h, triglyceride levels were decreased across all tested doses of NAFC (Figure 2F).

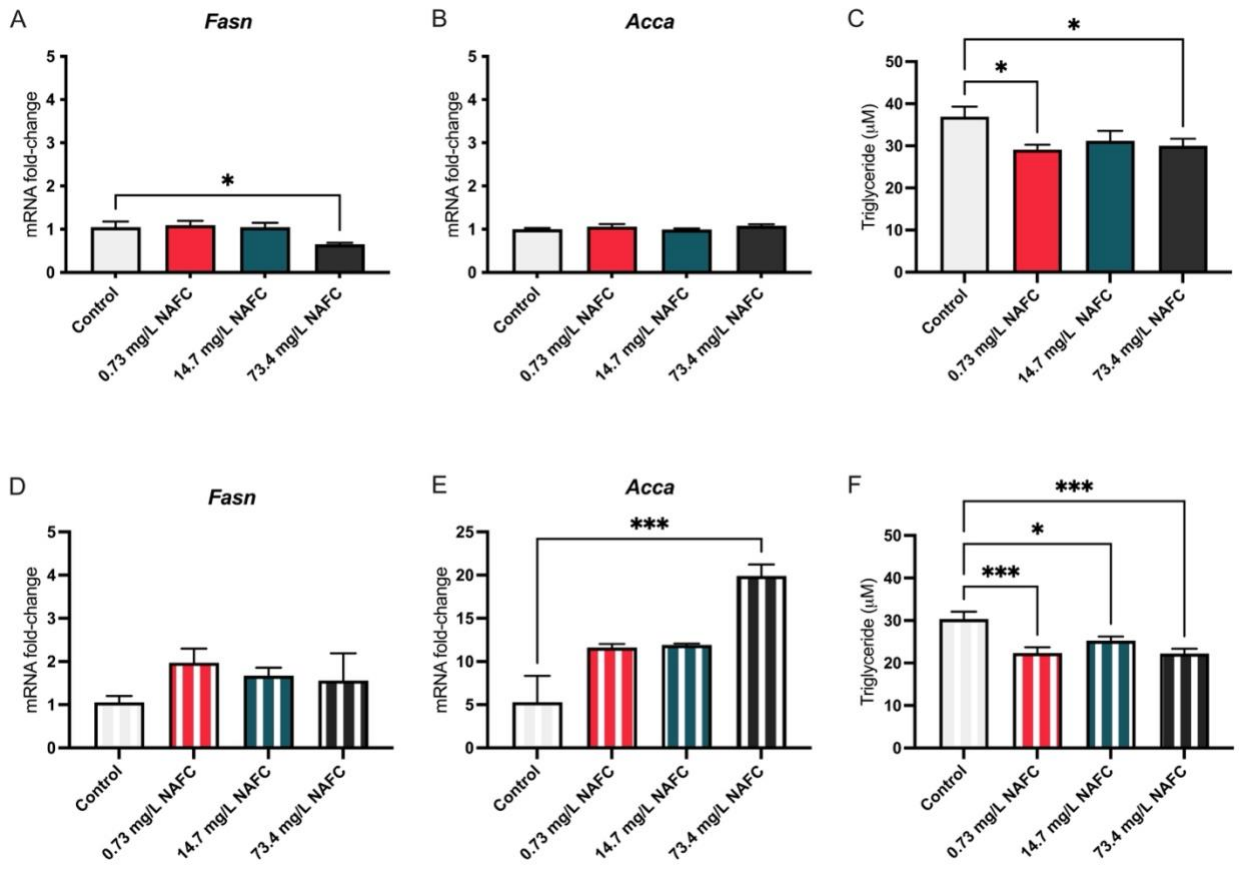


*Figure 5. mRNA expression of de novo lipogenesis pathway genes Fasn (A, D) and Acca (B, E), and triglyceride accumulation (C, F) in McA-RH7777 cells after 24 h (colored bars) and 48 h (striped bars) exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. Data are presented as mean ± SEM (N=6 gene; N=9 assay). Statistical significance compared to control is denoted by asterisks: *p < 0.05, ***p < 0.005.*

The expression of *Cd36*, a key regulator of fatty acid uptake, was significantly increased at both 14.7 mg/L and 73.4 mg/L NAFC at 24 and 48 h (Figures 3A and 3D). Similarly, *Cpt1α* expression increased at both 14.7 mg/L and 73.4 mg/L at 24 h (Figure 3B), but by 48 h, this increase was observed only at the higher dose of 73.4 mg/L (Figure 3E). These gene expression changes translated into distinct functional outcomes in fatty acid uptake. At both 24 and 48 h, the 14.7 mg/L dose resulted in decreased fatty acid uptake (Figure 3C), despite the upregulation of *Cd36*. In contrast, at the higher dose of 73.4 mg/L, fatty acid uptake was significantly increased at both time points (Figure 3F).

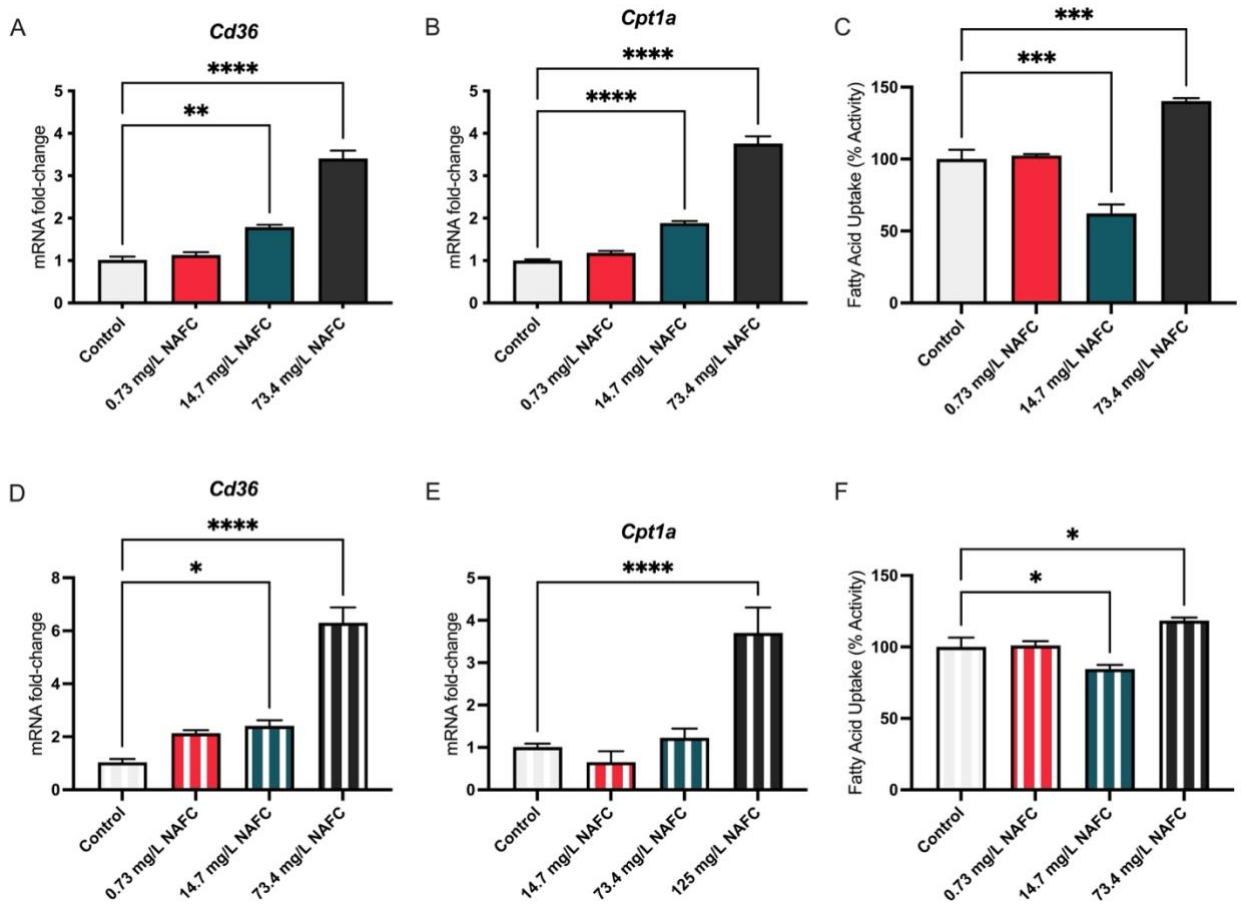


*Figure 6. mRNA expression of fatty acid oxidation pathway genes Cd36 (A, D) and Cpt1α (B, E), and fatty acid uptake (C, F) in McA-RH7777 cells following 24 h (colored bars) and 48 h (striped bars) exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. Data are presented as mean ± SEM (N=6 gene; N=5 assay). Statistical significance compared to control is denoted by asterisks: *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.*

3.4.2 Metabolic shifts in NAFC-exposed McA-RH7777 cells suggest alterations in mitochondrial function.

NAFC exposure also had a significant impact on cellular energy production, as indicated by changes in ATP levels. Across both 24 and 48 h of exposure, ATP levels were significantly increased in McA-RH7777 cells (Figure 4).

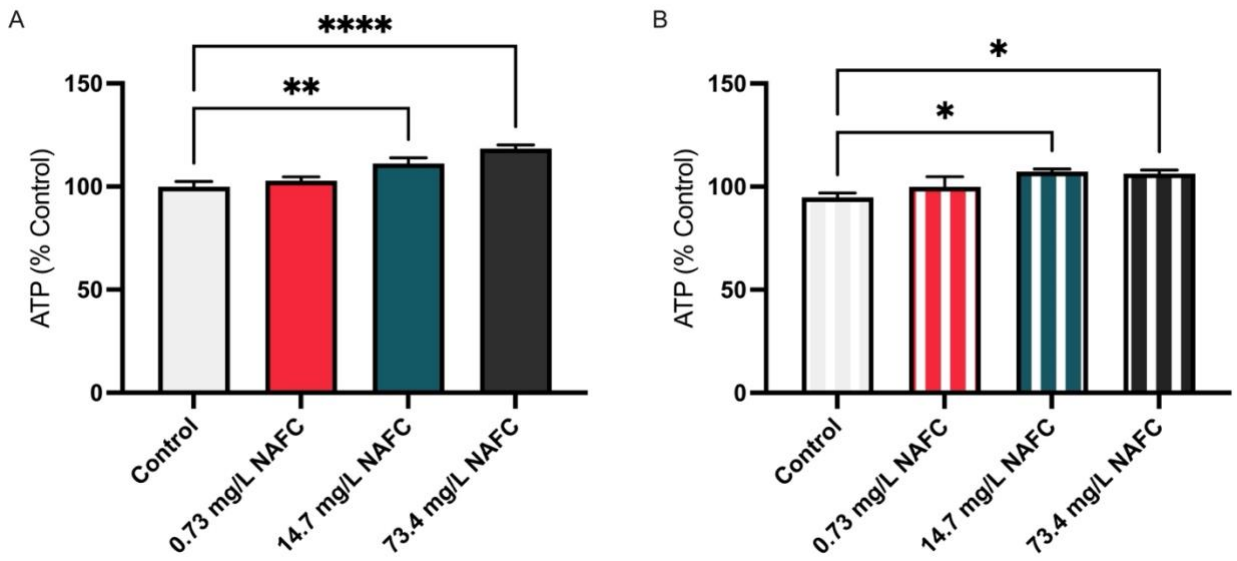


*Figure 7. ATP levels in McA-RH7777 cells following exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L] measured at (A) 24 and (B) 48 h. Data are presented as mean ± SEM (N=6). Statistical significance compared to control is denoted by asterisks: *p < 0.05, **p < 0.01, ****p < 0.001.*

In addition to changes in ATP levels, we assessed mitochondrial membrane potential (Δψm) using the JC-1 assay. Exposure to 73.4 mg/L NAFC resulted in a significant increase in JC-1 fluorescence at both 24 and 48 h (Figure 5).

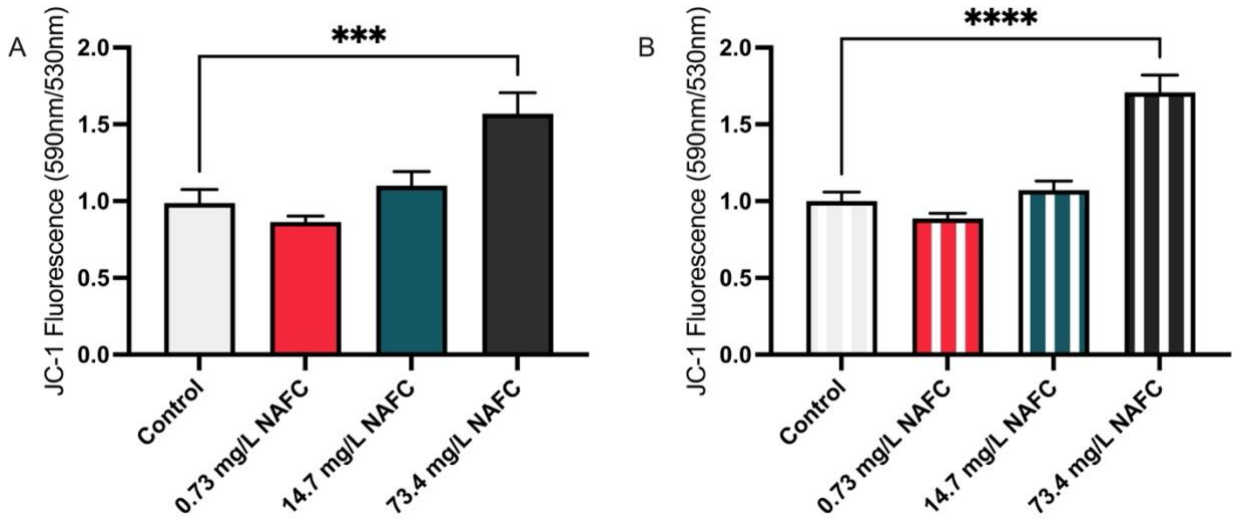


*Figure 8. Mitochondrial membrane potential in McA-RH7777 cells assessed by JC-1 assay following (A) 24 h and (B) 48 h exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. Fluorescence intensity ratios of JC-1 aggregates to monomers were quantified, indicating changes in mitochondrial health. Data are presented as mean ± SEM (N=8). Statistical significance compared to control is denoted by asterisks: ***p < 0.005, ****p < 0.001.*

This increase in mitochondrial ATP production and Δψm was accompanied by increased apoptosis, as measured by the TUNEL assay. Both 14.7 mg/L and 73.4 mg/L NAFC induced a significant increase in TUNEL-positive cells at both time points (Figure 6).

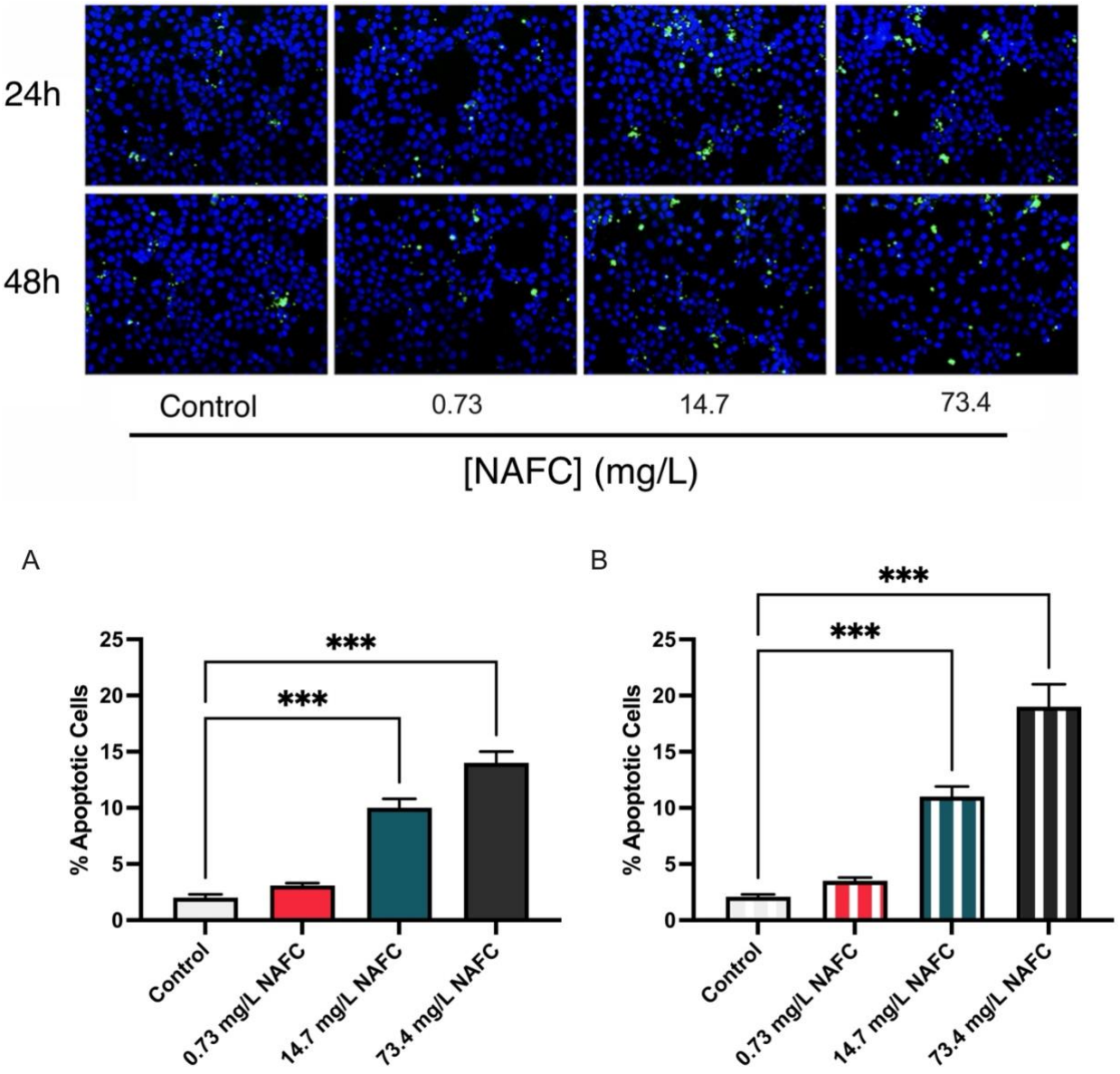


*Figure 9. Apoptotic cell detection in McA-RH7777 cells using the TUNEL assay after 24 h (A) and 48 h (B) exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. TUNEL-positive cells were quantified to assess the extent of apoptosis induced by the treatment. Data are presented as mean ± SEM for six replicates (N=4). Statistical significance compared to control is denoted by asterisks: ***p < 0.005, ****p < 0.001.*

3.4.3 The kynurenine-tryptophan ratio was altered in association with changes in substrate utilization and metabolic alterations in McA-RH7777 cells following exposure to 73.4 mg/L NAFC.

Lastly, we assessed the impact of NAFC exposure on the TRP-KYN pathway. NAFC exposure led to higher KYN levels and an elevated KTR at the 48 h time point following exposure to 73.4 mg/L NAFC (Figures 7E and 7F). Interestingly, at 24 h, the 14.7 mg/L dose led to a decrease in KYN levels, and both 0.73 mg/L and 14.7 mg/L doses resulted in a decreased KTR, despite no significant changes in TRP or KYN levels at the 0.73 mg/L dose (Figures 7B and C).

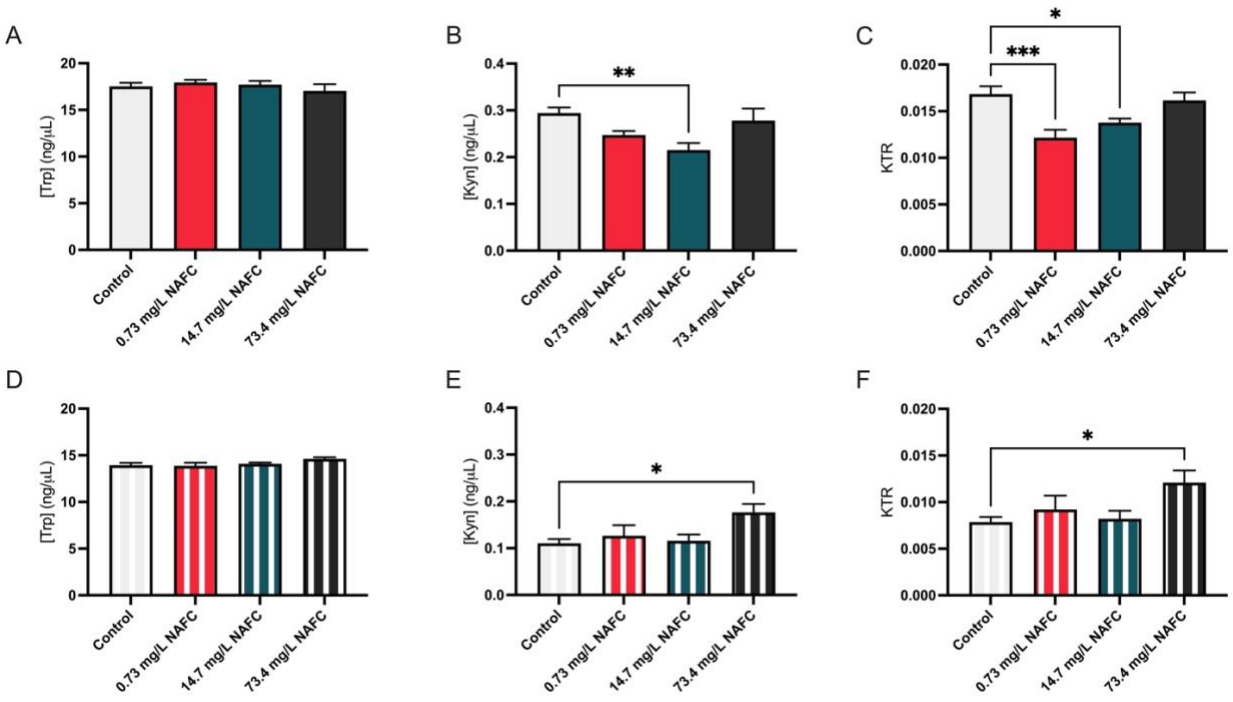


*Figure 10. TRP levels (A, D), KYN levels (B, E) and KTR (C, F) in McA-RH7777 cells following 24 h (colored bars) and 48 h (striped bars) exposure to NAFC [0, 0.73, 14.7, 73.4 mg/L]. Data are presented as mean ± SEM (N=6). Statistical significance compared to control is denoted by asterisks: *p < 0.05, **p < 0.01, ***p < 0.005.*

3.5 Discussion

The toxicity of OSPW and its constituents, particularly NAFCs, remains largely unexplored in mammals. Given the critical role that metabolic processes play in organismal health and survival, it is essential to understand how exposure to these chemical components might be disrupting energy homeostasis, and in turn, mammalian health. The present study used an NAFC extract consistent with previous laboratory studies on exposure effects.[23,24,38,40,46] In the 2024 reanalysis of this NAFC extract for the present study, the Merichem technical mixture served as the instrument calibrant, selected for its role as a traceable reference material. In earlier studies, concentrations ranges were based on previous calibration standards, with Merichem now providing a reliable, updated calibration reference. Using the same NAFC extracts, we previously reported significant changes in mammalian bone metabolism at concentrations ranging from 12.5 – 125 mg/L.[40] With the most recent analysis of the concentration in this mixture, these results correspond to 7.34 – 73.4 mg/L, which are consistent with levels found in wetlands impacted by tailings seepage and groundwater contamination.[17,28]

Metabolic substrate utilization in response to NAFC exposure

NAFC exposure led to significant metabolic alterations in McA-RH7777 cells. Specifically, exposure to 73.4 mg/L NAFC resulted in a marked decrease in triglyceride levels and glucose uptake at both 24 and 48 h. These changes were accompanied by decreased expression of the lipogenesis genes, *Fasn* and *Pgc1α*, suggesting a suppression of glucose-driven lipid synthesis. These findings are consistent with previous research on invertebrates, where NAFCs were shown to disrupt metabolic processes, leading to altered lipid profiles (Reviewed in:[3,19]). In aquatic species, naphthenic acids (NA) have been shown to reduce metabolic activity in rainbow trout (*Oncorhynchus mykiss)* hepatocytes[47], and disrupt liver and cholesterol metabolism in frog embryos. *Lithobates pipiens* tadpoles exposed to NA exhibited decreased growth and development, reduced glycogen stores and increased triglycerides, indicating significant disruptions in energy metabolism and hepatic glycolysis.[48] Similarly, frog *Silurana (Xenopus) tropicalis* embryos exposed to NA, had reduced body size and perturbations in liver and cholesterol metabolism.[36]

While the adverse effects of NAFCs on aquatic life are well-documented, less is known about their impact on mammalian and terrestrial wildlife.[49,50] Recent observations of altered body condition and diminished growth in wildlife within the AOSR suggest that these contaminants may similarly affect terrestrial species.[49–53] In the limited studies that do exist, there is evidence that NAFCs can adversely affect metabolic homeostasis. Rogers *et al*. (2002)[54] exposed rats to an organic fraction of acidified OSPW which included NAFCs and low amounts of polycyclic aromatic hydrocarbons (PAHs). The dosages [300 (high dose), 30 (medium dose), or 3 (low dose) mg/kg body weight NAFC] were designed to reflect 50, 5, or 0.5 times, respectively, a worst-case, single-day exposure for wild mammals. Rats exposed to high doses of NAFCs exhibited significant metabolic changes, including a notable increase in hepatic glycogen storage and a small but significant reduction in body weight in males. Interestingly, the livers of NAFC-exposed

rats were 36% heavier than those of controls, and this was accompanied by the presence of pericholangitis, an inflammation of the bile ducts observed in both male and female rats. These findings indicate that the liver is a primary target of NAFC toxicity, with structural and functional changes manifesting as increased organ weight and hepatic glycogen storage. While glycogen storage is a normal metabolic function, the abnormally high intracellular accumulation suggests hepatotoxicity or disruptions in carbohydrate metabolism.[54] Similarly, our study found changes in carbohydrate handling with a reduction in glucose uptake at both 24 and 48 h following 73.4 mg/L NAFC exposure. The observed decrease in glucose uptake suggests an early-stage metabolic adaptation, where the cells switch to alternative energy sources, such as fatty acids, to maintain energy homeostasis.

Altered utilization of glucose and triglycerides could have significant implications for organismal health and fitness. In mammals, efficient metabolic flexibility—the ability of the liver to switch between glucose and fatty acid utilization—is essential for maintaining energy homeostasis during periods of varying nutrient availability, such as times of abundance versus scarcity.[55,56] In our study, we observed decreased glucose uptake in association with evidence of increased mRNA expression of fatty acid beta-oxidation genes (i.e., *Cpt1α* and *Cd36)* in cells treated with 73.4 mg/L NAFC. *Cpt1α* and *Cd36* are critical for the transport and oxidation of fatty acids. This suggests that in response to reduced intracellular glucose uptake, hepatocytes may upregulate fatty acid oxidation pathways as an adaptive mechanism to sustain ATP production and overall energy homeostasis.[57] Indeed, during periods of nutrient scarcity, organisms typically break down fat stores (triglycerides) into free fatty acids, which are then released into the circulation and taken up by metabolically active tissues for oxidation.[56,58] Interestingly, fatty acid uptake decreased at 14.7 mg/L but increased at 73.4 mg/L, suggesting a dose-related response. The initial decrease at 14.7 mg/L could signal early mitochondrial dysfunction, while the increase at 73.4 mg/L may represent a compensatory mechanism to maintain energy homeostasis in response to impaired glucose metabolism.

Mitochondrial dysfunction as a 'switch' for NAFC-induced metabolic reprogramming

This shift towards fatty acid utilization, coupled with the increase in mitochondrial membrane potential (Δψm) at the 73.4 mg/L concentration, supports the hypothesis of mitochondrial hyperpolarization and stress. The continuous influx of fuel and incomplete utilization can lead to the accumulation of reducing equivalents within the mitochondria, increasing the mitochondrial membrane potential.[55,59] Elevated Δψm fosters the excessive generation of reactive oxygen species (ROS), which can cause oxidative damage, deleterious protein modifications, and ultimately, metabolic reprogramming and disease.[56,58]

In our study we saw an increase in both ATP production and mitochondrial membrane potential at the same concentrations where we observed an increase in markers of fatty acid oxidation (i.e., *Cpt1α* and *Cd36*). While increased mitochondrial membrane potential allows for enhanced ATP synthesis, as we observed with NAFC, at high ΔΨm the electron

transport chain produces more reactive oxygen species which are known to cause oxidative damage to mitochondrial components leading to apoptosis.[59] Indeed, we saw a concomitant increase in apoptosis at both 14.7 mg/L and 73.4 mg/L NAFC concentrations. In this study the increase in ΔΨm and ATP production are contrary to other studies where NAFC have been shown to reduce mitochondrial electron transport chain activity and mitochondrial membrane potential.[47,60,61] This discrepancy might be related to the differences in experimental models (rainbow trout mitochondria vs. rat hepatocytes) or the specific conditions of exposure. Additionally, the observed increase in mitochondrial membrane potential could indicate an initial compensatory response to NAFC-induced stress, where the mitochondria attempt to maintain ATP production despite underlying dysfunction. Moreover, both increased and decreased ATP and ΔΨm are indicative of mitochondrial dysfunction which can have profound effects on cell function and survival.[59] Collectively, these studies suggest that the mitochondria are an important target of NAFC toxicity in multiple vertebrate species, including mammals.

These oxidative stresses and subsequent cellular injuries can drive metabolic reprogramming, contributing to decreased growth rates and reduced overall fitness.[56] This aligns with findings from Rogers *et al*. (2002), who reported decreased body weight and increased liver weights in rats exposed to high doses of NAs, indicative of hepatic stress and compromised growth.

Kynurenine-Tryptophan Ratio as a Marker of Metabolic Reprogramming

There is now evidence demonstrating some environmental xenobiotics, known to alter glucose and lipid homeostasis, can also alter tryptophan (TRP) metabolism,[44] an essential amino acid involved in numerous physiological processes including growth and the synthesis of important biomolecules such as serotonin, melatonin, and niacin. For example, the prototypical AhR ligand, benzo[a]pyrene (BaP) has been shown to induce metabolic reprogramming alongside disruptions in amino acid metabolism in various mammalian cancer models.[62–64] A recent study investigating early life exposure to PCB126 in mice demonstrated acute alterations in hepatic metabolism, with significant reductions in TRP levels observed by the 6th day, although downstream KYN metabolites or KTR were not assessed.[65] In aquatic species, similar disruptions in tryptophan metabolism have been reported following exposure to naphthenic acids. For example, the common reed (*Phragmites australis*) exposed to NAs showed widespread metabolic alterations, including changes in amino acid metabolism, with tryptophan being identified as a significant marker of NA exposure ($p=0.022$).[66] Similarly, in a human leukemia monocytic cell line (THP1) exposed to OSPW [25% v/v OSPW in cell culture medium], the upregulation of kynureninase (KYNU)—an enzyme in the kynurenine pathway—was observed, further linking NA exposure to altered tryptophan metabolism.[67] While compounds that act as AhR ligands have been shown to affect tryptophan metabolism, other pathways have also been implicated including GR signaling, peroxisome proliferator-activated receptor (PPAR) activation, oxidative stress and inflammatory pathways. Given that NAFCs are a complex chemical mixture, they likely interact with multiple signaling pathways to alter tryptophan metabolism, with some evidence suggesting limited AhR-mediated activity[68],

GR signalling[40], and PPAR-related pathways[69]. Regardless, the changes in KTR, altered lipid and glucose metabolism and mitochondrial function all occur in concert suggesting that KTR may have utility as a hepatic marker of metabolic responses to NAFC exposure.

3.6 Conclusion

These findings provide valuable insights into how NAFCs may disrupt metabolic homeostasis in mammals. The observed metabolic reprogramming, including shifts in substrate utilization, mitochondrial dysfunction, and alterations in tryptophan metabolism, highlights a possible mechanism by which NAFCs impact the health of wildlife in the AOSR. The link between these cellular disruptions and reported cases of diminished growth and altered body condition in wildlife exposed to NAFCs suggests that these metabolic changes could be contributing to broader ecological consequences. By identifying the KTR as a potential biomarker of NAFC-induced metabolic alterations, this research offers a valuable tool for assessing the impact of bitumen-derived organic compounds on wildlife. Furthermore, these findings can be integrated into adverse outcome pathways (AOPs), which serve as structured frameworks that link molecular-level events to adverse biological outcomes. By mapping the metabolic and cellular disruptions caused by NAFCs onto established AOPs, this research contributes to the development of more robust environmental biomonitoring strategies. The ability to detect early molecular changes, such as metabolic reprogramming and KTR, allows for more proactive assessment of dynamic environmental conditions, including climate change. Therefore, this work not only advances our understanding of how NAFCs affect wildlife but also provides practical tools for environmental evaluation and protection.

3.7 References

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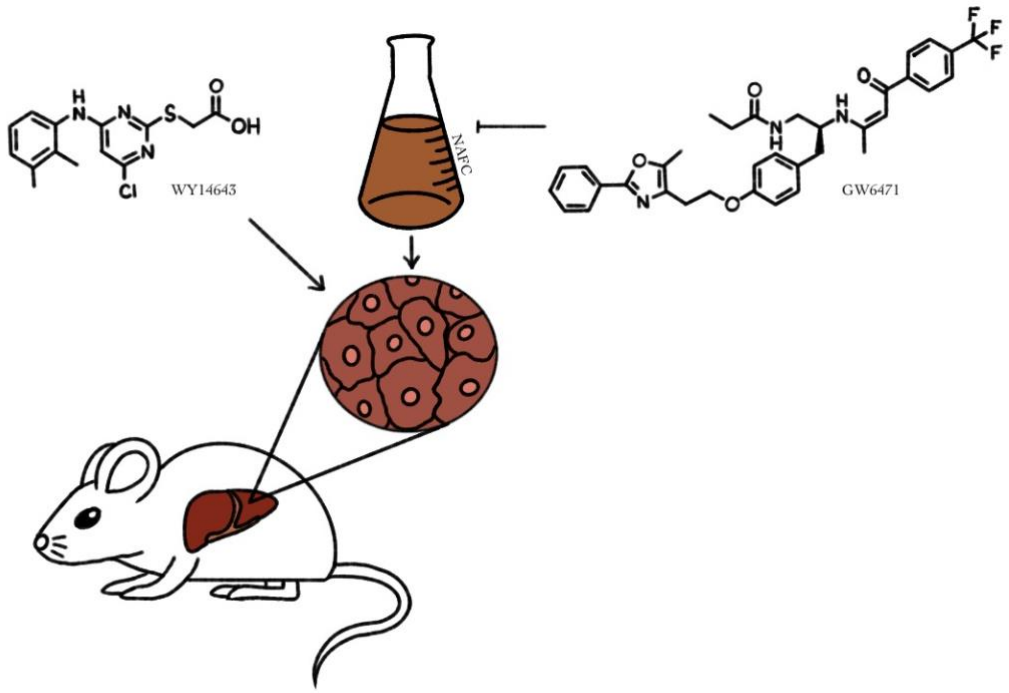
CHAPTER IV: NAPTHENIC ACID FRACTION COMPONENTS ACT AS PPARα AGONISTS TO REGULATE THE TRYPTOPHAN-KYNURENINE PATHWAY

This chapter has been submitted as an original article with the same title and is currently in peer-review, 2024 December.

4.1 Abstract



Graphical Abstract. NAFC and the PPARα agonist WY14643 regulate similar transcriptional pathways. NAFC-induced Tdo2 mRNA expression was completely blocked by PPARα antagonist GW6471, while the expression of other PPARα target genes were only partially inhibited, suggesting that NAFC may transcriptionally regulate the kynurenine-tryptophan pathway via PPARα. Together, these finding suggest a potential mechanism by which NAFC alter the kynurenine-tryptophan ratio and metabolic homeostasis in mammals.

There is concern about the impact of mining and extracting bitumen on water quality and wildlife health in the Athabasca Oil Sands Region (AOSR). Compounds found in bitumen-influenced waters, including oil sands process affected water (OSPW), can impact metabolic health, resulting in aberrant lipid accumulation and altered glycemic control. Our group recently showed that exposure to naphthenic acid fraction components (NAFCs) leads to significant changes in hepatocyte triglyceride levels and glucose uptake. Furthermore, this metabolic shift was associated with alterations in tryptophan metabolism. While the exact mechanisms underlying these effects remain unclear, there is evidence that naphthenic acids can act via peroxisome proliferator-activated receptors (PPARs). In the

current study we assess the extent of PPARα activation on NAFC-induced alteration of the kynurenine-tryptophan pathway and lipid homeostasis. McA-RH7777, rat hepatoma cells were treated with NAFCs at concentrations ranging from 0.73 to 73.4 mg/L, encompassing the ranges of reported ambient surface water and tailings pond concentrations. The highest dose of NAFC [73.4 mg/L] was also examined in combination with a PPARα-selective antagonist (GW6471) and compared to a PPARα agonist (WY14643). Exposure to 73.4 mg/L NAFC caused a significant increase in the mRNA expression of the tryptophan-metabolizing enzyme *Tdo2*. With a similar transcriptional profile to NAFC, the PPARα agonist WY14643 induced *Tdo2*, *Cpt1a*, and *Angptl4*. Co-treatment with the PPARα antagonist GW6471 fully blocked the NAFC-induced *Tdo2* increase. Our findings indicate that PPARα activation may play a role in mediating changes in gene expression involved in the tryptophan-kynurenine pathway and lipid homeostasis following exposure to NAFCs. Given the critical roles of the kynurenine-tryptophan pathway and PPARα in maintaining metabolic homeostasis and overall organismal health, these findings further emphasize the potential metabolic impact of OSPW-derived compounds on mammalian health.

4.2 Introduction

In the Athabasca Oil Sands Region (AOSR; Alberta, Canada), the storage of oil sands process-affected water (OSPW) in tailings ponds present potential environmental and health concerns, particularly due to the risk of leaching beyond containment systems, breaches of wildlife deterrents, and accidental spills and releases.[1–4] Exposure to OSPW has been shown to elicit adverse effects in various species, including freshwater invertebrates, fish, amphibians, and aquatic plants (Reviewed In:[2]). However, due to the complex nature of OSPW, the specific mechanisms by which its components exert these biological effects remain poorly understood. OSPW contains high levels of salts, metals, and dissolved organic compounds, including naphthenic acid fraction components (NAFCs) [3,5–7]. Known as a significant contributor to the toxicity of OSPW, NAFCs represent a complex mixture of acid-extractable organic compounds present in bitumen influenced waters and OSPW.[8,9] This complex mixture includes classical (O2), oxidized ($\geq$O3), nitrogen- and sulfur-containing, alicyclic species and aromatic NAs[10–12]. NAFC concentrations in OSPW tailings ponds typically range from 6 to 80 mg/L, and wetlands influenced by surface run-off from tailings ponds seepage can reach concentrations as high as 70 mg/L.[13] Importantly, NAFCs, are naturally present in the environment with concentrations ranging from <1.0 mg/L to 12 mg/L, reported in bitumen influenced waters including the Athabasca River, groundwater and wetlands in the AOSR. [1,3,13–17] Given the persistence of NAFCs in the environment, there is a growing need to better understand the mechanisms through which these compounds affect biological systems and wildlife. This understanding is critical for future monitoring efforts and assessing risks, especially in the AOSR, where distinguishing between the impacts of natural deposits and industrial activities presents unique challenges.

While the effects of NAFCs on aquatic species are better understood, studies of its impact on mammalian models are limited. Recent observations in wildlife, including mammals within the AOSR have shown altered body condition and diminished growth[18–22], which may be reflective of disruptions in metabolic processes and energy homeostasis pathways. Importantly NAFCs have been found to impact metabolic homeostasis, causing dyslipidemia and evidence of liver damage, such as aberrant lipid accumulation, altered glycemic control, and increased liver weight in rats[2,23,24]. More recently, our group has shown that exposure to NAFCs alters triglyceride levels and glucose uptake in hepatocytes, suggesting that NAFC exposure can directly impact hepatic glucose and lipid homeostasis (Chapter 3). Further, these changes were associated with altered tryptophan metabolism. As an essential amino acid, tryptophan (TRP) can be metabolized into a variety of compounds necessary for hormone secretion, immune and inflammatory responses, and metabolic homeostasis[25]. Notably, 95% of tryptophan metabolism proceeds through the kynurenine (KYN) pathway. Alterations in this pathway have been implicated in metabolic diseases such as obesity, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and liver steatosis (Reviewed In:[26]). The TRP-KYN pathway rate limiting enzymes (tryptophan 2,3-dioxygenase, TDO; and indoleamine 2,3-dioxygenase, IDO) are expressed in all mammalian tissues necessary for energy homeostasis including adipose, skeletal muscle, liver, pancreas, and heart, and are regulated by nutritional and inflammatory signals as well as by chemical exposures.[26] While the precise mechanisms behind NAFC-induced changes in tryptophan metabolism remain unclear, emerging evidence suggests that they may be mediated through peroxisome proliferator-activated receptor (PPAR) signaling pathways.[27,28]

PPARs (-α, -β/δ, -γ) are a group of nuclear receptors critical for the regulation of lipid and glucose metabolism. Among the PPAR subtypes, PPARα has been extensively studied in the context of energy homeostasis and metabolic diseases[29–31]. PPARα is primarily expressed in tissues with high energy demands, such as the liver, heart, and skeletal muscle, where it regulates the expression of genes involved in fatty acid oxidation, ketogenesis, and glucose metabolism[30,31]. PPARα activation promotes the uptake and catabolism of fatty acids, leading to decreased triglyceride accumulation, decreased glucose uptake, and enhancement of high-density lipoprotein synthesis, effects observed in in hepatocytes following NAFC exposure.[32] Additionally, studies have also found that PPARα can regulate amino acid metabolism in the liver[33]. Importantly, OSPW-derived compounds have also been found to induce expression of genes that are transcriptionally regulated by PPAR ligands [5,28,34–37]. Moreover, PPARα agonists have been found to increase the expression of key enzymes involved in tryptophan metabolism including indoleamine 2,3-dioxygenase, kynurenine aminotransferases and kynurenine hydroxylase[38,39]. Taken together, these studies suggest that the NAFC-induced changes in tryptophan metabolism may be a result of PPARα activation.

In an effort to further understand the mechanisms by which NAFCs in the AOSR exert toxicity in mammals, this study aimed to assess the role of PPARα activation in NAFC-induced alterations of tryptophan metabolism.

4.3 Methods

4.3.1 Cell culture maintenance

McA-RH7777 cells, an immortalized rat hepatoma cell line (CRL-1601; ATCC, Manassas, VA) were cultured at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM 1X with 4.5 g/L glucose, L-glutamine, and sodium pyruvate; DMEM; Corning, Manassas, VA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Unless otherwise noted, experimental protocols were carried out in DMEM media supplemented as described.

McA-RH7777 cells were grown in 10mm dishes (Corning). Fresh medium was provided every 48h and cells were sub-cultured when ~80% of the cells were fully adhered and cells showed a spindle-like morphology. Cells were considered confluent when 80% of cells were attached to the plate after a minimum of two passages. Confluent cells from passages 11-13 were used for all experiments.

4.3.2 Preparation of NAFC

Preparation of the NAFC stock solution has been previously described (Frank *et al*., 2006). Briefly, approximately 2000 L of OSPW were collected from an active tailings pond at Industry A in 2009. The OSPW was stored at 4°C in the dark until extraction commenced within 6 months of sample collection. NAFC were extracted, purified[7], quantified following the method of Brunswick *et al*. (2015)[40], and were chemically characterized in Marentette (2015).[41] NAFC extracts were stored in amber bottles at 4°C.

4.3.3 Cell culture treatments

McA-RH7777 cells were treated for 24h with vehicle (DMEM, control), or NAFC [0.73, 14.7, 73.4 mg/L] (N = 6 independent experiments)[7] in supplemented-DMEM as described above. The concentrations of NAFC [0.73, 14.7, 73.4 mg/L] were chosen to be within the range of naphthenic acid concentrations reported in surface waters, wetlands, tailings ponds, and OSPW from the Athabasca Oil Sands Region[17,41–45]. The 24h exposure represented a timepoint where changes in gene expression and function were previously observed[46].

We have previously reported that exposure to 73.4 mg/L NAFC exposure significantly increased steady-state mRNA levels of *Tdo2* and altered the Kynurenine:Tryptophan Ratio in spent media (Chapter 3). To compare the effects of NAFC [73.4 mg/L] against the known PPARα agonist WY14643 (Cayman Chemical, Ann Arbor, MI), cells were exposed to NAFC and WY14643 [10 µM] in supplemented-DMEM as described above. To examine whether the increased Tdo2 expression could be causally attributed to PPARα-mediated signalling, cells were pre-treated for 1h with 10 µM GW6471, a potent PPARα-selective antagonist (Tocris Biosciences, Toronto, ON) in supplemented-DMEM as described above, followed by the highest dose of NAFC [73.4 mg/L] for 23h. Both GW6471 and WY14643 we dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions; the final concentration of DMSO in the supplemented-DMEM media did not exceed 0.1% (v/v).

4.3.4 Real-time quantitative PCR

Real-time quantitative polymerase chain reaction (qPCR) was performed to determine changes in gene expression of the key rate-limiting enzymes in tryptophan metabolism [*Tdo2, Ido2*][47] and serotonin biosynthesis [*Tph1*][48]. Although TRP is critical precursor for serotonin synthesis, only 1-2% of dietary TRP is converted to serotonin via TPH1, while ~95% of dietary TRP is metabolized by TDO2 and IDO2 into kynurenine and its metabolites pathway.[47] Additionally, qPCR was used to assess the mRNA expression of validated PPARα-target genes involved in lipid metabolism [*Cpt1a, Cd36, Angptl4*].[49,50] ANGPTL4 is a circulating protein that plays a critical role in modulating the release of free fatty acids, and their subsequent uptake into peripheral tissues, thereby playing a significant role in lipid metabolism, glucose homeostasis, and energy balance. Similarly, CD36 plays a crucial role for fatty acid uptake. CPT1α, on the other hand is located on the outer mitochondrial membrane and serves as a rate-limiting enzyme that facilitates mitochondrial β-oxidation.

Following cell treatment, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentrations were measured using the NanoDrop One™ Microvolume UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. RT-qPCR was performed using PerfeCta® SYBR® green FastMix® (Quanta Biosciences, Gaithersburg, MD) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). The cycling conditions included polymerase activation (95°C for 10 min), followed by 40 cycles of denaturing (95°C for 15 sec) and annealing/elongation (60°C for 1 min). Levels of gene expression were calculated using the ΔΔCt method[51] and normalized using the geometric means of reference gene expression: beta-2-microglobulin (*B2m*) and peptidylprolyl isomerase A (*Ppia*). Primer sequences are supplied in Table 1.

Table 1. Table of Primer Sequences.

Accession number	Gene name	Symbol	Forward Sequence (5'-3')	Reverse sequence (5'-3')
NM_017101.1	Peptidylprolyl isomerase A	Ppia	CCGCTGTCTCTTTTCGCC	GCTGTCTTTGGAACTTTGTCTGC
NM_012512.2	Beta-2-microglobulin	B2m	AATTCACACCCACCGAGACC	GCTCCTTCAGAGTGACGTGT
NM_022403.2	Tryptophan 2,3-dioxygenase	Tdo2	GCTGCTCTGCTTGTTCGATG	TCGAGGCTCCTCCCTGTAAA

NM_001419674.1	Indoleamine 2,3-dioxygenase 2	Ido2	TGCTGTGCTGAGCTTCTTGA	ATGCAGGACGTGACTGCTAA
NM_001100634.3	Tryptophan hydroxylase 1	Tph1	GCGGCATGACCTCGATGT	CTGGGCCACCTGCTGACT
NM_199115.2	Angiopoietin Like 4	Angptl4	CAGGACTGGGATGGCAATG	CCTCACCCCCCAAATGG
NM_031559.2	Carnitine Palmitoyl transferase 1A	Cpt1a	CACCCCAACCCATATCCAGG	TCCTCACGGTCTAATGTGCG
NM_031561.2	CD36 Molecule	Cd36	TCTCACACAACTCAGATACTGCT	GCACTTGCTTCTTGCCAACT

4.3.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (v.9.5.1, GraphPad Software, San Diego, CA). Data were tested for outliers (Grubb's Test), normality (Shapiro-Wilke), and equal variance. For outcome measures that required comparisons between control and multiple treatment groups, a one-way ANOVA followed by Dunnett's test was used. For the dose-response and agonist experiments, Dunnett's test was used to compare each treatment group to the control; for the antagonist experiment, pairwise comparisons using Dunnett's test was conducted. Statistical significance was indicated at $p \leq 0.05$. Data that failed normality were analyzed using the Kruskal-Wallis one-way ANOVA on ranks, followed by post-hoc analysis if significance was indicated. All data are presented as mean ± SEM and were considered significant when $p \leq 0.05$.

4.4 Results

4.4.1 NAFC selectively induced Tdo2 expression.

NAFC exposure [73.4 mg/L] resulted in a significant increase in the expression of the rate-limiting enzyme tryptophan-metabolizing enzyme *Tdo2* but there was no significant effect of NAFC at any dose tested on the mRNA expression of either *Tph1* or *Ido2*.

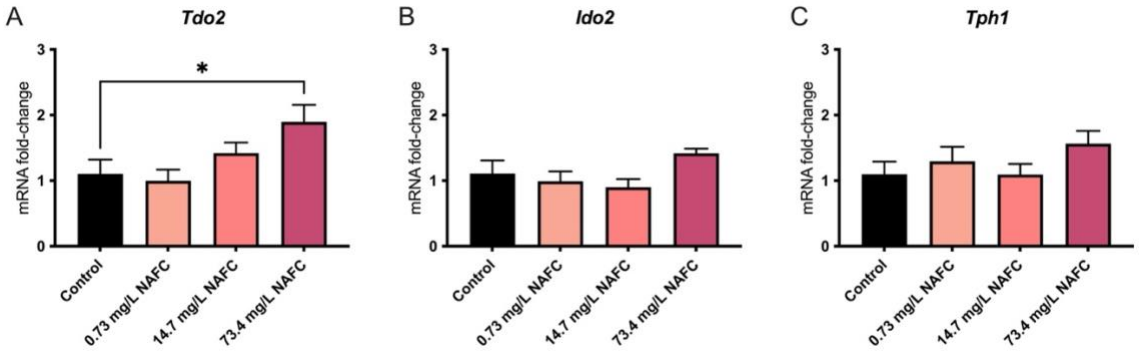


*Figure 1. mRNA expression of key rate-limiting tryptophan-kynurenine pathway enzymes Tdo2 (A), Ido2 (B), and Tph1 (C) in McA-RH7777 cells following 24h exposure to NAFC [0 (control), 0.73, 14.7, 73.4 mg/L] (n=6). Values represented are mean ±SEM, n=6. Values with asterisks * are significantly different (p<0.05) compared to control.*

4.4.2 NAFC acts similarly to PPARα agonist WY14643.

Similar to 73.4 mg/L NAFC, the PPARα agonist WY14643, induced *Tdo2*, *Cpt1a* and *Angptl4* (Figure 2). While *Cd36* showed a significant induction with 73.4 mg/L NAFC exposure, the WY14643-induced increase in *Cd36* was not statistically significant (p=0.09). Interestingly, the induction of Tdo2 expression was not different between NAFC and WY14643 (p=0.6039). However, for the PPARα target genes involved in lipid metabolism (*Cpt1a, Angptl4)*, there were significant differences in mRNA fold-change between NAFC and WY14636. Specifically, NAFC induced higher levels of *Cpt1a*, while WY14643 induced higher levels of *Angptl4.*

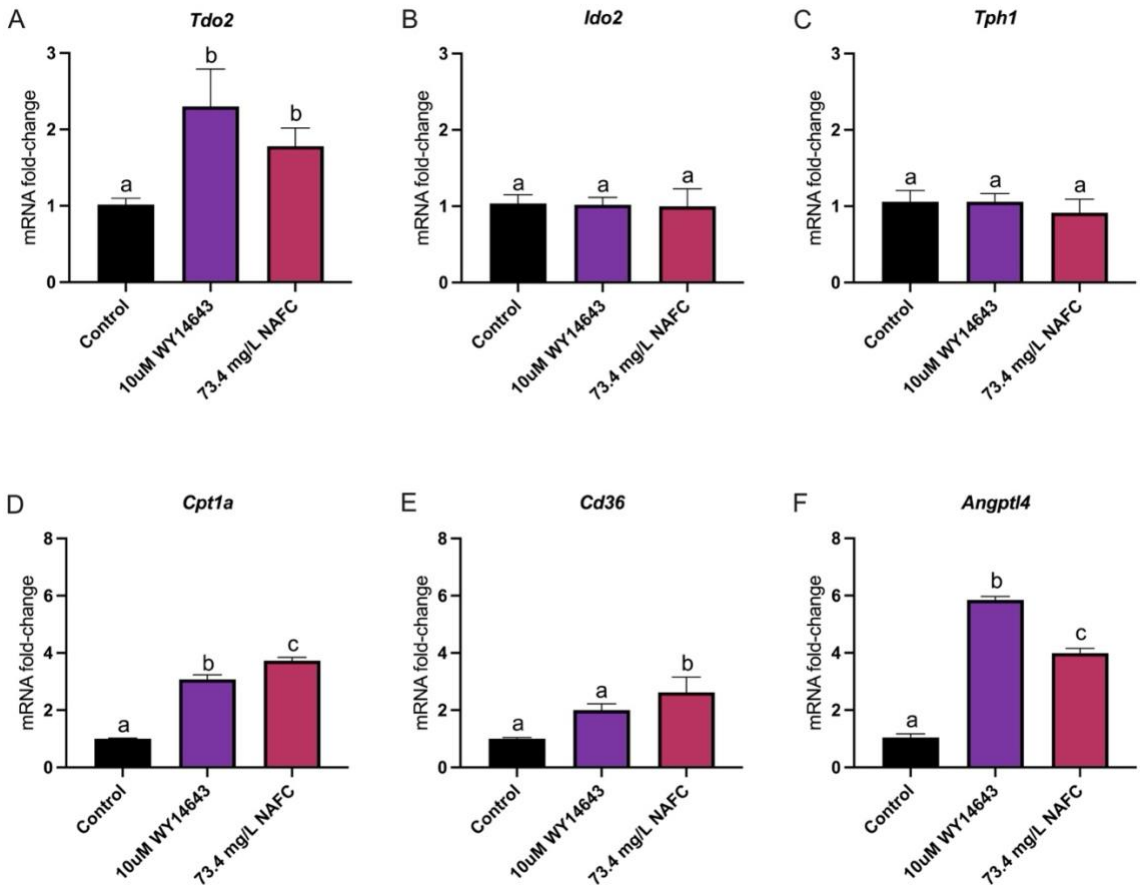


Figure 2. mRNA expression of key rate-limiting tryptophan-kynurenine pathway enzymes Tdo2 (A), Ido2 (B), and Tph1 (C) and peroxisomal proliferator activating receptor alpha-target genes Cpt1a (D), Cd36 (E), and Angptl4 (F) in McA-RH7777 cells following 24h exposure to 0 (control), NAFC [73.4 mg/L], and 10μM WY14643 (n=6). Values represented are mean ± SEM, n=6. Values with different superscripts are significantly different (p<0.05) from each other.

4.4.3 PPARα antagonist GW6471 blocked the NAFC-induced increase in Tdo2.

Co-treatment with PPARα antagonist GW6471 fully blocked the NAFC-induced increase in *Tdo2* (Figure 3A). The PPARα target gene *Cpt1a*, which plays a role in fatty acid beta-oxidation, was significantly upregulated by NAFC exposure. Although the effect was diminished by a PPARα antagonist, it was not completely restored to control levels (Figure 3D). The expression of *Cd36* and *Angptl4* were both significantly induced by NAFC; however, co-treatment with GW6471 only attenuated the increased expression of *Cd36*, with no effect on *Angptl4* (Figures 3E and 3F).

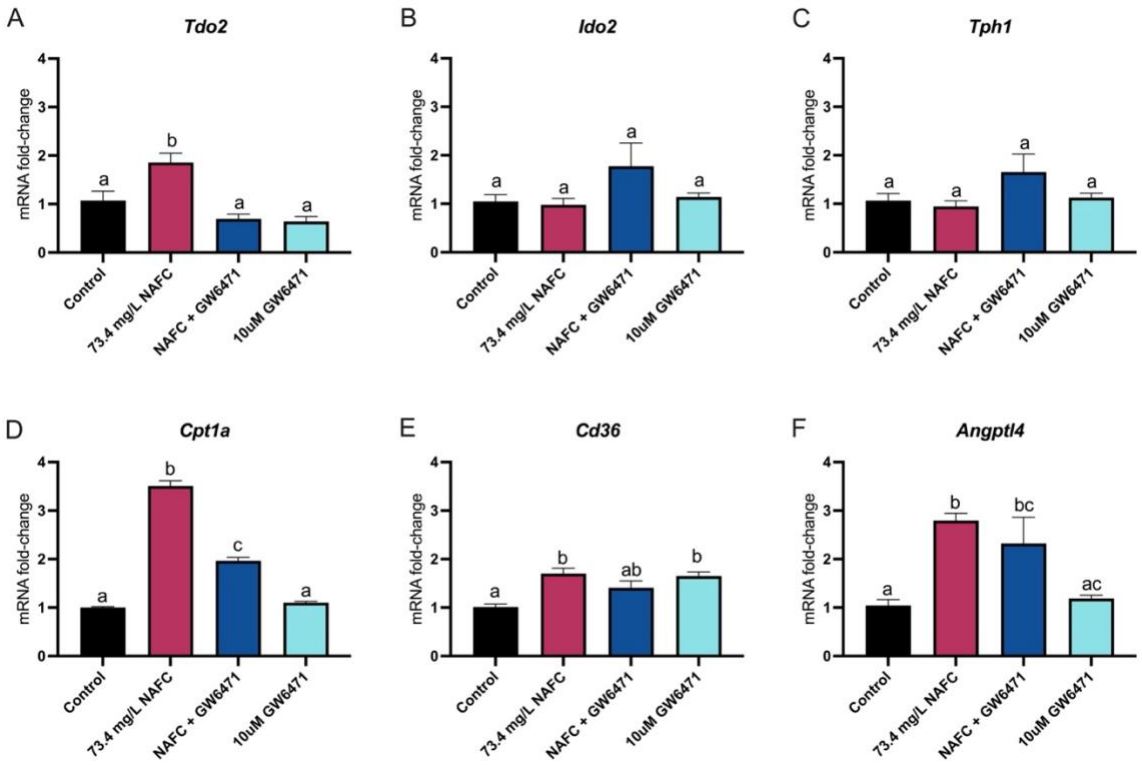


Figure 3. mRNA expression of key rate-limiting tryptophan-kynurenine pathway enzymes Tdo2 (A), Ido2 (B), and Tph1 (C) and peroxisomal proliferator activating receptor alpha-target genes Cpt1a (D), Cd36 (E), and Angptl4 (F) in McA-RH7777 cells following 24 h exposure to 0 (control), 10μM GW6471, NAFC [73.4 mg/L], and co-treatment of 73.4 mg/L NAFC and 10μM GW6471 (n=6). Values represented are mean ± SEM, n=6. Values with different superscripts are significantly different ($p<0.05$) from each other.

4.5 Discussion

Tryptophan metabolism in the liver plays a crucial role in metabolic regulation and immune response, particularly in protecting against metabolic and inflammatory disturbances in disease states and exposure to xenobiotics (Reviewed in:[25]). As the primary site for systemic kynurenine pathway metabolism, hepatic TDO and IDO are pivotal for controlling the flow of 90% of the body's tryptophan.[47] Kynurenine metabolites, have been shown to modulate the immune response, and are linked to metabolic disorders including insulin resistance[52], type 2 diabetes[53], and non-alcoholic fatty liver disease[54,55]. Although TRP metabolism has been shown to be altered by inflammation and glucocorticoids, there is also evidence that chemical exposures can affect key components of the TRP-KYN pathway.[25] We have previously shown that NAFC exposure altered the tryptophan-kynurenine ratio as well as expression of *Tdo2*, a key enzyme important for tryptophan metabolism (Chapter 3). However, the molecular pathways by which NAFC could impact TRP-KYN are unknown.

There is evidence that components of OSPW and other bitumen-influenced waters, including NAFC, may act as PPAR ligands.[27,56] While these reports were limited to assessing PPARγ mediated effects of OSPW components, there is considerable overlap between PPARγ and PPARα signalling in regards to the regulation of systemic and cellular metabolism and energy balance. PPARα regulates genes involved in all processes associated with lipid metabolism, including fatty acid uptake, mitochondrial and peroxisomal fatty acid β-oxidation, alongside the biosynthesis and catabolism of triglycerides and lipid droplets.[57] Interestingly, these pathways align with previous alterations observed following OSPW-derived NAFC exposure in rat hepatocytes (Chapter 3). Given the observed increase in the kynurenine-tryptophan ratio and PPARα's established regulatory functions in lipid metabolism, we further explored the impact of NAFCs on PPARα-mediated transcriptional regulation of the key hepatic enzymes important for tryptophan metabolism.

Results from this study identified that exposure to OSPW-derived NAFCs at 73.4 mg/L significantly upregulated *Tdo2* expression, similar to the effects observed with PPARα agonist WY14643. Both compounds induced *Tdo2*, without affecting *Ido2* or *Tph1* mRNA expression. Importantly, the lack of statistically significant difference in *Tdo2* induction between NAFCs and WY14643 ($p=0.6039$) suggests that NAFC may be acting as a PPARa agonist. This is consistent with evidence from studies showing that PPARα activation by WY14643 in rats shifted tryptophan metabolism towards increased TRP-KYN pathway activity and niacin synthesis by altering downstream enzymes (i.e., ACMSD: α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; QAPRT: quinolinic acid phosphoribosyltransferase).[58] Although TDO2 was not directly examined in these studies[38,59], the observed changes in downstream KYN enzymes suggest that PPARα activation redirects tryptophan enzymatic flux towards enhanced KYN pathway activity. Furthermore, metabolomics and genetic analyses using PPARα agonists in rodents have shown a relationship between the tryptophan-NAD+ pathway and PPARα-dependent pathways.[60–62] Delaney *et al.* (2004)[60] constructed a multivariate statistical model of peroxisome proliferation in rats using various urinary metabolites. Despite the PPARα ligand library showing variable effects on the mRNA expression of downstream TRP-KYN enzymes, the urinary metabolites from the tryptophan-NAD+ pathway still correlated strongly with peroxisome proliferation.[60] This suggests that these metabolites can serve as reliable predictive markers for hepatocellular peroxisome count, regardless of the changes in enzyme expression. Similarly, in PPARα-null mice fed WY14643[62], the most elevated urinary biomarkers were from the tryptophan-niacin pathway. However, these investigations have focused on the downstream metabolites or the mRNA expression of downstream enzymes rather than the rate-limiting enzymes, TDO or IDO. Our study is the first to describe the role of *Tdo2*, the rate-limiting enzyme, in this context, providing new insights into how PPARα agonists may influence TRP metabolism.

To confirm the role of PPARα regulating the observed increase in *Tdo2* following NAFC exposure, we employed GW6471, a potent PPARα-selective antagonist, to block NAFC-induced PPARα activation. GW6471 acts as a competitive antagonist, binding to the ligand-

binding domain of PPARα and preventing the receptor from initiating the transcription of its downstream target genes.[63] GW6471 treatment successfully blocked the induction of *Tdo2* seen with NAFC exposure, returning *Tdo2* expression to control levels, suggesting *Tdo2* expression is mediated via PPARα activation. This finding aligns with previous studies in cancer and tumour xenograft models, in which PPARα-knockout (KO) mice and PPARα-knockdown mice exhibited decreased levels of kynurenine metabolites.[64,65] In fact, He *et al*. (2011)[64] compared metabolomics data from the liver and intestine of PPARα-KO and wild-type (WT) mice and demonstrated that tryptophan metabolism was downregulated in PPARα-KO models, consistent with decreased activity of TDO, IDO and TPH enzymes.[64] They further analyzed a Liver-WY14643 (KO/WT) dataset, in which PPARα-KO and WT mice were treated with WY14643, to determine whether the altered metabolomics pathways were PPARα-dependent. Their findings confirmed that tryptophan metabolism is indeed PPARα-dependent. Together, these studies suggest that PPARα plays a crucial role in regulating TRP metabolism. Our results confirm this role, indicating that NAFC may be acting via PPARα to increase *Tdo2* expression and thus promote TRP catabolism towards KYN metabolites.

To confirm that the induction of *Tdo2* by NAFCs could be attributed to PPARα signalling, we also explored the effects of NAFC on the expression of the validated PPARα target genes *Cpt1a*, *Angptl4*, and *Cd36*. While *Cpt1a* and *Angptl4* expression levels mirrored those of *Tdo2* and were increased by both NAFC and WY14643; NAFC elicited a higher fold change increased in *Cpt1a* expression, whereas *Angptl4* was more strongly induced by WY14643. This differential response suggests that some components within the NAFC mixture can mimic the effects of WY14643 on TRP metabolism, while other components may have distinct PPARα-independent effects on lipid metabolism pathways.

Both ANGPTL4 and CPT1α have been found to be transcriptionally regulated by PPARα activation.[66–68] However, Angptl4 expression has been found to be induced by multiple factors including fasting[69], activation by PPAR agonists[66,70] and exposure to polycyclic aromatic hydrocarbons (PAHs) present in cigarette smoke[71], particulate emissions[72], and industry effluent.[73] Additionally, ANGPTL4 expression is known to be regulated by various receptor signalling pathways including PPARs, aryl-hydrocarbon receptors (AhRs) and glucocorticoid receptors (GRs). This broader regulatory landscape for *Angptl4* might explain why WY14643, a specific PPARα agonist induces higher mRNA expression compared to NAFC, which is a complex mixture of bitumen-derived compounds. Some of these compounds may also act as ligands for AhR or GR, potentially modulating ANGPTL4 expression differently. In fact, long-term glucocorticoid treatment has been shown to decrease *Angptl4* expression in human HepG2 hepatocytes.[74] Furthermore, data from our group has shown that this NAFC mixture exhibits glucocorticoid-like effects in bone cells.[34] This cross-receptor co-activation in our NAFC-exposed McA-RH7777s may explain the lower *Angptl4* induction when compared to WY14643. Furthermore, our inability to fully block NAFC-induced *Angptl4* expression, and the partial inhibition of NAFC-induced *Cpt1a* and *Cd36* using the PPARα-antagonist GW6471, suggests that NAFC might exert additional PPARα-independent effects.

While we have demonstrated in this study that NAFC can function as a PPARα ligand to regulate *Tdo2*, a key enzyme important for tryptophan metabolism, NAFC is a highly complex mixture which complicates efforts to compare binding affinities and determine which compounds might bind more strongly to PPARα, thereby influencing the expression of target genes. This complexity further suggests that the compounds within NAFC may interact with PPARα in ways that are not entirely counteracted by GW6471, potentially due to competitive binding or activation through alternative ligands in the mixture. Generally, our data suggests that PPARα ligands regulate the TRP-KYN pathway, and that NAFC-induction of TRP-KYN metabolizing enzymes occurs via PPARα activation. As NAFC significantly impact the TRP-KYN pathway and PPARα-mediated gene expression, this dual regulatory role suggests a potential mechanism by which NAFC have the potential to influence metabolic processes. While many studies have not specifically investigated TDO-dependent tryptophan metabolism in the liver, our study is the first to show that PPARα-antagonism can block TDO transcription in the liver. Given the critical roles of the TRP-KYN pathway and PPARα in maintaining metabolic homeostasis and overall health, the mechanisms identified in this study warrant further study on the effects of NAFC compounds on mammals.

4.6 Conclusion

Our investigation establishes a transcriptional connection between PPARα activation and tryptophan metabolism, with potential implications for understanding and treating metabolic, and inflammatory disorders. These findings add to the literature that exposure to NAFCs in mammals lead to significant metabolic alterations, including changes in the TRP-KYN pathway and lipid homeostasis, and that PPARα activation may play a role in mediating these effects. These findings further emphasize the potential metabolic impact of bitumen- and OSPW-derived compounds and the importance of understanding the complex interplay of multiple receptor pathways when assessing the metabolic impact of mixtures such as NAFCs. Currently, there is a zero-discharge policy for treated OSPW, and no guidelines exist on its treatment and release into AOSR environments. However, there is the potential for future federal regulations to establish safe discharge criteria into the Athabasca River.[75] This regulatory effort includes assessments to establish baseline conditions and predict the impact of releasing treated OSPW. The current study reinforces the need for careful consideration regarding OSPW release, as bitumen-derived compounds and components may disrupt metabolic and immune functions in exposed higher-trophic level organisms, which could have broader implications for ecosystem health. Importantly, KTR offers a mammalian-based endpoint that extends beyond acute lethality, allowing for a more comprehensive understanding of sublethal effects. If KTR is biomarker of exposure to NAFCs regardless of source (ie. NAFCs from both naturally occurring bitumen influenced waters or OSPW) this may be a useful endpoint for environmental monitoring efforts. However, if the molecular pathways by which KTR is altered depend on the source of NAFCs then KTR has potential to be used to detect contamination sites and potentially initiate forensic investigations to determine whether the source is natural bitumen or industrial in origin. Furthermore, as this research focuses on mammals, it carries implications for Indigenous communities in the AOSR that rely on local wildlife for

sustenance. As NAFCs can affect KTR, a marker of metabolic health, this may have consequences for the health of wildlife populations and, subsequently, the communities that depend on these species.

4.7 References

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CHAPTER V: ASSESSING THE KYNURENINE-TRYPTOPHAN RATIO (KTR) AND CYP1 ACTIVITY IN LONGNOSE (CATOSTOMUS CATOSTOMUS) AND WHITE SUCKERS (CATOSTOMUS COMMERSONII) EXPOSED TO PETROLEUM-DERIVED CONTAMINANTS FROM THE ATHABASCA OIL SANDS REGION

5.1 Abstract

Environmental stressors from industrial activities in the Athabasca Oil Sands Region (AOSR) have significant but species-specific impacts on local wildlife. This study evaluated the kynurenine-tryptophan ratio (KTR) as a potential biomarker for environmental exposure in longnose suckers (*Catostomus catostomus*) and white suckers (*Catostomus commersonii*) collected from various locations within the AOSR. The relationship between KTR and CYP1 enzyme activity (EROD) was assessed alongside biometric indices, including liver somatic index (LSI), gonadosomatic index (GSI), and fat content. Both species exhibited increased EROD activity when exposed to oil sands industry activity, indicating significant polycyclic aromatic compound (PAC) exposure. However, KTR changes were species-dependent: longnose suckers showed an inverse relationship between KTR and EROD, while white suckers displayed a direct correlation. Longnose suckers downstream of industrial activity exhibited significant increases in GSI and fat content, with KTR varying more consistently by location rather than sex, making it a more reliable marker for location-based exposure. Species-specific differences in KTR and EROD relationships may be influenced by the distinct environmental requirements of each species, as well as by environmental factors during the sample collection periods. These findings illustrate the complexity of interpreting environmental biomarkers in wildlife and emphasize the need to consider ecological requirements and environmental conditions. Further research is necessary to validate these biomarkers across different years and conditions, enhancing their applicability in environmental monitoring and conservation efforts.

5.2 Introduction

In Canada, environmental monitoring is a key component of the Fisheries Act conditions for authorization to deposit effluent.[1] Monitoring of fish is conducted by measuring levels of chemicals, indicators of reproductive success (e.g., gonad weight, fecundity) and overall health (e.g., liver weight, condition factor, age) in native species as indicators of ecosystem and individual health.

In the past decade, the oil sands region in Alberta has garnered attention for the potential cumulative ecological effects of oil sands industrial activity on local wildlife and the aquatic environment. Environment and Climate Change Canada (ECCC) has been collaborating with the Alberta government and local Indigenous stakeholders to ensure that the oil sands region is developed in an environmentally responsible way.[2] Freshwater fish and other aquatic organisms are particularly susceptible to the impact of oil sands industrial activity due to chemical inputs into the watershed.[3] Environmental monitoring for these

vulnerable populations is crucial to determine the degree of contamination and its downstream effect on the local ecosystem.

The longnose sucker (*Catostomus catostomus*) and the white sucker (*Catostomus commersonii*) are native species commonly used for environmental monitoring in Canada due to their widespread abundance, robustness, and adaptability.[4,5] In environmental monitoring programs using fish, common biometric tools to assess energy metabolism and reproductive condition are the liver somatic index (LSI), the gonadal somatic index, and fat content.[6,7] While these measures provide valuable insights into aspects of fish health, they may not capture the full range of physiological responses to environmental stressors.[2,8] This limitation prompted us to explore more integrative markers of health. Tryptophan metabolism, which is involved in energy regulation, protein excretion, and serotonin production, plays a crucial role in growth, reproduction, and survival, suggesting that tryptophan or its kynurenine pathway metabolites could serve as a more comprehensive biomarker of overall health in fish.[9]

Recent research has highlighted the kynurenine-tryptophan ratio (KTR) as a potential cross-species indicator of organism health.[10,11] The KTR measures the relative proportion of key metabolites, kynurenine and tryptophan, that are involved in generating and managing stress and immunological responses in mammals, fish, and birds. Additionally, alterations in the KTR have been implicated in responses to stress and environmental pollutants.[10,12–17] Given that the oil sands region is being heavily monitored and various biometric data is being collected in ongoing biomonitoring efforts, we hypothesized that the KTR may provide additional information as an integrated marker of organismal health. Therefore, the objective of this study was to determine the effects of the oil sands deposit and industrial activity in longnose suckers and white suckers from the oil sands region using metabolite levels, enzyme activity, and established biometric tools.

5.3 Methods

5.3.1 Fish Collection

Suckers (*Catostomus*) species are widespread in North America and can be found in the Athabasca river in Alberta, Canada. [1] The fish sampling for this study was conducted as described in McMaster *et al*., (2018) [2] and Arciszewski and McMaster (2021). [3] Briefly, white suckers (*Catostomus commersonii*) and longnose suckers (*Catostomus catostomus*) were collected in the fall (September – October) of study years (2013 and 2014). White and longnose sucker were collected within the river system at two sites outside of the oil sands deposit (Athabasca and Poacher's Landing, collectively termed Reference), one site within the deposit upstream of development (Northland, termed Deposit), and two or three sites within the deposit and within oil sands development (Suncor, Muskeg, collectively termed Development). For this study we used adult female and male longnose suckers (N=20 per sex per site) that were collected in 2013 at each of the study locations using boat electrofishing under scientific collection permits issued by Alberta Environment and Parks (13-0445, 14-0456) and following the Canadian Council on Animal Care approved Animal Use protocols (1315, 1415) issued by the National Water Research Institute Animal Care

Committee. The white suckers assessed in this study were collected in 2014. In this sample set there were 10 animals per site; this number included female, male and immature fish. For all fish, after electroshock, fish were captured using dip nets (approx. 0.5-cm mesh size), placed in the livewell and were transported to the portable on-site laboratory for processing. In the mobile laboratory, fork length (FL; ±1 mm), body weight (BW; ±0.1 g), liver weight (LW; ±0.01 g), gonad weight (GW; ±0.01 g), and sex were recorded. Opercula were also removed for aging (±1 y). Portions of the livers were placed in vials and frozen in liquid nitrogen, then later stored at -80°C prior to analysis. Hepatic ethoxyresorufin-O-deethylase (EROD) activity was measured as an indicator of mixed-function oxygenase (MFO) activity, following the methods of Van den Heuvel *et al.* (1995). [4]

5.3.2 Kynurenine-Tryptophan Ratio (KTR) Assessment

Materials and Reagents

Unlabeled analytical standards of L-tryptophan (TRP, ≥ 98% HPLC) and L-kynurenine (KYN, ≥ 98%) were purchased from Sigma- Aldrich Canada Co. (Oakville, ON, CA). Mass labeled L-tryptophan- 2′,4′,5′,6′,7′-d_5 (indole-d_5) (98%, TRP-d_5), L-kynurenine-d_4 [4-(2-aminophenyl-3,5-d_2)] (99%, KYN-d_4) were purchased from CDN Isotopes (Pointe-Claire, QB, CA). All solutions were stored at 4◦C. L-Acetonitrile (ACN) and water were purchased from Fisher-Scientific and of HPLC-grade. Ultrapure Milli-Q was obtained from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA).

Sample Preparation and Extraction

Homogenized fish tissue (~0.02 g) was placed into a 2.0 mL microcentrifuge tube and spiked with 10 µL of the mass-labeled internal standards TRP-d_5 and KYN-d_4 (each at 25 ng/uL). Extractions were performed by adding 1.35 mL of 35:65 H_2O:ACN to each tube, sonicating in an ice bath for 12 mins, vortexing for 1 min and centrifuging at 14 800 rpm for 15 minutes. The supernatant was removed, and the remaining tissue was re-extracted as before. Extracts were combined and reduced in volume to 1.5 mL with a gentle stream of ultra-high purity N_2 prior to high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) analysis.

High Performance Liquid Chromatography Tandem Mass Spectrometry

The detection and quantitation of TRP and KYN in fish tissue was based on a previously published validated method.[5] Separations of mass labeled and native KYN and TRP were performed using a SEQUANT ZIC^R HILIC PEEK coated HPLC column (4.6 mm x 100mm, 3.5 µM particle size, 200 A pore size) (Merck, Darnstadt, Germany). The HPLC was coupled to a Sciex 365 triple quadrupole mass spectrometer retrofitted with an HSID Ionics EP$^+$ orthogonal ionization source. Ionization was performed in the positive ion mode and the multiple reaction monitoring ion transitions used for detection and quantitation were: TRP: *m/z* 205.1 → 188.2; KYN: *m/z* 209.4 → 94.1, TRP-d_5: *m/z* 201.2 → 192.4 and KYN-d_4: *m/z* 213.3 → 196.2. Method detection limits for KYN and TRP, determined according to the procedure described in Eurachem Guide for Method Validation[6], were 133.9 and 89.8 ng/g, respectively.

5.3.3 Data and Statistical Analysis

All statistical analyses were conducted using GraphPad Prism (v.9.5.1, GraphPad Software, San Diego, CA). Data were assessed for outliers using Grubb's Test, tested for normality with the Shapiro-Wilk test, and checked for equal variance. For outcome measures requiring comparisons between control and multiple locations, a one-way ANOVA was performed, followed by Dunnett's test for post hoc comparisons when significant differences were detected ($p \leq 0.05$). Data that did not pass tests for normality or equal variance were analyzed by Kruskal-Wallis one-way ANOVA on ranks. Dunn's post-hoc test was applied for comparisons against the control when significant differences were found. All results are presented as mean ± SEM and were considered significant at $p \leq 0.05$.

To investigate the relationships between variables, Pearson's correlation analysis was performed to compare ethoxyresorufin-O-deethylase (EROD) activity and Kynurenine-Tryptophan Ratio (KTR) levels using simple linear regression. Further profiling included Pearson's correlations between EROD activity, KTR levels, weight, condition factor, liver somatic index (LSI), gonadosomatic index (GSI), and fat content for each fish type.

Additionally, variation based on sex and location was analyzed using two-way ANOVA to assess the influence of these factors on the outcome measures. Interactions between sex, location, and physiological parameters were also explored to determine site-specific and sex-specific responses

5.4 Results & Discussion

5.4.1 Comparative Analysis of Kynurenine-Tryptophan Ratio and EROD Activity in Longnose and White Sucker

In field studies, fish exposures serve as critical tools for monitoring the quality of aquatic environments. The use of fish in eco-epidemiology has become increasingly prominent, particularly in pollution monitoring programs.[7] Typical assessments focus on screening for visible signs of disease and abnormalities, alongside biochemical markers, including tissue residue concentrations of contaminants and metabolic stress indicators (e.g., lipid content and condition factor), to provide insights into the health of fish populations and their environments.[8] Biomonitoring of fish health is of considerable importance particularly in regions where there is potential for industrial activities to affect aquatic environments.

Catostomid fishes are a diverse family of over 76 freshwater species distributed across North America in a variety of habitats. These fish face numerous challenges, including migration barriers, variations in water levels and temperatures, environmental contamination, habitat degradation, exploitation, and the impacts of introduced species.[9] In Alberta, *Catostomus* species are particularly affected by temperature fluctuations and environmental pollutants making them valuable indicator species.[2,9,10] To evaluate the impact of oil sands industrial activity on the KTR ratio, fish were collected from five sites within the Athabasca River system, including two sites outside of the oil sands deposit (Athabasca and Poacher's Landing, collectively termed Reference), one site within the

deposit upstream of development (Northland, termed Deposit), and two sites within the deposit and within oil sands development (Suncor, Muskeg, collectively termed Development) (Figure 1).

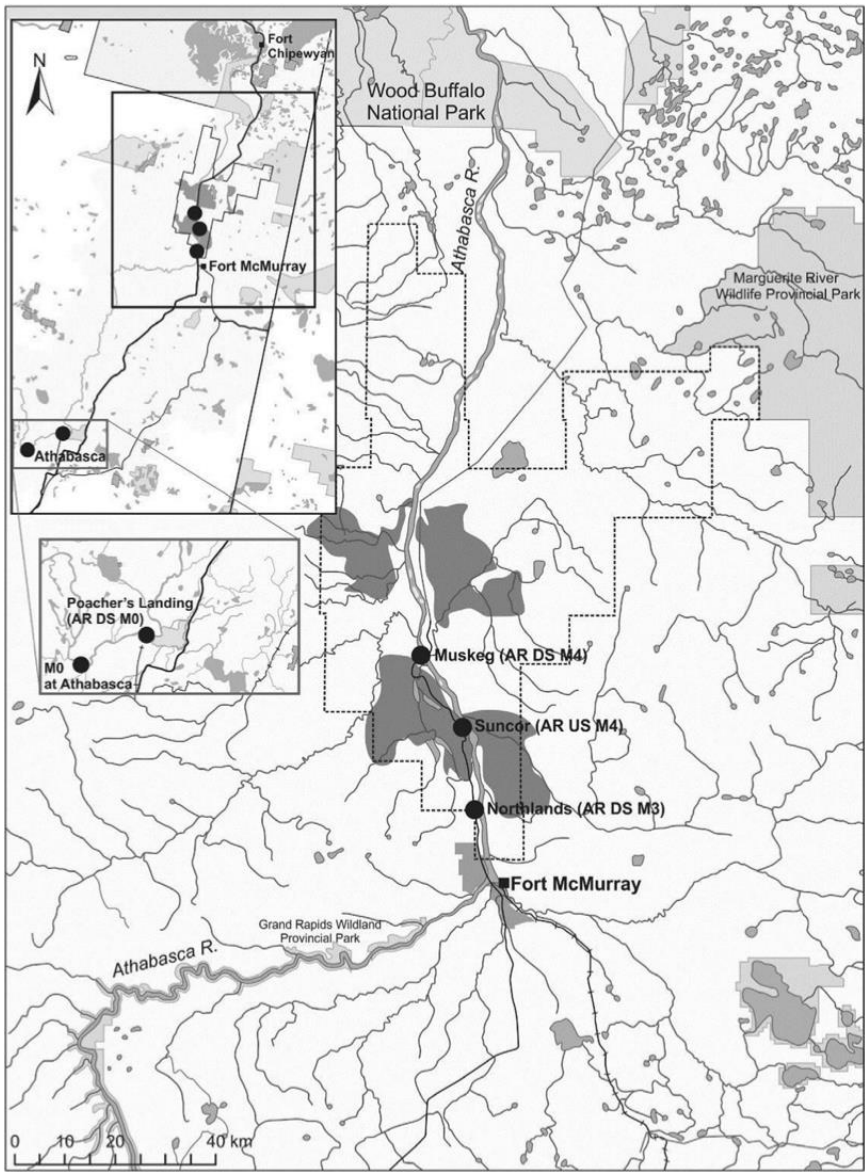


Figure 1. Map of Fish collection sites, two sites outside of the oil sands formation, one site in the formation upstream of development and two sites in the formation downstream of oil sands development. McMaster and Arciszewski (2021).

Longnose suckers (*Catostomus catostomus*) were assessed across all collection sites, pooling data from both sexes (Figure 2). Significant differences in KTR were observed between reference sites and both deposit and industry sites. This decrease in KTR was primarily driven by reduced kynurenine levels, while tryptophan levels remained

unchanged. Fish from the Northland, Suncor, and Muskeg sites exhibited significantly lower KTR compared to reference sites (Figure 2C), indicating location-specific alterations in the kynurenine pathway. In contrast, white sucker (*Catostomus commersonii*) did not show statistically significant changes in KTR across collection sites (Figure 3).

Fish react to environmental stress through two primary hormonal axes: the sympatho-chromaffin (SC) axis and the hypothalamic-pituitary-interrenal (HPI) axis.[11] Cortisol is a main hormonal mediator of the stress response in fish. Importantly, stress has been shown to alter the KTR in fish.[5] Our group previously found that rainbow trout (*Oncorhynchus mykiss*) subjected to acute stress exhibited increased KTR in liver and brain tissues 48 hours post-exposure, which correlated with elevated cortisol levels.[5] Although we have not measured cortisol levels in this study, it is plausible that the longnose suckers captured in the sites influenced by industrial activity may have higher levels of stress which are associated with changes in the KTR.

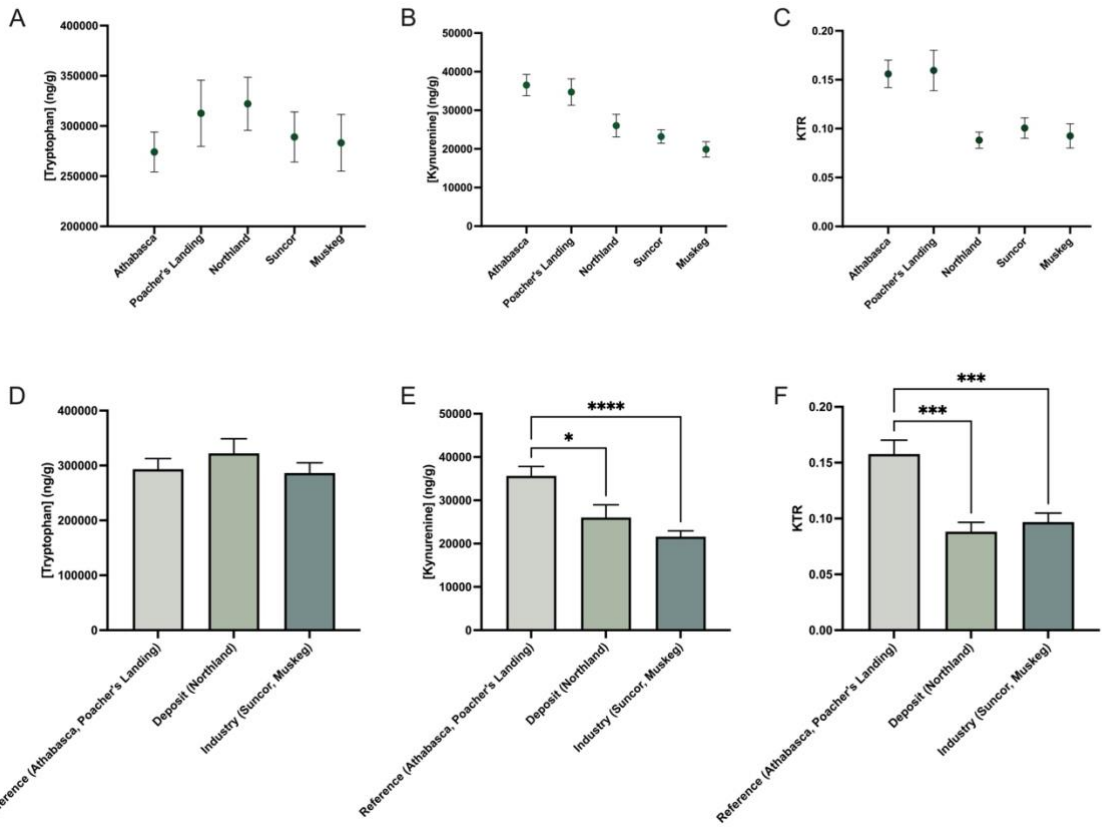


Figure 2. Kynurenine-tryptophan ratio (KTR) of longnose sucker (Catostomus catostomus) across five collection sites (A, B, C), pooled male and females (~40), and then grouped according to reference (Athabasca, Poacher's Landing), oil sands deposits (Northland), and industry activity (Suncor, Muskeg) (D, E, F).

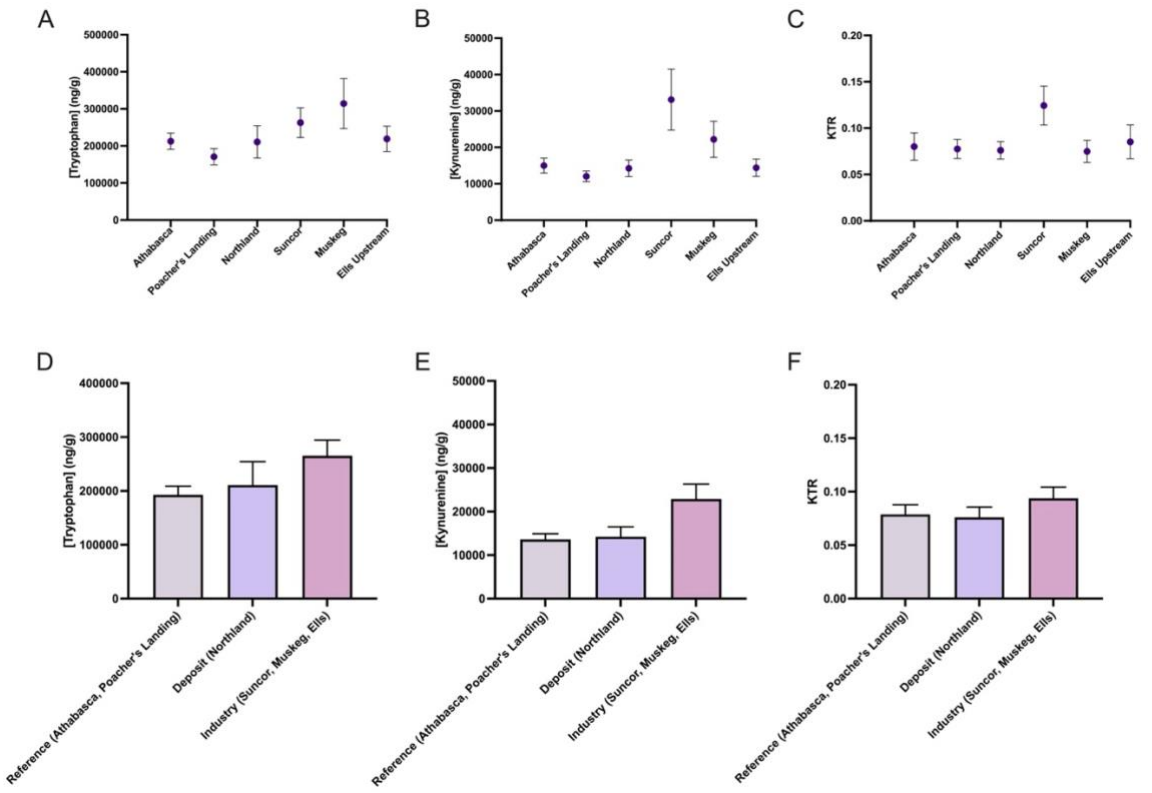


Figure 3. Kynurenine-tryptophan ratio (KTR) of white sucker (Catostomus commersonii) across five collection sites (A, B, C), pooled male and females (~10), and then grouped according to reference (Athabasca, Poacher's Landing), oil sands deposits (Northland), and industry activity (Suncor, Muskeg, Ells) (D, E, F).

The observed decrease in KTR in longnose but not white suckers may reflect the differing ecological requirements and sensitivities of the two species. Longnose suckers are known to require more specialized environments, often favoring cold, clear waters and specific latitudinal ranges, where they thrive in stable environments with minimal turbidity.[12–14] Changes to their environment, such as increased temperature, pollution, or altered flow conditions, can disrupt longnose sucker's stress response, potentially overwhelming their ability to maintain homeostasis and leading to shifts in metabolic pathways like the tryptophan-kynurenine pathway. In contrast, white suckers did not show significant changes in KTR. Studies have shown that white suckers can tolerate a range of environmental conditions, including variations in nutrient loading.[2,15] Indeed, Quinn *et al*. (2010)[10], examined Mountain Whitefish and White Sucker exposed to temperature and agrochemicals in Alberta's Oldman River, found species-specific differences in AChE activities and responses to physiological stress – suggesting that white suckers were more resistant to temperature and pesticide stress. The lack of change in KTR in the white suckers may be a reflection of this resilience and the lack of a stress response to industrial inputs in

the different sampling regions. However, without cortisol levels to test this hypothesis, this remains speculative. An alternative explanation for changes in KTR in the different sampling regions is related to the impact of bitumen-related compounds on aryl hydrocarbon receptor (AhR) signalling pathways.

AhR is activated by various environmental pollutants, including polycyclic aromatic hydrocarbons (PAHs), which are abundant in the AOSR and industry effluent.[16,17] Activation of AhR leads to downstream metabolic changes, including the induction of cytochrome P450 enzymes, particularly CYP1A. Xenobiotics and endogenous compounds which act as AhR ligands increase expression of CYP1, which can be monitored by assessing ethoxyresorufin-O-deethylase (EROD) activity, a common biomarker of AhR activation and CYP1A activity. Numerous studies have demonstrated the value of EROD as a biomarker in both laboratory and field studies, particularly in assessing exposure to industrial effluents, contaminated sediments, chemical spills and PACs (Reviewed In:[18,19]).

In this study, EROD activity was significantly elevated in longnose sucker from both the deposit and industry sites compared to reference locations (Figures 4A, C). This increase in EROD activity suggests a heightened level of AhR activation and CYP1A induction in longnose sucker in response to bitumen exposure, regardless of source. In white sucker, EROD activity was significantly higher only at the industry sites, with no significant change observed at the deposit site (Figures 4B, D). Interestingly, AhR ligands have also been demonstrated to increase tryptophan catabolism leading to increased levels of kynurenine metabolites which are also AhR ligands. Indeed, it is well-established that derivatives of tryptophan metabolism, particularly those that are photo-oxidized, can bind to AhR with high affinity and serve as ideal substrates for CYP1 enzymes, suggesting that tryptophan metabolism can influence AhR activity.[20,21] In this study, there was a statistically significant negative correlation was observed between EROD activity and KTR levels in longnose suckers ($r = -0.9910$, $p < 0.05$) (Figure 5), whereas in white suckers, a statistically significant positive correlation between EROD activity and KTR levels was found ($r = 0.8154$, $p = 0.048$). These differences may indicate species-specific sensitivity to contaminants or a differing threshold for inducing CYP1A in white suckers.[10]

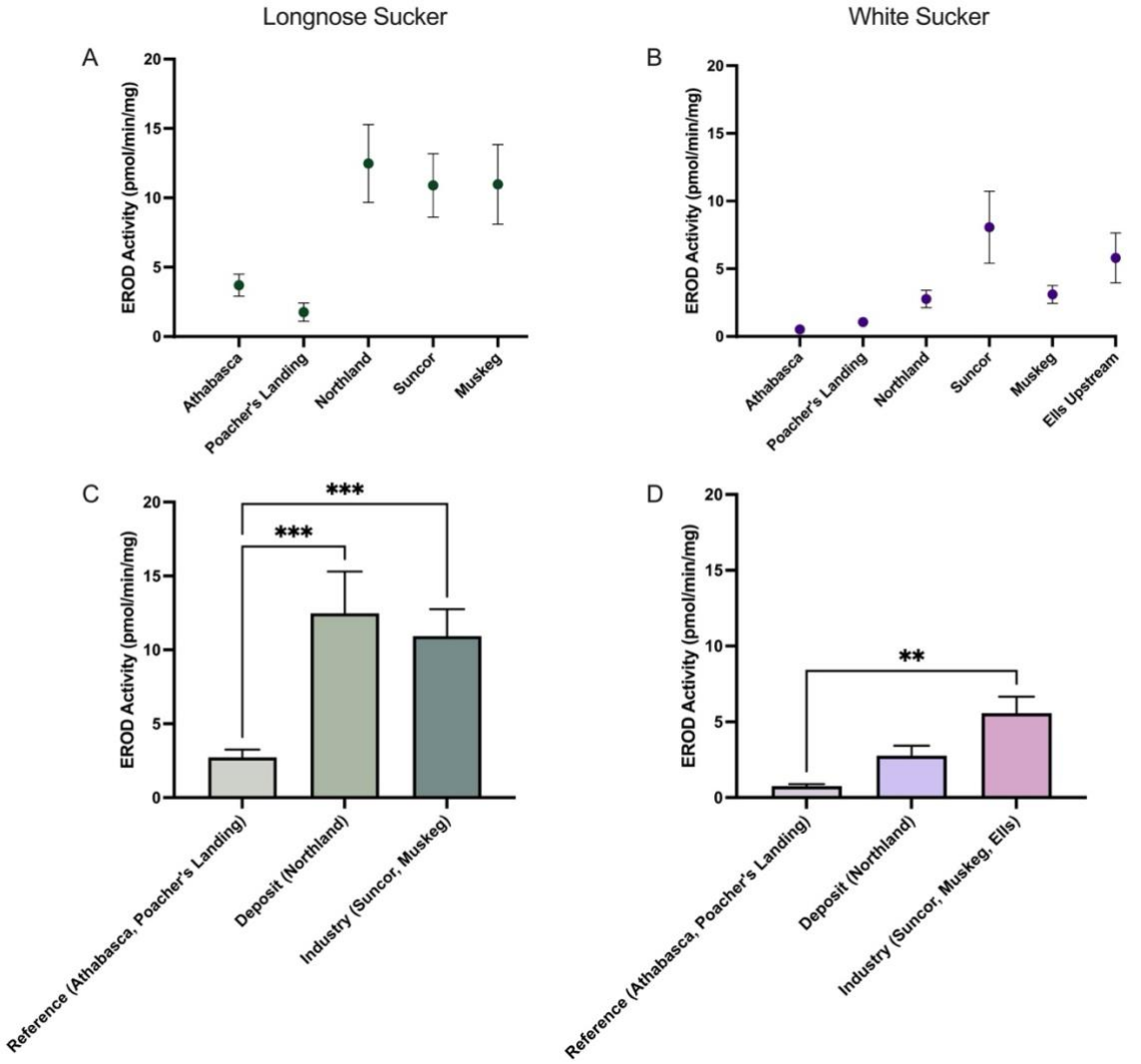
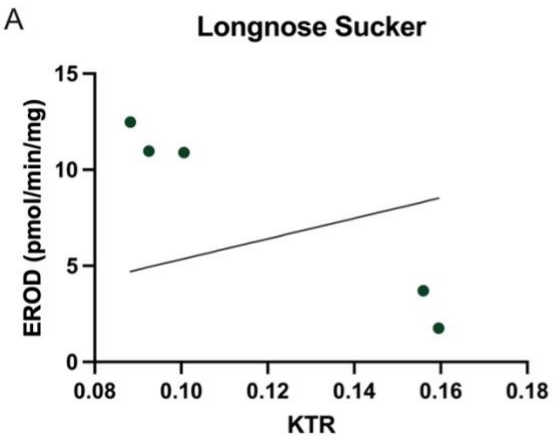
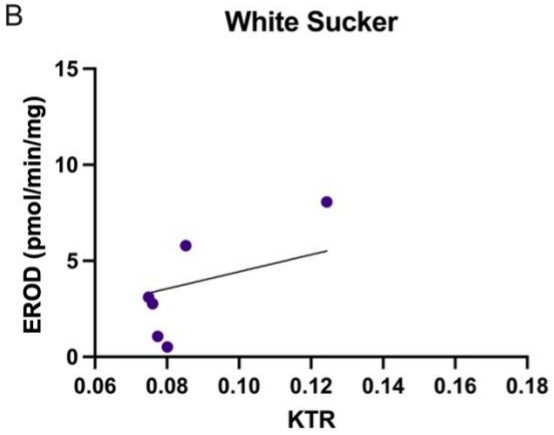


Figure 4. EROD activity (pmol/min/mg) of Longnose Sucker (Catostomus catostomus) (A,C) pooled male and females (~40), and White Sucker (Catostomus commersonii) (B, D) pooled male and females (~10), across 5 collection sites and grouped according to reference (Athabasca, Poacher's Landing), oil sands deposits (Northland), and industry activity (Suncor, Muskeg, Ells) (D, E, F).



Pearson r	
r	-0.9910
95% confidence interval	-0.9994 to -0.8646
R squared	0.9820
P (two-tailed)	0.0010



Pearson r	
r	0.8154
95% confidence interval	0.01129 to 0.9791
R squared	0.6648
P (two-tailed)	0.0480

Figure 5. Pearson's correlation analysis between longnose sucker (Catostomus catostomus) and white sucker (Catostomus commersonii) in relation to ethoxyresorufin-O-deethylase (EROD) activity and Kynurenine-Tryptophan Ratio (KTR) levels.

However, it is essential to consider the differences in the timing and sample sizes when interpreting these results. Longnose suckers were collected in 2013, with a robust sample size of 20 males and 20 females at each site. In contrast, white suckers were collected in 2014, a year characterized by the lowest flow and warmest temperatures of the six years of available data, with only 10 total fish sampled per site.[3] This combination of low flow and high temperatures could have affected water quality and bitumen-derived contaminant distribution, potentially influencing the physiological responses observed in the white suckers.[22] This difference in sampling year and size introduces variability that complicates direct comparisons of exposures between the two species, particularly for endpoints measured in 2014. Further investigations with larger sample sizes collected at the same time would need to confirm that the observed site-specific changes in KTR are species dependent.

5.4.2 Does KTR correlate with biometric data?

In longnose sucker, correlation analysis revealed that KTR and EROD activity were the only variables significantly correlated (Figure 6A). While EROD activity varied significantly by both sex and location, KTR was influenced only by location, indicating that environmental contaminants are the dominant factor driving changes in tryptophan metabolism in this species (Figure 7). LSI, GSI, and fat were significantly different between reference and industry sites, but not reference and deposit (Figures 6D, E, F). The marked reduction in GSI, which reflects the energy invested in gonadal development, at industry sites compared to both reference and deposit sites suggests that reproductive health is being adversely impacted by environmental contaminants specifically associated with industry activity. Although increased tryptophan catabolism to kynurenine has the potential to reduce the amount of tryptophan used as substrate for serotonin, an important mediator of fish reproduction[23], there was no relationship between KTR and GSI.

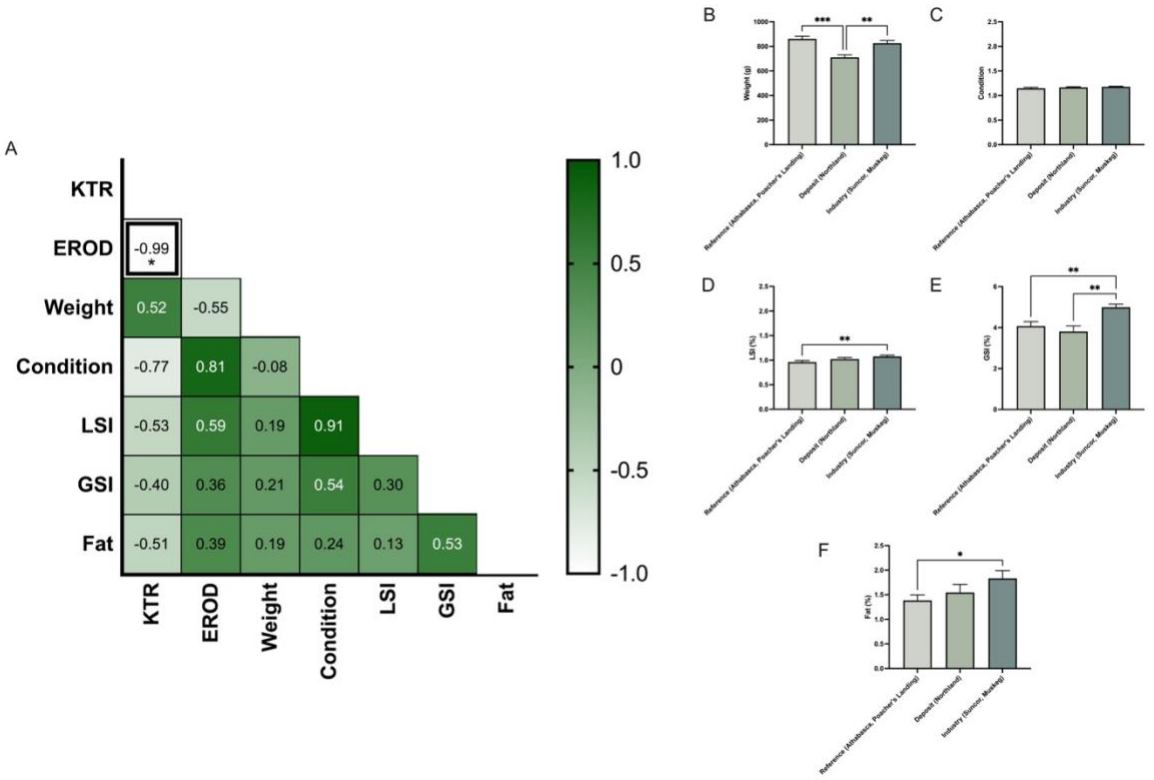
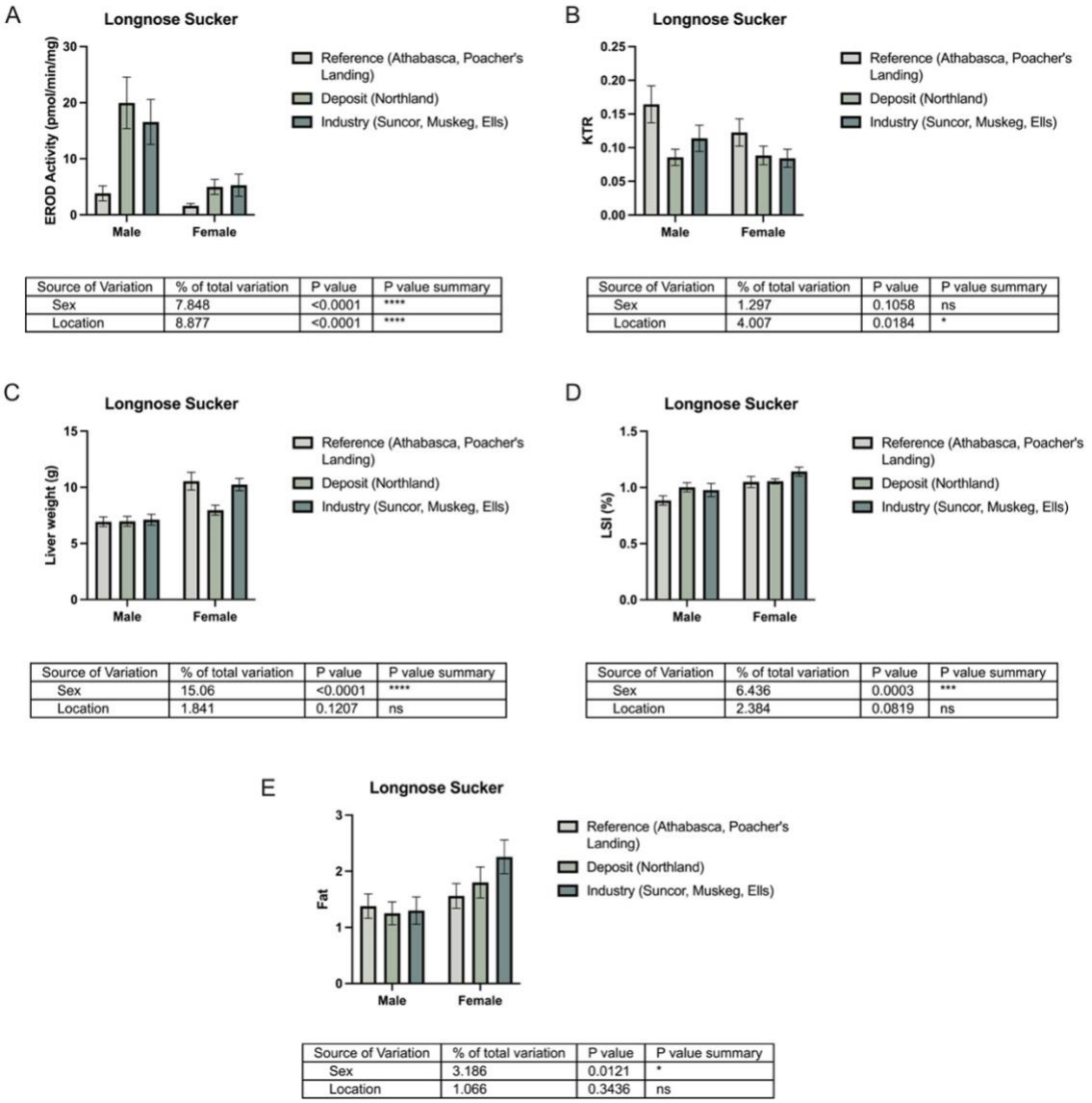


Figure 6. Profiling the longnose sucker (Catostomus catostomus): Pearson's correlation matrix analysis between ethoxyresorufin-O-deethylase (EROD) activity, Kynurenine-Tryptophan Ratio (KTR) levels, weight, condition, liver somatic index (LSI), gonadosomatic index (GSI), and fat.



Source of Variation	% of total variation	P value	P value summary
Sex	7.848	<0.0001	****
Location	8.877	<0.0001	****

Source of Variation	% of total variation	P value	P value summary
Sex	1.297	0.1058	ns
Location	4.007	0.0184	*

Source of Variation	% of total variation	P value	P value summary
Sex	15.06	<0.0001	****
Location	1.841	0.1207	ns

Source of Variation	% of total variation	P value	P value summary
Sex	6.436	0.0003	***
Location	2.384	0.0819	ns

Source of Variation	% of total variation	P value	P value summary
Sex	3.186	0.0121	*
Location	1.066	0.3436	ns

Figure 7. Profiling the longnose sucker (Catostomus catostomus): Two-way ANOVA analysis comparing sex and location among ethoxyresorufin-O-deethylase (EROD) activity, Kynurenine-Tryptophan Ratio (KTR) levels, liver weight, liver somatic index (LSI) and fat.

In white suckers, there was a significant decrease in GSI at deposit sites (Figure 8) suggesting that reproductive stress in white sucker is linked to environmental conditions at the deposit sites, rather than being solely driven by anthropogenic activity associated with the oil sands industry. However, similar to what was observed in longnose suckers, there was no significant correlation between GSI and KTR in white suckers (Figure 9).

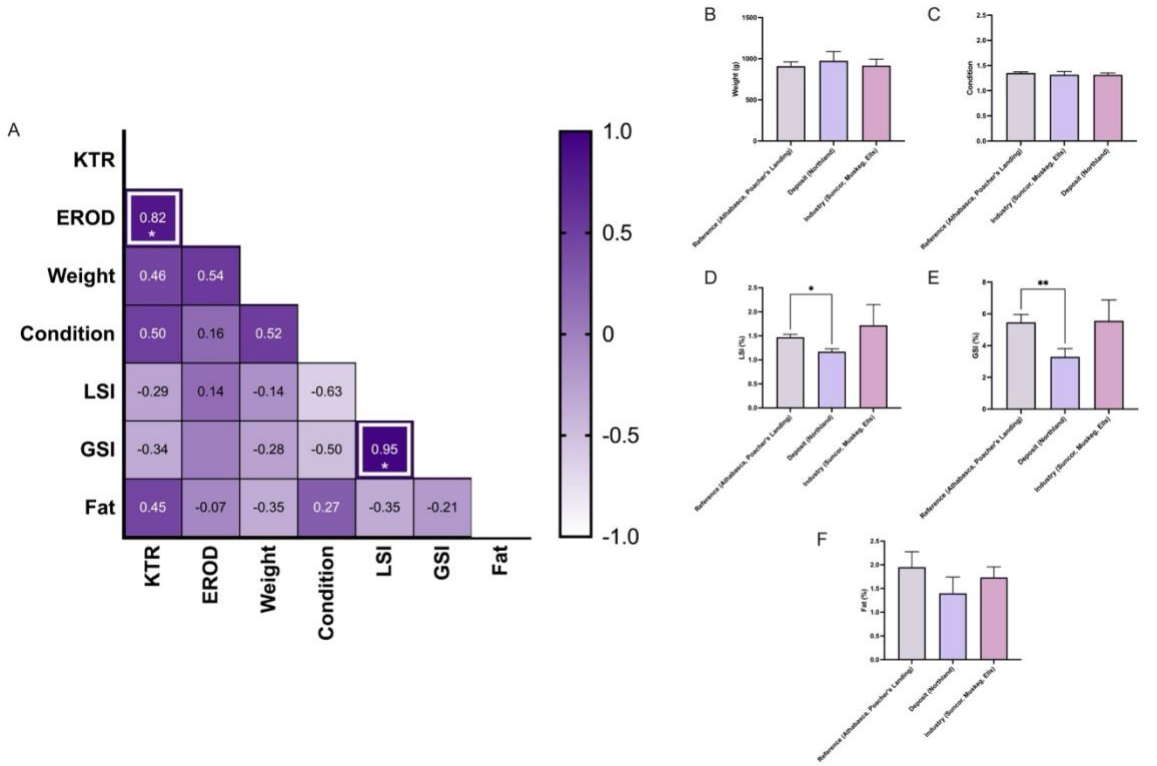
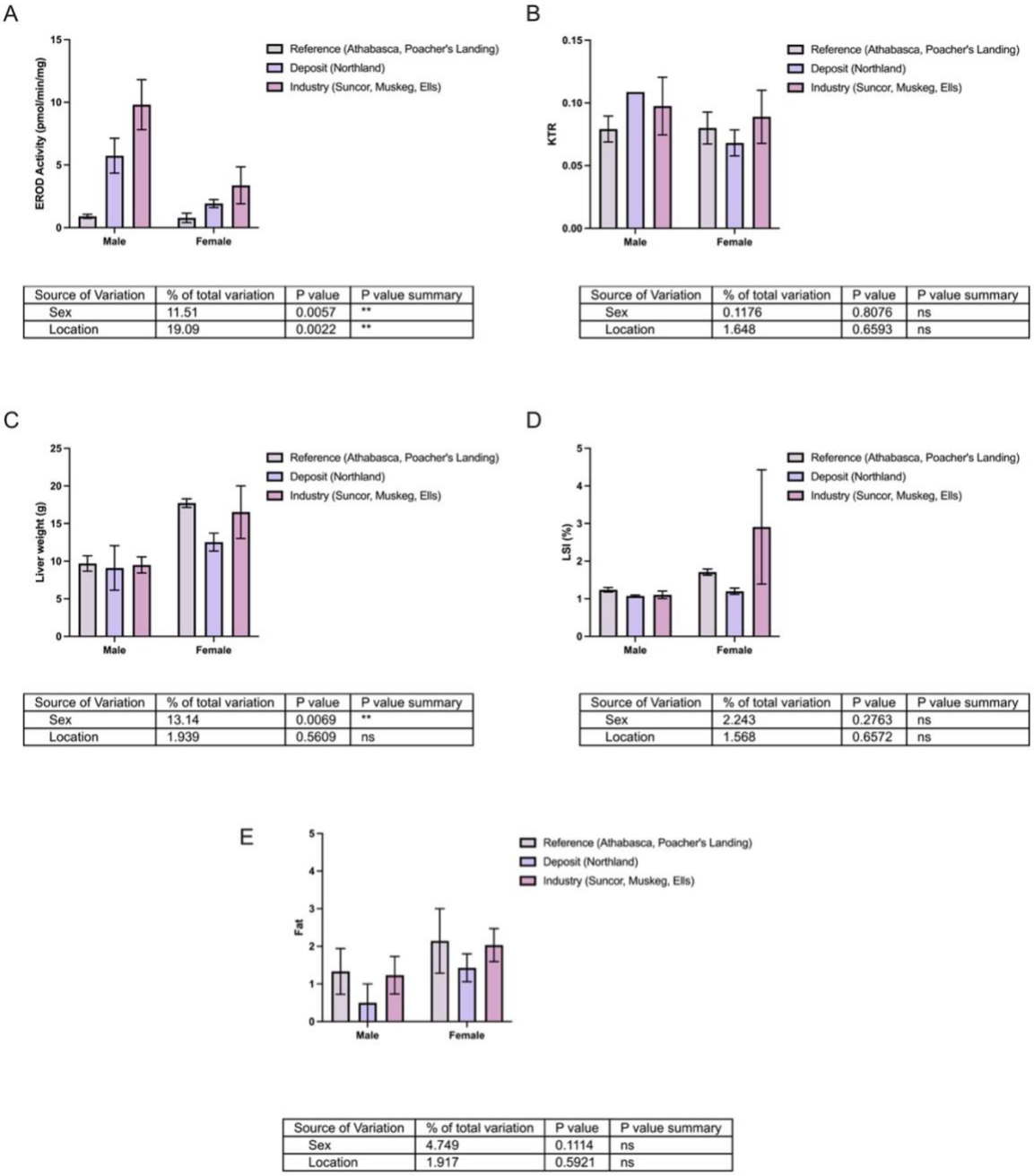


Figure 8. Profiling the white sucker (Catostomus commersonii): Pearson's correlation matrix analysis between ethoxyresorufin-O-deethylase (EROD) activity, Kynurenine-Tryptophan Ratio (KTR) levels, weight, condition, liver somatic index (LSI), gonadosomatic index (GSI), and fat.



Source of Variation	% of total variation	P value	P value summary
Sex	11.51	0.0057	**
Location	19.09	0.0022	**

Source of Variation	% of total variation	P value	P value summary
Sex	0.1176	0.8076	ns
Location	1.648	0.6593	ns

Source of Variation	% of total variation	P value	P value summary
Sex	13.14	0.0069	**
Location	1.939	0.5609	ns

Source of Variation	% of total variation	P value	P value summary
Sex	2.243	0.2763	ns
Location	1.568	0.6572	ns

Source of Variation	% of total variation	P value	P value summary
Sex	4.749	0.1114	ns
Location	1.917	0.5921	ns

Figure 9. Profiling the white sucker (Catostomus commersonii): Two-way ANOVA analysis comparing sex and location among ethoxyresorufin-O-deethylase (EROD) activity, Kynurenine-Tryptophan Ratio (KTR) levels, liver weight, liver somatic index (LSI) and fat.

5.5 Conclusion

This study validates the potential of the kynurenine-to-tryptophan ratio as a valuable biomarker for assessing polycyclic aromatic compound exposure in aquatic environments. By linking KTR with EROD activity, a well-established marker of CYP1A enzyme induction and polycyclic aromatic compound exposure, we provide evidence that KTR effectively reflects metabolic disruptions in response to environmental stressors linked to both natural bitumen deposits and industrial activity in the AOSR. This is the second study confirming that KTR assessments can be performed in fish, and, importantly, in a species-specific manner. Given that the goal of the ECCC is to advance, refine, and improve environmental regulation, safety, and industry, the KTR may be used as an encompassing, species-specific marker of environmental and organismal health.

Future work should aim to expand sample sizes and further validate KTR across diverse environmental conditions to establish its utility as a reliable biomarker. The location-dependent nature of KTR observed in longnose sucker reinforces the need for broader temporal and spatial studies to understand the variability of KTR responses across different ecosystems and under varying levels of industrial activity and natural deposits. Additionally, while EROD activity remains a key marker for ecological assessments, KTR offers complementary insights into energy homeostasis and downstream biological responses to xenobiotics. Future studies should investigate how KTR reflects long-term metabolic impacts and reproductive health in aquatic species exposed to oil sands-related contaminants.

Ongoing work in our group is focused on exploring the use of KTR in non-lethally collected tissues, such as mucus or scales, to enhance its application in environmental monitoring without harming fish populations. These efforts will help solidify KTR as a practical and non-invasive biomarker for assessing environmental contamination and fish health in the AOSR and beyond. Taken together, these data suggest that KTR can be used as an indicator for environmental contamination and fish health, which can further provide insights to the consequences of industrial activity on aquatic species.

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CHAPTER VI: DISCUSSION AND FUTURE DIRECTIONS

6.1 Summary of Work

This thesis aimed to investigate the kynurenine-tryptophan ratio (KTR) as a biomarker for environmental stress and toxicant exposure across species, focusing on petroleum-derived compounds from the Athabasca Oil Sands Region (AOSR). By examining both *in vitro* and *in vivo* models, this work explored the disruption of tryptophan metabolism in response to complex environmental mixtures, including polycyclic aromatic compounds (PACs), acid-extractable organics like Naphthenic Acid Fraction Components (NAFCs), and an in-lab exposure of bitumen-influenced waters: Bitumen Water Accommodated Fraction (BitWAF). Altered KTR was linked to receptor-mediated disruptions in tryptophan metabolism and metabolic reprogramming, offering new insights into the broader effects of environmental toxicants on cellular metabolic pathways.

In vitro studies with mammalian hepatocytes revealed that KTR increases were driven primarily by elevated kynurenine levels, pointing to disruptions in tryptophan metabolism via receptor pathways including the aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor alpha (PPARα). The findings indicate that environmental toxicants, including those which are found in the AOSR, can influence receptor signaling pathways, altering the expression of key enzymes such as *Tdo2*, which plays a central role in regulating the production of downstream kynurenine metabolites. In association with changes in KTR, significant metabolic reprogramming was observed. NAFC exposure led to mitochondrial dysfunction and disruptions in glucose and fatty acid uptake, impairing the cell's ability to maintain energy homeostasis. Further assessments revealed that disruptions these disruptions in lipid metabolism and energy homeostatic pathways coincided with impaired mitochondrial function, as these toxicants triggered a cascade of metabolic shifts that were marked by changes in KTR. Overall, these findings establish a foundation for using KTR as a marker of disruptions in tryptophan metabolism and metabolic disturbances in energy homeostasis, critical for adaptive responses to environmental stress.

Importantly, this cellular framework of assessments, developed in a mammalian *in vitro* model, was extended to *in vivo* environmental-exposure assessments to validate the ecological relevance of KTR alterations in the context of biomonitoring. KTR was correlated with known markers of environmental stress, and PAC exposure, such as CYP1 enzyme activity (measured by EROD activity), in local fish species from the AOSR. I demonstrated that KTR can be used to track species-specific responses to location, which captures the complex influences of environmental and ecological factors such as potential exposure to contaminants, predation pressure, water flow, temperature, and anthropogenic or industrial activity. Notably, longnose suckers (*Catostomus catostomus*) and white suckers (*Catostomus commersonii*) showed differing responses, with KTR displaying an inverse relationship with EROD activity in longnose suckers and a direct relationship in white suckers. This species-dependent variability highlights the importance of tailoring biomonitoring approaches to individual species. These results suggest that KTR could serve

as a valuable biomarker for environmental monitoring, although species-specific calibration may be necessary for broader application.

Overall, this thesis provides foundational knowledge in 1) the pathways affected by exposure to complex mixtures, as marked by KTR, 2) the mechanisms through which mammalian KTR is altered in response to such mixtures, and 3) the utility of KTR as a reliable and species-sensitive marker for detecting adaptive changes to environmental stressors. However, while these findings significantly advance our understanding of KTR as a biomarker, several limitations must be acknowledged. These include the use of a single mammalian cell line for the initial mechanistic studies, which may not fully capture systemic responses; the inherent complexity of the mixtures tested, which poses challenges in pinpointing the specific drivers of observed effects; and the variability in environmental conditions when extending the framework to fish.

In the following sections, several key areas will be explored: alternative mechanisms of action observed through metabolic reprogramming and energy homeostasis in response to these toxicants, the complexity of environmental mixtures and its implications for toxicity studies, and the value of *in vivo* validation and cross-species comparisons for monitoring environmental stress. Future studies should build on these findings to further refine the use of KTR as a tool for biomonitoring and policy development in toxicant exposures and environmental stress.

6.2 Alternative Receptor Pathways and Ligand Binding in Response to Environmental Mixtures

A key challenge in understanding the broader implications of this thesis lies in the inherent complexity of environmental mixtures. The organic constituents in Oil Sands Process-Affected Water (OSPW) and related contaminants are complex, non-uniform mixtures that evolve and vary both spatially and temporally [1,2]. This complexity demands a more nuanced approach to studying their biological effects.

Metabolic reprogramming was identified as a significant adaptive response to NAFCs, a complex component of OSPW (Chapter 3: NAFCs). Disruptions in glucose and fatty acid uptake, coupled with altered mitochondrial function, suggest that exposure to these acid-extractable organics directly impacts cellular energy homeostasis. As the central hub of energy production, mitochondria are particularly vulnerable to metabolic disruptions [3]. The kynurenine-tryptophan ratio plays a crucial role in mitochondrial health and energy metabolism, as it is intricately linked to NAD+ biosynthesis–a molecule essential for energy regulation. NAD+ biosynthesis is synthesized via five key precursors/intermediates: tryptophan, nicotinamide, nicotinic acid, nicotinamide riboside, and nicotinamide mononucleotide. *De novo* NAD+ synthesis occurs through the conversion of tryptophan to nicotinic acid mononucleotide (NaMN), via the kynurenine pathway across all vertebrates [4]. In fact, the TRP-KYN pathway was historically referred to as the “tryptophan-NAD+ pathway” after NAD+ was discovered in 1906, with its relationship to tryptophan metabolism gaining recognition in the 1960s and 1970s.

NAD+ functions as a cofactor in multiple metabolic processes, including glycolysis, the tricarboxylic acid cycle, and fatty acid oxidation [5,6]. In its reduced form (NADH), it donates electrons for ATP production through mitochondrial oxidative phosphorylation. Beyond energy metabolism, NAD+ also acts as a co-substrate for enzymes involved in DNA repair (such as poly(ADP-ribose) polymerases), epigenetic regulation (through sirtuins), and stress resistance. Disruptions in NAD+ dynamics and availability due to cellular stress can rewire biological processes *via* post-synthesis modifications of DNA, RNA, and proteins (Reviewed In:[3,5]). Importantly, this metabolic reprogramming can be marked by changes in NAD+, which can then be reflected in KTR. While we did not directly measure NAD+ levels in our study, the observed alterations in KTR, along with the switch from glucose to fatty acid oxidation substrate usage, changes in mitochondrial membrane potential, and increased ATP levels, strongly suggest that disruptions in NAD+ metabolism are occurring. These shifts in energy substrate preference and mitochondrial function are hallmarks of metabolic reprogramming, likely driven by altered NAD+ biosynthesis through the kynurenine pathway.

The kynurenine pathway, and consequently NAD+ biosynthesis, is initiated by the conversion of TRP to N-formylkynurenine, catalyzed by the rate-limiting enzymes: indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). These enzymes are often overexpressed in pathological conditions, contributing to an increase in kynurenine metabolites, which serve as secondary messengers in signaling pathways regulating adaptive stress responses, immune tolerance, and energy homeostasis [7–10]. In the context of environmental exposures, both exogenous chemical contaminants and endogenous KYN metabolites, produced in response to contaminant exposures, can bind to and activate various receptor pathways including aryl hydrocarbon receptors (AhRs) and peroxisome proliferator-activated receptors (PPARs), which were investigated in this thesis.

AhR activation is the first line of investigation when assessing compounds containing polycyclic aromatic compounds, as it plays a critical role in sensing xenobiotics and regulating the expression of genes involved in their metabolism, including drug transporters and cytochrome P450s (CYPs) [11–13]. I demonstrated that NAFC, but not BitWAF altered *Cyp1a1* and *Cyp1b1* mRNA expression, indicating AhR activation. In the case of NAFC, this activation may occur directly, through AhR ligands present in these complex mixtures, or indirectly, via downstream KYN metabolites that also act as AhR ligands [14].

Several tryptophan metabolites, such as kynurenine, kynurenic acid, indoxyl sulfate, indolepropionate and xanthurenate, have been identified as weak AhR ligands, contributing to basal endogenous AhR activation [11]. Granados and colleagues further demonstrated that AhR knockout (Ahr-/-) mice exhibited significant metabolic alterations, notably in fatty acid metabolism, the tricarboxylic acid cycle, and tryptophan metabolism, mirroring some of the metabolic changes we observed following NAFC (Chapter 3) and BitWAF

exposure (Supplemental Figure 2). Moreover, chemoinformatic analyses of AhR-/- mice suggest that AhR influences the hydrophobicity and transport of metabolites, in which Granados *et al*. observed enhanced export of carnitine-bound fatty acids and tryptophan metabolites, including L-kynurenine and glutaryl-CoA. Taking their study and the results from this thesis together, it is clear that AhR activation plays a pivotal role in modulating metabolic pathways, particularly tryptophan metabolism and mitochondrial fatty acid oxidation, in response to environmental exposures. As such, there is a critical need to further investigate these metabolic pathways and their associated signaling responses to different AhR ligands, particularly in the context of complex environmental mixtures, to better understand their role in mediating metabolic and toxicological outcomes.

Moreover, it is important to consider that complex mixtures, such as BitWAF may contain both AhR agonists and antagonists, and that the presence of antagonists could inhibit or modulated receptor activation, potentially contributing to the lack of AhR-mediated response observed in an otherwise expected PAC-mixture. When modelling bitumen-influenced waters, we used the CROSERF BitWAF preparation protocol and expected that the resulting mixture contained a significant concentration of PACs (Supplemental Figure 1), along with other organic constituents such as aliphatic hydrocarbons, sulfur- and nitrogen-containing heterocyclic compounds, and dissolved organics. These complex mixtures are known to activate receptor pathways, particularly AhR, leading to the induction of *Cyp1a1, Cyp1b1,* or *Tiparp*. However, variability in the biological response to BitWAF (Chapter 2), including the inconsistent correlation between KTR and *Cyp1a1* expression, suggests the involvement of (1) differing chemical composition or (2) non-canonical pathways in KTR regulation.

It is important to consider that complex mixtures, such as BitWAF may contain both AhR agonists and antagonists, and that the presence of antagonists could inhibit or modulated receptor activation, potentially contributing to the lack of AhR-mediated response observed in an otherwise expected PAC-mixture. Moreover, the widely used CROSERF protocol introduces challenges during preparation, including the loss of volatile and insoluble constituents [15]. As a result, critical components, such as certain PAHs or other exogenous AhR ligands, may be underrepresented or entirely absent in the final test solution. The selective solubility of individual constituents is further influenced by preparation conditions, as even minor variations in technique can lead to significant changes in the WAF composition compared to the original bitumen/oil/fuel used [15]. These challenges reduce the fidelity of WAF as a proxy for real-world mixtures and introduce variability in chemical exposure within test systems. This is particularly relevant in cell-line studies, where WAF testing was conducted for the first time in this study, unlike the extensive validation work done using loading rates in aquatic models such as algae, crustaceans, mollusks, and fish (Reviewed In: [15]).

Beyond the challenges associated with WAF preparation and the potential loss of AhR ligands, it is also important to consider the role of non-canonical pathways in regulating KTR and other observed biological responses. These non-canonical pathways could include

activation of alternative receptors, such as glucocorticoid receptors (GRs) or PPARs, which are known to modulate metabolic and immune responses. PPARα and PPARγ are involved in lipid metabolism and mitochondrial function, while GRs modulate glucose and lipid metabolism during stress responses [16–20]. In Chapter 4, we demonstrated that PPARα transcriptionally regulates *Tdo2*, linking PPARα activation to KTR modulation and energy metabolism. PPARα's role in regulating fatty acid oxidation and mitochondrial function—via genes such as *Cpt1a*, *Cd36*, and *Angptl4*— suggests that disruptions in KTR could alter receptor signaling and downstream metabolic pathways. Additionally, GRs are key regulators of stress responses and energy metabolism [21,22]. Dexamethasone, a synthetic glucocorticoid, has been shown to induce *Tdo2* expression (Supplemental Figure 3), suggesting a connection between environmental stress, glucocorticoid signaling, and TRP metabolism. Additionally, the crosstalk between AhR, PPARs, and GRs under stress conditions may influence how KTR responds to environmental contaminants, with downstream effects on energy production, immune regulation, and metabolic adaptation.

There are additional receptor pathways beyond those examined in this thesis that could play a significant role in KTR regulation and environmental adaptive response. For example, nuclear receptors such as the pregnane X receptor (PXR), constitutive androstane receptor (CAR), and liver X receptor (LXR) are all involved in the regulation of xenobiotic metabolism, lipid homeostasis, and inflammatory responses (Reviewed In: [13,23,24]). While these pathways were not directly investigated in this thesis, they hold potential as key regulators of biotransformation, metabolism, and drug/xenobiotic/metabolite transport. Importantly, given their roles in modulating gene expression in response to lipophilic compounds, these xenosensitive receptors, may also play crucial roles in the response of cells to environmental contaminants. PXR and CAR, in particular, play crucial roles in the metabolism and clearance of xenobiotics, potentially influencing the TRP-KYN pathway in response to environmental contaminants [23]. LXR, involved in cholesterol and lipid metabolism, may also modulate immune and inflammatory responses under environmental stress [25]. Together, these nuclear receptors provide additional regulatory complexity and could influence KTR, making them valuable targets for future studies on the metabolic effects of environmental exposures.

Recent progress in *in silico* studies has provided insights into Toll-like receptor (TLR) binding to polycyclic aromatic hydrocarbons, particularly in the context of cancer development [26–28]. TLRs, with their broad spectrum of pathogen recognition, initiate signaling pathways that are highly conserved across a wide range of invertebrate and vertebrate species [29] including mammals [30], fish [31] and birds [32,33]. In humans, TLRs (2, 3, 4, 7/8, 9) have been found to modulate the TRP-KYN pathway, resulting in increased kynurenic and quinolinic acids in peripheral monocytes [34]. Given that TLRs are known to influence KTR in response to infection and inflammation, it is plausible that they could also be activated by environmental compound exposures such as BitWAF and NAFC. Exploring these alternative receptor pathways—PXR, CAR, LXR, and TLRs—will provide a more comprehensive understanding of how environmental exposures affect KTR and related metabolic pathways, with future research needed to fully map the regulatory

mechanisms and networks that modulate the TRP-KYN pathway in response to xenobiotics and environmental stressors.

6.3 Complexity of Environmental Mixtures

One of the primary challenges in assessing the biological effects of environmental contaminants is the inherent complexity of mixtures present in ecosystems such as the AOSR. Industrial activities in the region produce effluents that contain diverse mixtures of compounds, such as PACs, NAFCs, volatile organic compounds, metals, ions, and BTEX (benzene, toluene, ethylbenzene, and xylenes) [35]. The chemical composition of bitumen and OSPW varies significantly depending on source, producer, extraction methods, and spatial and temporal conditions within tailings ponds [1]. Moreover, these mixtures are not static; environmental factors such as weathering and microbial degradation, and time alter their chemical profiles, further complicating predictions about their effects on biological systems. This dynamic nature of environmental mixtures makes it difficult to predict and assess their bioavailability and toxicity. As such, single-compound studies are valuable for characterizing the toxicity of AOSR contaminants, often focusing on the most abundant compounds and those that are considered *key drivers* of toxicity. However, they overlook how these compounds interact within the full mixture, potentially missing synergistic or antagonistic effects. The variability and chemical diversity of these mixtures create challenges in assessing their impact on various biological systems, including organ-specific responses and species differences over time (acute vs. sub-chronic vs. chronic exposure).

In this thesis, the use of prototypical-positive controls, such as benzo[a]pyrene for AhR activation and dexamethasone, a GR agonist, for *Tdo2* induction, served as a validation framework for the experiments. These controls helped isolate specific pathways and responses, providing insights into how environmentally relevant PAH mixtures can affect cellular responses linking back to the TRP-KYN pathways. Using well-characterized controls, such as compounds that serve as agonists or antagonists for specific pathways of interest, we can better isolate the contributions of individual components within these mixtures. For instance, parent PACs commonly found in environmental mixtures or compounds present at the highest concentrations in the mixture being studied could serve as reference compounds that should be added to the experimental set up. These controls help to 1) validate pathway-specific responses and allow for clearer differentiation between the effects of individual components and the combined effect of the full mixture, and 2) pinpoint individual chemical drivers and their contribution to the observed biological responses.

Importantly, for complex mixtures like BitWAF, NAFCs, OSPW, or water effluents, the effectiveness of this approach relies heavily on comprehensive chemical fingerprinting of these mixtures. To determine which controls or compounds to assess, it is essential to evaluate the mixture in its entirety. PACs are often the primary focus of chemical assessments, and this is largely driven by regulatory frameworks, specific compounds of interest, and technical limitations. Screening for all potential compounds in a mixture is both expensive and labor-intensive, so many laboratories prioritize the 16 priority PAHs,

BDEs, or PCBs, depending on regulatory guidelines and current environmental mandates. However, for long-term environmental monitoring, it is essential to establish standardized approaches to expand chemical analyses to a broader range of contaminants, so that we can identify all *active* constituents.

In 2017, Nie and colleagues used Py-GC-MS technique to investigate thermal cracking fractions of oil sands bitumen and found that by changing reaction temperatures, they could extract more heteroatomic compounds, naphthalenes, and indoles [36]. Notably, indoles and indole derivatives, which can directly influence KTR by binding to IDO, were previously overlooked in standard chemical assessments. This is a critical gap in our understanding of and exploration of environmental mixtures, as traditional screening methods only capture a limited set of pre-selected compounds. Without a comprehensive analysis that includes indoles (and other not-yet identified compounds), our ability to make accurate hypotheses about mechanisms of action—such as KTR modulation—is constrained. Therefore, expanding our chemical assessments of mixture constituents would allow for a more accurate understanding of how complex environmental mixtures affect biological pathways and systems, and more comprehensive cross-comparisons between different mixtures and their sources.

This broader knowledge of chemical constituents not only improves our understanding of exposure risks but also opens new avenues for investigating their impact on various biological pathways including enzymatic activity. Given that TDO's enzymatic binding pocket is specific to tryptophan while IDO binds more broadly to indole-containing compounds [37]—including downstream kynurenine metabolites and exogenous indole compounds—there is significant potential to uncover novel KTR modulators present in oil sands-related mixtures. Moreover, these indole derivatives, both natural and synthetic, have been shown to regulate glucose homeostasis and lipid metabolism (Reviewed In: [38]), processes that were altered in BitWAF and NAFC exposure. While this thesis has focused primarily on PACs and acid-extractable organics, we have not yet identified or investigated the indole components present in bitumen from the AOSR. However, these analyses would be crucial to understanding the mechanisms of action of these complex mixtures on the TRP-KYN pathway and, ultimately, KTR. The differential binding affinities and functions of TDO and IDO in response to environmental mixtures remain an underexplored area that could provide valuable insights into how xenobiotics influence tryptophan metabolism, energy balance, and immune responses through interactions with the pathway's rate-limiting heme-enzymes.

Recently, *in silico* ligand-binding approaches have enabled efficient screening of environmental mixtures, assessing how their individual components bind and interact with receptors [26]. These computational methods are valuable for identifying potential health risks and understanding both receptor-ligand and enzyme-ligand interactions. As different ligands—including indoles and KYN metabolites created in response to exposures—bind to IDO, *in silico* approaches offer a powerful tool to predict their effects on the TRP-KYN pathway. This provides a foundation for enzymatic assessments, where *in silico* predictions

can guide experimental validation of ligand-protein interactions. Importantly, this type of assessment relies heavily on detailed chemical composition, the chemical structures of individual compounds, and data from existing protein-ligand complexes, which can be obtained from freely available databases, such as the Protein Data Bank [39].

NAFCs pose a challenge in this type of assessment due to their complex carboxylic acid composition. The structural diversity of these carboxylic acids makes it difficult to fully separate and analyze them with currently available technology, limiting our ability to assess individual components of NAFCs [40]. However, in this case, forensic comparison of NAFCs can still provide valuable insights. By comparing NAFCs extracted from different sources—such as commercial versus OSPW-derived NAFCs or aged versus fresh samples [1,41]—we can better understand how their composition changes over time and between sources. These comparisons can help identify key compounds driving toxicity and provide insights into the environmental persistence and degradation patterns of NAFCs. With NAFCs, comparisons with other mixture assessments, can also help refine our understanding of composition. Techniques like whole water equivalence solid phase extraction (ENV+ and Soxhlet) have been designed to extract organics from seepage water into fractions which can then be further chemically characterized by polarity separating out NAFC-like mixtures from oxygenated compounds, from poly-oxygenated NAs [42–45]. As such, the technical ability to fractionate and characterize mixtures is crucial for our understanding of biological effects.

Together, applying multi-factorial, *in silico*, and forensic chemical fingerprinting approaches to environmental mixtures would build a strong foundation for targeted experimental research by identifying key ligands and their potential effects on metabolic and signaling pathways. Importantly, this thesis demonstrated that, despite the potential complexity of interactions between environmental pollutants and cellular pathways, the outcome—altered KTR—remains consistent in both mammalian *in vitro* models and fish exposed to industry-influence and natural-deposits of bitumen.

6.4 Strategies for Assessing Environmental Toxicity: *In Vitro* Advances, *In Vivo* Insights, and Emerging Biological Matrices

Advancing in vitro *models: the role of 3D culture and co-culture systems*

In this thesis, all assessments were conducted using traditional two-dimensional (2D) cell cultures. While 2D models are widely accepted and commonly used in toxicological studies, they have inherent limitations in replicating the complex cellular architecture and interactions of *in vivo* environments. The constrained flat monolayer structure of 2D models restricts cellular interactions, metabolic gradients, and signaling pathways, reducing the model's ability to capture subtle, dose-dependent responses to environmental toxicants, particularly at lower concentrations (Reviewed in: [46–48]). As such, 2D models provide a useful foundation, they do not fully account for the complexity of cellular behavior in more physiologically relevant environments.

Given these limitations, future studies should consider incorporating three-dimensional (3D) culture systems, which offer a more physiological environment that mimics the spatial organization and intercellular signaling present in tissues. Recently, I demonstrated 3D spheroid cultures are more sensitive and exhibit more pronounced gene expression changes, particularly in response to complex mixtures like NAFCs [48]. Indeed, we found that 3D McA-RH7777 spheroids demonstrate enhanced sensitivity and more uniform dose–response patterns in gene expression related to xenobiotic metabolism (AhR and PPARs) for both single compounds and complex mixtures. Specifically, 3D cultures showed varying degrees of sensitivity and resistance to the apoptotic effects induced by NAFCs, suggesting that the structured 3D environment may offer protection against toxicant-induced apoptosis [46]. This protective effect may be influenced by the specific methods used to culture 3D spheroids and holds significant potential for optimizing culture conditions to more accurately reflect *in vivo* responses [49,50]. Additionally, advancing these models further through the integration of co-culture systems, which combine multiple cell types, would provide an even more comprehensive approach to studying the crosstalk between different cellular populations in response to toxicants. Future *in vitro* validation of KTR as a toxicology marker and to assess its mechanisms of action, co-culturing hepatocytes with immune, endothelial, or adipose cells would provide deeper insights into how environmental toxicants influence both metabolic and immune signaling pathways. This approach could further establish KTR's role as a key indicator of cellular stress and system-wide toxicological responses, helping to better investigate the broader impacts of environmental exposures.

Going forward, multi-organ systems, such as organ-on-a-chip technologies, represent an even more advanced approach [51]. These systems allow for the study of toxicant effects across different tissues and organs simultaneously, replicating the systemic interactions present in living organisms. Incorporating 3D, co-culture, and multi-organ systems in future environmental toxicology research could provide more accurate and predictive models for assessing the health risks associated with environmental exposures.

In Vivo *Assessments: Field-based studies and laboratory comparisons*

Despite the growing importance of *in vitro* models [52–54], *in vivo* assessments remain essential for understanding the complex biological responses to environmental toxicants. Although this thesis did not include traditional *in vivo* animal models, field-based environmental exposure studies provide a relevant assessment of the impact of industrial activity and pollutants on wildlife. The fish collection studies conducted in this thesis offer critical insights into KTR validation [55] and how environmental stressors affect holistic biological responses across species. Field-based work allows for real-world assessments that capture the complexity of natural ecosystems, including the interactions between species, pollutants, and their environments.

Longnose suckers (*Catostomus catostomus*) and white suckers (*Catostomus commersonii*) were collected from multiple locations across a gradient of exposure which included two reference locations outside of the oil sands deposit, one deposit site upstream of industrial

development, and two or three sites within the oil sands development region (Chapter 5). These field assessments revealed species-specific differences in how the fish responded to changes in their environments, particularly in relation to KTR and EROD activity, once again linking changes in KTR to PAC exposure. Interestingly, longnose suckers exhibited an inverse relationship between KTR and EROD activity, while white suckers showed a directly proportional relationship. These findings suggest that the two species metabolize tryptophan and respond to environmental stressors differently, possibly due to differences in habitat requirements, physiological adaptations, or environmental preferences.

Importantly, this species-specific variability highlights the importance of conducting *in vivo* assessments in natural and environmentally controlled settings, as they provide contextual insights that controlled laboratory environments alone may not capture. However, it is also important to recognize the limitations of field-based work, as they cannot isolate the specific effects of environmental exposure, making it challenging to tease apart factors like environmental adaptations, contaminants, temperature, and water levels.

Conducting *in vivo* studies on complex mixtures like OSPW, BitWAF, and NAFCs presents additional challenges, particularly when determining environmentally relevant concentrations in aquatic ecosystems. Adjusting for water equivalences is difficult, as tailings ponds are typically equipped with deterrents to prevent animals from entering them directly. However, indirect exposure pathways still exist. Birds may land on contaminated waters, and mammals may consume fish downstream of the affected areas, introducing ecosystem and food-web effects. This complexity of exposure scenarios in natural environments, where contamination can propagate through various trophic levels and across ecosystems is difficult to study in traditional *in vivo* experiments. In our study, we investigated fish upstream and downstream of oil sands deposits, providing a preliminary assessment of contaminant exposure at lower trophic levels. While these assessments offer insight into the bioavailability of pollutants within the aquatic ecosystem, it is crucial to continue exploring the impacts of such contaminants on higher trophic levels, including mammals and birds, that may be exposed through consumption of contaminated prey. Understanding how pollutants bioaccumulate and biomagnify through the food web will provide a more comprehensive understanding of ecosystem-wide impacts.

Alternative Biological Matrices

Commonly used biological matrices in environmental toxicology studies include blood, tissue samples, and feathers (Reviewed In: [56–59]). Each of these matrices offers valuable insights but also comes with limitations. Blood is frequently used because it provides real-time data on the organism's physiological state, yet it can be invasive and offers only a snapshot of exposure. Tissue samples (liver, muscle) are effective for assessing contaminant accumulation, but they are lethal, making them unsuitable for long-term monitoring or endangered species. Feathers are often used for non-invasive sampling, but they may not always provide an accurate representation of the temporal exposure due to molting cycles and other biological factors.

Furthermore, the selection of sentinel species is critical when developing a biomonitoring strategy using wildlife, particularly for species that forage across multiple trophic levels, such as those feeding on both aquatic and terrestrial food webs [60,61]. Birds are excellent indicators of local environmental quality due to their unique biology, foraging habits, and physiology [60,62]. As such, our next steps include a pilot study using eggs of Red-throated Loons (*Gavia stellata*), to determine if changes in KTR in eggs can be an alternative biological matrix for assessing long-term exposure to environmental pollutants.

Homogenized bird eggs present an ideal matrix for environmental toxicology studies due to several key factors. Firstly, they are non-invasive, as eggs can be collected without harming the adult bird or disrupting its natural behaviors. Logistically, egg collection is relatively easy in the field, reducing the need for resource-intensive, specialized equipment, or specially trained personnel, often associated with wildlife studies. More importantly, eggs represent an integrated exposure over time, reflecting both maternal and environmental conditions during the period of yolk formation, encapsulating the contaminants and stress biomarkers the mother has been exposed to over an extended period [56]. This makes them a comprehensive matrix for detecting environmental toxins, offering insights into both immediate and cumulative long-term exposures. By analyzing homogenized eggs, researchers can detect a wide range of contaminants, including persistent organic pollutants, heavy metals, and endocrine-disrupting chemicals, which may be transferred from the mother to the offspring [56]. Together, screening contaminant levels and stress biomarkers of exposure, including KTR, this type of sampling will be especially useful for assessment of the health of bird populations in contaminated regions like the AOSR, where birds may land on or feed near polluted water sources.

Similar to bird eggs, fecal samples are another valuable non-invasive biological matrix [57,63,64]. Feces provide a snapshot of recent dietary intake and contaminant exposure, making them a practical and ethical choice for assessing environmental pollutants. Fecal samples can offer insights into the metabolic and physiological state of animals without the need for invasive sampling, and they are often used in conjunction with other matrices to provide a broader picture of exposure and health impacts in wildlife populations [57]. However, in the context of KTR, fecal samples pose a challenge. Any changes in KTR observed in feces may be influenced by gut tryptophan metabolites produced by the microbiota or by host metabolism [65]. Currently, we lack the methods to differentiate between these sources, making it difficult to interpret KTR results from fecal samples accurately. While fecal samples can still provide valuable information about recent dietary intake and exposure to environmental contaminants, they are less reliable for assessing KTR due to the difficulty of attributing changes in KTR to host health vs those related to altered microbiome function. That said, we are currently validating approaches to differentiate between microbiota- and host-derived metabolites, which could make fecal samples a more viable matrix for KTR assessment in the future.

6.5 KTR as a biomonitoring marker for forensic analysis and policy

This thesis demonstrates the potential of the kynurenine-to-tryptophan ratio to be utilized in biomonitoring and forensic analysis, particularly in regions affected by industrial activity. KTR has emerged as a promising biomarker for detecting environmental exposure, as it reflects metabolic alterations in *in vitro* and *in vivo* systems exposed to complex mixtures of pollutants found is waste-waters and exposed waters in the AOSR. By integrating KTR into biomonitoring frameworks, it is possible to move beyond acute lethality endpoints and focus on more sensitive, early indicators of stress, which are critical for understanding long-term ecological impacts. However, while KTR demonstrates significant promise, further validation is necessary across a wider range of species and biological matrices to fully establish its utility. As discussed, different species may respond to environmental stressors in varying ways, signaling pathways of effects remain to be determined, and matrices like feces, tissues, or homogenized eggs offer different windows into exposure and biological response. Expanding KTR assessments across these matrices and species will be essential for confirming its robustness as a biomarker in diverse ecological contexts.

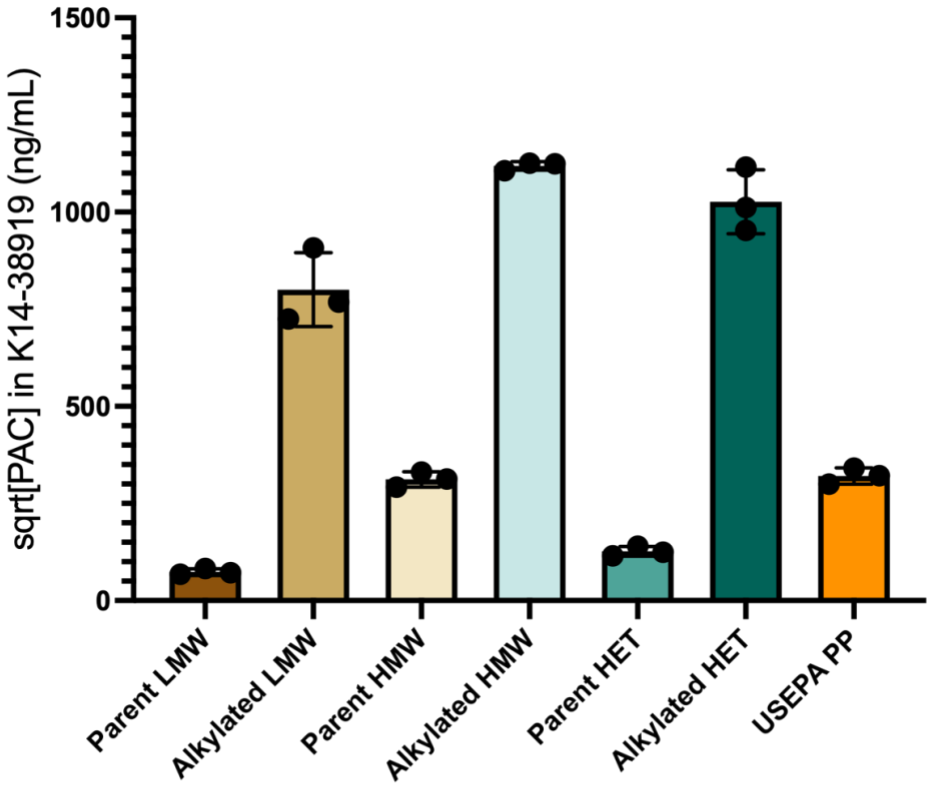
In regions like the AOSR, where industrial development is closely intertwined with natural ecosystems, forensic analysis using KTR could help distinguish between natural and industrial sources of pollution. The ability to detect elevated KTR levels in wildlife or environmental samples could be used as a trigger for forensic investigation to determine whether contaminants are stemming from OSPW or natural bitumen deposits. These investigations would contribute to environmental sustainability by providing critical data to better understand the impacts of industrial activity and effluent storage/usage, inform management practices, and protect ecosystems, wildlife, and human health.

By offering a biomarker that is sensitive to sub-lethal, chronic exposures, this research contributes to improving environmental protection efforts. Unlike traditional endpoints that often focus on mortality or overt toxicity, KTR offers a more nuanced tool for detecting early biological effects, which can inform timely interventions before large-scale damage occurs. While more species-specific and matrix-specific validation is required, KTR has the potential to be a valuable addition to environmental monitoring programs, enabling a more comprehensive and preventive assessment of ecosystem health, and in doing so, would allow for new and updated guidelines for permissible exposure limits. Moreover, identifying sources of contamination through forensic analyses could drive the development of policies aimed at more stringent industrial practices, improving both environmental and human health outcomes.

Together, my work aligns with a broader shift in environmental toxicology towards using sensitive, non-lethal endpoints for long-term ecological monitoring. While the long-term goal is to fully validate KTR as a key biomarker across species and matrices, this research is foundational in demonstrating its potential to detect early biological changes that could inform more effective management of industrial pollutants. KTR has the potential to serve as a biomarker that not only identifies elevated contaminant exposure but also contributes

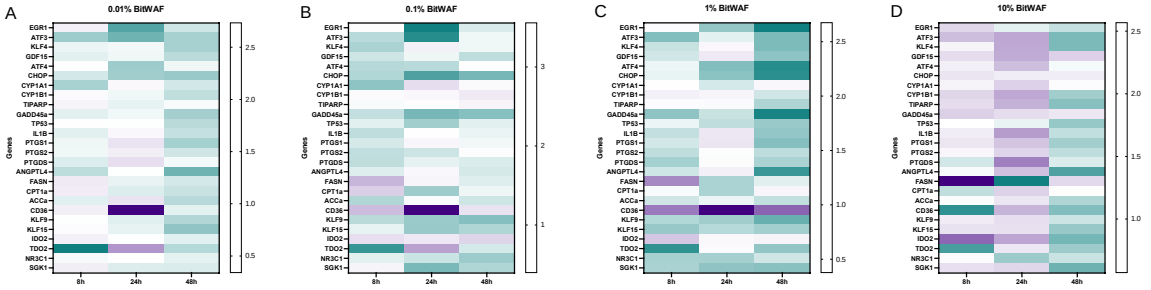
to the early identification of ecological risks, guiding both scientific and policy responses to environmental threats.

6.6 Supplemental

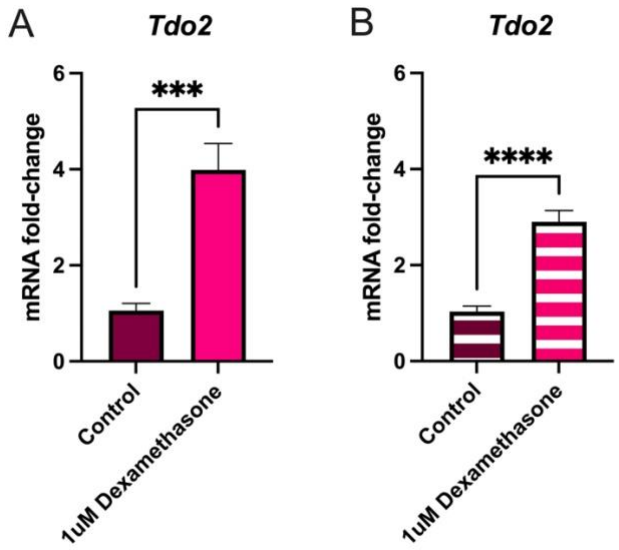


	Rep 1	Rep 2	Rep 3	Mean	Std.Dev
Parent LMW	6812.05	4585.57	5142.67	5513.43	1158.62
Alkylated LMW	823537.85	525751.58	590735.09	646674.84	156576.20
Parent HMW	109507.34	85111.90	98064.66	97561.30	12205.50
Alkylated HMW	1223667.73	1263349.33	1268148.02	1251721.69	24413.64
Parent HET	19581.58	13287.09	15493.76	16120.81	3193.75
Alkylated HET	1244631.59	908331.35	1023265.87	1058742.94	170933.99
USEPA PP	116319.39	89697.47	103207.32	103074.73	13311.45

Supplemental Figure 1. Average Normalized PAC concentrations (ng/mL) in Bitumen K14-38919 in DMSO. The table presents the concentrations of the sum of Parent and alkylated low molecular weight (LMW), high molecular weight (HMW), and heterocyclic aromatic compounds (HET), as well as the USEPA priority pollutants (USEPA PP).



Supplemental Figure 2. Heat map illustrating the mRNA fold change of genes involved in stress response (EGR1, ATF3, KLF4, GDF15, ATF4, CHOP), xenobiotic metabolism (CYP1A1, CYP1B1, TIPARP), DNA damage (GADD45A, TP53), inflammation (IL1B, PTGS1, PTGS2, PTGDS), lipid metabolism (ANGPTL4, FASN, CPT1A, ACCA, CD36), transcription regulation (KLF9, KLF15), and tryptophan metabolism (IDO, TDO2), as well as glucocorticoid response (NR3C1, SGK1), following BitWAF exposure at three time points (8, 24, 48 hours) and across four doses. Color intensity corresponds to fold changes, with higher expressions in red and lower in blue, highlighting the temporal and dose-dependent regulatory effects of BitWAF.



Supplemental Figure 3. mRNA fold change of TDO2 following treatment with dexamethasone at 24 hours (A) and 48 hours (B).

6.7 References

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