Assessing nutrient toxicity in fathead minnows using a multi-omics approach.

by

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Abstract

The rising demand for nutritious and low-fat foods has made aquaculture one of the fastest-growing food industries worldwide. Inland finfish aquaculture operations produce wastewater high in phosphorus from uneaten feed and ammonia from nitrogenous excretions. Northern wild rice (*Zizania palustris)*, a nutritious and valued wetland crop, benefits from large quantities of phosphorus and ammonia-based nitrogen. Applying aquaculture wastewater (AWW) as a fertilizer for wild rice paddies presents a sustainable system for the recycling of nutrient rich wastewater while supporting optimal plant growth. Yet, introducing uncharacterized wastewater to an ecosystem presents substantial ecological risks. This includes the addition of ammonia, which is toxic at low concentrations, and phosphorus, which can contribute to eutrophication resulting in oxygen depletion within aquatic ecosystems. In Chapter 2, we assessed the ecotoxicological effects of adding aquaculture wastewater to an aquatic ecosystem using targeted transcriptomics with liver tissues of adult fathead minnows (FHM; *Pimephales promelas*) chronically exposed to incremental additions of AWW in simulated wild rice paddy mesocosms (n = 16). In Chapter 3, we assessed the effects of chronic nitrogen and phosphorus exposures, at concentrations relevant to AWW applications, on the health and cellular stress response of FHMs using non-targeted proteomics on mucus and liver tissues from FHMs exposed for 30 days in the laboratory. The objective was to determine if adequate nutrient loadings would adversely impact fishes in the ecosystem. We found that increasing quantities of AWW and nutrients did not cause lethality or affect morphometric endpoints, however oxidative stress, immunological, and metabolic responses were observed in both transcriptomic and proteomic evaluations of liver tissues. Mucus proteins were not effective in detecting cellular stress responses between treatments, however temporal trends were observed. Environmentally-relevant AWW and

nutrient exposures were sufficient to elicit cellular stress responses, however, this concentration would likely not be great enough to impair the health and survival of FHMs. This study may aid in understanding the ecotoxicity of aquaculture wastewater effluent and may further develop the use of mucus in omic analyses to limit lethal sampling in biological and ecotoxicological research.

Dedication

This thesis is dedicated to my dog Merry, who provided me with the support, love, inspiration, and motivation I needed to write this thesis. Merry passed away while I was writing the final chapter. I'm not sure how I would have gotten through it without his loving cuddles, rounds of fetch, and walks to break up the workday. When I look back on writing this thesis, I will remember the short, but special time I had with my best friend.

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Chapter 1: Introduction and Background

1.1 Introduction

As human populations continue to grow and the demand for nutritious food intensifies, food production industries must grow rapidly to meet food security challenges. In 2009, the United Nations stated that food production must double by 2050 to meet the demands of the exponentially growing population, and with that innovative strategies are required to accomplish this task (United Nations, 2009). A reduction in wild-caught fish and a rising demand for fish and seafood products has resulted in aquaculture becoming the fastest growing food production industry globally (Osterblom, 2016). Aquaculture is defined as the farming of aquatic organisms such as algae, and animals including finfish, molluscs, and crustaceans, occurring in either fresh or saltwater (Osterblom, 2016). In 2018, the United Nations' Food and Agriculture Organization (FAO) found that finfish farming exceeded other methods of aquatic animal production. Within finfish production, inland aquaculture produced the majority of farmed fish, equating to 62.5% of global fish production, generating over 51 million tonnes (FAO, 2020).

Like any animal farming operation, finfish aquaculture generates waste products. Aquaculture wastewater commonly consists of left-over feed, nitrogenous metabolites, and decaying fish (Dauda, 2019). Aquaculture feed is high in protein resulting in solid and dissolved waste from uneaten feed that is high in nitrogen and phosphorus (Dauda, 2019). The main metabolic waste product of finfish is ammonia, constituting 80-90% of the dissolved nitrogen excreted, where urea represents the remaining 10-20% of the dissolved nitrogen production (Bureau, 2004). Importantly, the discharged effluents from inland aquaculture facilities can pollute neighboring water bodies, with ~10% of these facilities discharging effluent without any

treatment (Lalonde et al., 2015). The growth of the aquaculture industry has resulted in various environmental implications, including the spread of diseases, overuse of antibiotics, and eutrophication in receiving environments (Ye et al., 2021, Thomsen et al., 2020).

Like the aquaculture industry, wild rice production has increased in response to growing demand for nutritious food from health-conscious consumers. Wild rice is an aquatic grass, with grains that are low in fat, high in dietary fiber and essential amino acids, making it a desirable food product (Watts and Dronzek, 1981). Wild rice has been a primary food resource to North American Indigenous peoples for thousands of years, holding both cultural and spiritual significance (Moodie, 1991, Raster and Hill, 2017, Oelke and Porter, 2016). Indigenous communities hold a respectful relationship with wild rice in lakes and wetlands to help manage wild rice ecosystems for food production (Ahmed, 2020). Thus, the modern domestication of wild rice has been reliant on traditional knowledge and harvesting practices observed from Indigenous peoples (Moodie, 1991). The domestication of wild rice began in the 1950's with commercial cultivation beginning in the early 1990s (Oelke and Porter, 2016). Since the start of commercial production, the wild rice industry holds vast economic implications (Agricultural Utilization Research Institute, 2022). The majority of wild rice cultivation now is commercially grown in flooded paddy fields in Minnesota and California, with most of the historical and traditional habitats of wild rice in lakes and wetlands now lost in response to anthropogenic activities (Oelke and Porter 2016; Ahmed, 2020).

Wild rice requires large quantities of nitrogen and phosphorus to successfully obtain high crop yields (Oelke et al., 1982). Nitrogen is the limiting factor for inflorescences, seed quantity, and seed mass in wild rice. Phosphorus is also beneficial to increase the number of seedlings per plant in conjunction with nitrogen (Sims et al., 2012). Further, ammonia-based nitrogen

fertilizers are preferred due to their stability in flooded soils, and to prevent dissipation into nitrogen gas via denitrification (Oelke et al., 1982). In addition to wild rice's ability to assimilate nutrients for growth, research suggests that wetland plants, such as wild rice, can be used as a tool to remediate ecosystems polluted with wastewater effluents (Tepe and Temel, 2018; Abed et al., 2017). The rhizosphere of wetland plants provides a zone for microbial colonization, stimulating the degradation of contaminants (Bhatia and Goyal, 2013). In Northern wild rice (*Zizania palustris*), the root epidermal cells form iron plaques caused by oxidation of iron in the sediment and microbial activity (Jorgenson et al., 2013). These plaques can sequester phosphates by enhancing uptake and diffusion of phosphorus into plant roots (Xu et al., 2009). The ability of wild rice to act as a nitrogen and phosphorus sink and exhibit particular success with ammonia-based fertilizers makes aquaculture wastewater an ideal candidate for a sustainable fertilizing alternative to conventional fertilizers.

There exists a potential to couple inland finfish aquaculture systems to wild rice cultivation in a circular economy. By implementing a dual fertilizing and bioremediation step of aquaculture wastewater effluents, phosphorus and nitrogen may be sequestered, minimizing nutrient inputs into receiving environments whilst benefiting from the production of an economically and nutritionally important crop.

1.2 Composition and toxicity of aquaculture wastewater

1.2.1 Phosphorus

While recycling wastewater as fertilizer presents a viable method of repurposing a nutrient rich effluent, its application to flooded paddy fields poses ecological risks. Specifically, runoff into surrounding water bodies can threaten ecosystem health. Moreover, when the flooded

fields are eventually drained at the end of the growing season to facilitate harvesting, there is a possibility for adverse impacts on the receiving system. Although phosphorus is not inherently toxic to aquatic organisms at environmentally-relevant concentrations, it poses a significant threat to water quality and ecosystem dynamics (Kim, 2013; Correll, 1998). Phosphorus is an essential element to primary production, and therefore the high quantity of phosphorus found in aquaculture wastewater has the potential to create eutrophic conditions in freshwater systems (Schindler et al., 2006). Eutrophication is distinguished by a surplus in growth of algae and macrophytes due to the excess availability of phosphorus (Chislock et al., 2013).

The formation of algal mats and cyanobacteria blooms from excess phosphorus increases the microbial abundance, thereby increasing respiration rates leading to a hypoxic or anoxic water body (Correll, 1998). Low dissolved oxygen threatens aquatic life; fish species vary in their tolerance, but generally mortality is observed at concentrations below 2 mg L^{-1} (Francis-Floyd, 1992). Further, hypoxia has been shown to affect behaviour, energetics, cardiorespiratory and locomotive function in teleost fish (Lefrancois et al., 2005). Hypoxia has the potential to impact reproduction, where male and female carp (*Cyprinus carpio*) showed reduced gonad development, spawning and fertilization success, egg hatching and larval survival (Wu et al., 2003). Eutrophic conditions can result in increased turbidity, introducing visual challenges for fish species such as fathead minnows (*Pimephales promelas*), resulting in decreases in foraging efficiency and predator avoidance (Gray et al., 2014). Degraded water quality and ecosystem health is linked to the abundance of harmful algal blooms (HAB), which may increase in abundance upon increased phosphorus inputs and pose an additional risk to aquatic animals due to the production of toxins (Chislock et al., 2013).

1.2.2 Ammonia and its toxicity to fish

Another key component of aquaculture wastewater is ammonia, which is known to be toxic at nominal concentrations (0.06 mg L^{-1} NH_3) in fathead minnows (Armstrong et al., 2012). Ammonia exists in its unionized (NH_3) and ionized form (NH_4^+); the two forms are in equilibrium in water and the total of both is referred to as total ammonia nitrogen (TAN). Unionized ammonia is considered the principal toxic species, where NH_4^+ is considered significantly less toxic (Thurston,1981). Toxicity of NH_3 arises because it readily diffuses across cellular membranes, whereas NH_4^+ is a larger, charged molecule that does not diffuse freely (Thurston, 1981; Allen and Collinson, 2020). The relative concentrations of unionized and ionized forms of ammonia are largely dependent on environmental factors including pH, temperature, and ionic strength. Increasing pH and temperature of freshwater is found to shift the equilibrium towards unionized ammonia, increasing toxicity (Emmerson, 1975). Higher ionic strength, for example hard water, decreases the concentration of unionized ammonia when pH and temperature are held constant (Emmerson, 1975).

Ammonia both enters and is excreted from fish bodies through the gill epithelium. As a result, increasing concentrations of both internal or external ammonia can impact acute or chronic toxicity (Armstrong et al., 2012). Numerous fish can detoxify internal ammonia through conversion of NH_4^+ and glutamate to glutamine, catalyzed by the enzymatic reactions of glutamine synthetase (Randall and Tsui, 2002). Rainbow trout (*Oncorhynchus mykiss*) upregulate mRNA expression for glutamine synthetase when exposed to elevated environmental ammonia (Wright et al., 2007). However, when external ammonia levels are increased beyond a threshold, the ability of fish to reduce their internal ammonia concentration diminishes, resulting in toxicity (Armstrong et al., 2012). During acute exposure in vertebrates, ammonia acts by

targeting the central nervous system (CNS) causing 'ammonia intoxication' ultimately leading to convulsions and mortality (Randall and Tsui, 2002). The mechanism by which this transpires occurs in the brain through a type of glutamate receptor called N-methyl-D-aspartate (NMDA) which facilitates excitatory transmission through the CNS by mediating ion transport (Chen et al., 2010; Marcaida, 1992). The exact mechanism of ammonia toxicity is not completely understood, nonetheless it is suggested that ammonia triggers over activation of NMDA receptors in a voltage-dependent manner, depleting the brain of ATP, resulting in greater releases of glutamate (Monfort et al., 2002). The depolarization event caused by excess ammonia allows the Mg^{2+} block in the NMDA receptor to be released, thereby allowing an influx of Na^{+} and Ca^{2+} ions (Monfort et al., 2002). The consequence of this influx is activation of calcium-dependent enzymes which cause a cascade of reactions that ultimately lead to cell death (Monfort et al., 2002; Randall and Tsui, 2002). More specifically, increased intracellular Ca^{2+} in the postsynaptic neuron binds to calmodulin, which activates nitric oxide synthetase, leading to increased levels of nitric oxide which activates guanylyl cyclase and finally increases cyclic guanosine monophosphate, which causes cell death (Monfort et al., 2002).

The toxicity of ammonia is dependent on the frequency and duration of exposure. Low concentrations of ammonia may not cause adverse effects or result in mortality of fish within a short exposure period. However, chronic exposure under identical conditions may cause mortality or impairments (Levit, 2010). A one-year study on larval fathead minnows investigating chronic effects of ammonia found that concentrations ≥ 0.37 mg L^{-1} NH_3 (6.43 mg L^{-1} TAN) reduced hatching rate, and concentrations ≥ 0.21 mg L^{-1} NH_3 (3.5 mg L^{-1} TAN) caused brain lesions (Thurston et al., 1986). A more recent study investigating egg production found that 0.06 mg/L NH_3 (5 mg L^{-1} TAN) significantly reduced fecundity by 29% in adult fathead

minnows during a 20-day exposure period (Armstrong et al., 2012). Furthermore, sublethal concentrations of ammonia were found to impair fast-twitch muscles in teleost fish, reducing their ability to escape prey (McKenzie et al., 2009).

Ammonia toxicity in fish is influenced by several factors including exercise, feeding, and stress (Randall and Tsui, 2002). Internal ammonia levels increase following periods of exercise in several species of fish including salmon (*Oncorhynchus nerka)* where migration causes surges of protein catabolism resulting in elevated plasma ammonia (Randall and Tsui, 2002). Further, the LC_{50} of ammonia was significantly higher for salmon at rest compared to swimming, showing that exercise can augment ammonia toxicity (Wicks et al., 2002). Feeding habits may also increase plasma ammonia levels; yet some fish, such as rainbow trout, may have compensatory protective mechanisms from toxicity during feeding (Randall and Tsui, 2002). Although feeding can increase plasma ammonia levels to lethal levels, fish become less sensitive to external ammonia due to the upregulation of glutamine synthetase during feeding (Randall and Tsui, 2002). The result is that ammonia is quickly converted to glutamine, protecting fish from toxic internal concentrations (Wicks and Randall, 2002).

The role of stress in ammonia toxicity is an important and complex factor. Cortisol is the principal corticosteroid released from the adrenal cortex during stress in teleost fish (Mommsen et al., 1999). Plasma cortisol increases dramatically in response to ammonia induced stress, with internal cortisol levels rising between 2-fold and 15-fold (Zeitoun et al., 2016; Sanderson et al., 2010). Increased stress may cause further damage to fish in the presence of ammonia, and cortisol injections are shown to exacerbate ammonia toxicity in teleost fish (Randall and Tsui, 2002). Cortisol also plays an important role in carbohydrate, lipid, and protein metabolism. Therefore, increases in gluconeogenesis, glycogenesis, protein synthesis and catabolism are

possible outcomes of increased plasma cortisol levels (Mommsen et al., 1999; Randall and Tsui, 2002).

Finally, both stress and ammonia exposure have been found to affect reproduction in teleost fish. For example, interactions of both factors may exacerbate negative reproductive outcomes. Vitellogenin (*vtg*), a precursor in egg yolks, is often quantified at the mRNA and protein level as a measure of egg quality, reproductive potential, and serves as a biomarker for exposure to endocrine disrupting compounds (EDCs; Murphy et al., 2005). Surges in cortisol can reduce concentrations of plasma *vtg* in fish, which could be a potential consequence of ammonia stress (Giesy et al., 2003). However, Armstrong et al (2012) found no significant effects of ammonia on liver *vtg* mRNA in female fathead minnows. Furthermore, trace contaminants and eutrophication from aquaculture wastewater may also affect reproductive potential. Aquaculture effluents can contain estrogenic compounds including 17β-estradiol (E2), which is normally stored in oocytes of females and may cause feminization of male fish such as the synthesis of *vtg* (Kolodziej et al., 2004; Sweeney et al., 2021; Fang et al., 2021). Increasing nutrient concentrations have been found to affect the bioavailability of estrogen compounds; greater levels of nitrogen and phosphorus are shown to reduce the effects of a synthetic estrogen on *vtg* (Gordon et al., 2006). As previously mentioned, hypoxia from eutrophication may affect the endocrine function of fish species (Wu et al., 2003). Hypoxia influences the development of the ovaries and the mass of yolk produced, potentially impacting the quantity of *vtg* (Zhang et al., 2009). Therefore, *vtg* can be a useful indicator for changes in fecundity in fish exposed to aquaculture waste, while also serving as a reliable indicator of EDCs in aquaculture waste.

1.2.3 Nitrites and Nitrates

Ammonia is the principal toxic component of aquaculture wastewater, and its derivatives also result in toxicity to aquatic organisms. The nitrogen cycle in water bodies is mediated by nitrifying bacterial communities that break down and convert nitrogen products into various constituents. Primarily, bacteria convert ammonia to nitrite (NO_2^-), and eventually to nitrate (NO_3^-) during nitrification. Acute nitrite and nitrate toxicity in vertebrates results from the conversion of haemoglobin to a non-oxygen binding alternative, methemoglobin, resulting in anoxia among exposed aquatic organisms (Lewis and Morris, 1986). Chronic toxicity of nitrite includes oxidative stress, inhibition of growth, impaired metabolic function, and mortality from infection (Miao et al., 2018). Nitrite enters fish bodies through gill epithelium via chloride/bicarbonate exchange (Eddy and Williams, 1986). As such, the presence of chloride in the surrounding waters can alleviate the toxicity of nitrite as the two molecules will be competing for uptake by the same transporter across the gill epithelium (Lewis and Morris, 1986). The concentration of chloride in the environment is therefore an important factor to consider when assessing acute and chronic toxicity of nitrite.

1.2.4 Contaminants of Emerging Concern (CECs)

In some areas, the intensification of the aquaculture industry has led to the destabilization of good aquaculture practices including hygienic and biosecure systems that are in place when practicing good husbandry techniques (Schwartz et al., 2017). A consequence of this development is the inclusion of a series of additives required to ensure the health, stability, and yield of finfish stock. Some of these additives are referred to as contaminants of emerging concern (CEC), due to the lack of regulation on their usage, and causation of acute and chronic effects on animals who are exposed to wastewater effluents (Ahmad et al., 2022). One such CEC

includes antimicrobials. The frequency of pathogen exposure is increasing with the growth of aquaculture, propelling the usage of antibiotics in the industry (Schar et al., 2020). There are a series of antibiotics that are approved for usage in Canada, including florfenicol, formalin, bronopol, oxytetracycline (OTC) hydrochloride, sulfadimethoxine plus ormetoprim, emamectin benzoate, and trimethoprim with sulfadiazine powder (Health Canada, 2010). In 2021, the total reported antimicrobial usage in Canada was 6,448 kg, with the majority as florfenicol (71.5%), and oxytetracycline (28.1%) (Fisheries and Oceans Canada, 2022).

A major concern with the widespread use of antibiotics in aquaculture is the lack of proficient wastewater treatment and accompanying downstream effects on receiving environments including the incidence of antimicrobial resistance (Lalonde et al., 2012). In fact, an assessment of OTC downstream of several aquaculture facilities in Atlantic Canada found concentrations as high as 18 mg kg^{-1} in sediment, a concentration likely capable of producing resistant bacteria (Lalonde et al., 2012). Long term exposure to OTC produced behavioral impairments, oxidative damage, and alterations to gut microbiomes in zebrafish at environmentally relevant concentrations (Almeida et al., 2019). Rainbow trout displayed genotoxicity and antioxidant dysregulation upon chronic exposure to concentrations of OTC observed in aquatic environments (Rodrigues et al., 2017). Trimethoprim, also approved in Canada, is toxic to green algae, duckweed (*Lemna minor*), zooplankton (*Daphnia magna*), and zebrafish (Yang et al., 2020; Diogo et al., 2024). Chronic exposure of sulfadimethoxine to zebrafish at environmentally relevant concentrations resulted in behavioural and reproductive abnormalities, as well as transcriptional indications of oxidative stress (Yang et al., 2020).

Applications of antifouling agents is another source of CEC in aquaculture settings (Ahmad et al., 2022). Biofouling includes the growth of nuisance and damaging organisms on

the surface of equipment, which ultimately lead to reduced efficiency and profitability of aquaculture systems (Sievers et al., 2019). As a remedy, antifoulants are coated on equipment to prevent the growth and proliferation of harmful species (Ahmad et al., 2022). Due to their apparently lower environmental toxicity, copper in combination with inorganic zinc is commonly used as biocides (Guardiolo et al., 2012). However, the leaching of the biocides from equipment can facilitate accumulation of these metals into wastewaters. Copper toxicity is well studied in aquatic animals, with acute exposure known to affect sodium ion regulation (Guardiolo et al., 2012; Brix et al., 2022). Whereas chronic copper exposure elicits a stress response, escalating the release of cortisol leading to adverse systemic effects including oxidative stress, eventually initiating tissue damage and stress induced apoptosis (Brix et al., 2022). Zinc pyrithione (ZnPT) is often used in combination with copper oxide in aquaculture facilities. Although ZnPT has the capacity to be degraded under UV-light, it can still accumulate in water and sediment (Yang et al., 2019). In addition to acting as a biocide, ZnPT carries antifungal and antimicrobial properties and has been identified as a driver of oxidative stress induced male reproductive toxicity (Panga and Zhao, 2024). Acute toxicity tests have found the 96-hour LC_{50} of ZnPT to be as low as 2.6 μL^{-1} in fathead minnows (Yamada, 2005). In combination, copper and zinc work synergistically to cause greater toxicity in aquatic organisms (Bao et al., 2014).

The use of EDCs in aquaculture practices present an additional contaminant of concern. Endocrine disrupting compounds are defined by the ability to interfere with, imitate or inhibit an animal's natural hormonal regulations (NIH, 2024). Endocrine disrupting compounds are either natural or synthetic, where natural steroid hormones are primarily used in aquaculture practices and synthetics are generally controlled due to their potency (Hoga et al., 2018). Natural steroid hormones such as estrogens and androgens, are added into aquaculture feed for artificial

reproduction, increasing fish size, or to manipulate sex reversals when species exhibit size specific sexual dimorphism (Hoga et al., 2018). Canada permits the usage of natural steroid hormones including testosterone, progesterone, and 17β-estradiol (Health Canada, 2012). However, a series of banned EDCs and feed options are still abused in aquaculture practices as they largely affect growth rate (Hoga et al., 2018; Wu et al., 2016). A suite of reproductive, physiological, and behavioural impairments is possible in fish exposed to EDC pollutants (Ismail et al., 2017).

Phytoestrogens are an essential component of aquaculture feed. Derived from plants and primarily composed of alfalfa and legumes, phytoestrogens also exhibit endocrine disrupting properties (Muhammad et al., 2023). Phytoestrogens are non-steroidal, but mimic estrogen due to their structural similarities, including a phenolic ring and two hydroxyl groups which allow binding to estrogen receptors (Muhammad et al., 2023). Phytoestrogens act by affecting enzymes involved in regulating hormone pathways, imitating and competing with endogenous estrogen, and by inducing apoptosis independently from estrogen receptors (Muhammad et al., 2023). There are a variety of traits that influence the effect of phytoestrogen exposure, including the age, sex and species of the fish, but plainly phytoestrogens have the ability to influence fertility, fecundity and sexual differentiation (Muhammad et al., 2023).

The presence of EDCs in aquaculture presents a major concern; AWW effluents require sufficient treatment to protect the resident fish populations in environments receiving AWW effluent discharge. In aquaculture, fish are continuously fed a diet containing phytoestrogens, generating wastewater that is rich in an estrogen-mimicking compound, potentially causing widespread disruptions of wild fish populations. The implementation of novel treatment methods, such as UV irradiation, will be required to ensure effective eradication of EDCs from

AWW (Bennett et al., 2018). Without sufficient wastewater treatment, and continuous discharge from fish farming facilities, EDCs can persist and bioaccumulate in the environment, posing a threat to ecosystem dynamics, and long-term ecosystem health (Ismail et al., 2017).

1.3 Multi-omics approaches

1.3.1 Transcriptomics and the EcoToxChip

The term 'Omics' refers to several molecular techniques involved in studying biological molecules and includes the sub-disciplines of genomics, transcriptomics, proteomics, and metabolomics. Omics practices have become highly advanced and are now regularly used in toxicogenomic studies (Schirmer et al., 2010; Brinke and Buchinger, 2017). Transcriptomics is the study of gene expression at the RNA level, where transcriptomic profiling focuses on mRNA transcripts as the blueprints for protein expression that determine the physiological response of an organism. The characterization of RNA in toxicogenomic studies is valuable as it determines the active genes in a cell at a given moment in time, identifying activated or inhibited genes in response to a chemical or toxicant (Schirmer et al., 2010).

RNA has been used as an indicator for organism health and function for decades and several techniques have been developed to quantify transcripts. Quantitative polymerase chain reactions (qPCR) estimates transcript abundance in environmental samples using assay specific primers designed to investigate specific biological outcomes (Smith and Osborn, 2009). Although methods which provide a complete picture of gene transcription in tissues such as RNA-Seq are often preferred because of their high discovery power for novel transcripts, qPCR offers its own unique benefits as a cost-effective, rapid, and precise method for quantifying genes of interest rather than working with large datasets (Smith and Osborn, 2009). The EcoToxChip is

an example of a qPCR technique utilizing a 384-well microplate, with each well chosen to target a specific functional gene associated with a known adverse outcome pathway (AOP; Basu et al., 2019). Adverse outcome pathways are frameworks that provide links between molecular events and their known biological outcome in an environmental risk assessment framework (Basu et al., 2019). The AOPs implemented in the EcoToxChip project were chosen based on ecotoxicology literature, bioinformatics data, and target genes with regulatory relevance (Basu et al., 2019). The EcoToxChip introduces a commercialized tool to assess ecotoxicology risks in a consistent, commercialized, and affordable manner (Basu et al., 2019). Ewald et al., (2020) outlined 21 adverse outcome pathways for the fathead minnow and deemed them 'EcoToxModules'. These modules were chosen to reflect predefined molecular pathways using an ecotoxicology perspective by taking data from regulators while also using genes known to be influenced by environmental contaminants (Ewald et al., 2020). This approach simplifies complex transcriptomic data by focusing on significantly impacted genes, and reduces costs associated with traditional regulatory testing (Basu et al., 2019).

1.3.2 Proteomics

Proteomics, another useful molecular technique, quantifies and characterizes proteins translated in cells, tissues, and biofluids. When used in conjunction with transcriptomics, proteomics gives a thorough picture of the molecular and phenotypic responses. Although mRNA transcripts and translated proteins can be correlated, the analysis of both endpoints provides a fundamental understanding of the relationship between gene and phenotype, thus identifying specific gene expression controls (Buccitelli and Selbach, 2020). Furthermore, mRNA transcripts are not always translated to protein and therefore do not contribute to physiological function (Zapalska-Sozoniuk, 2019). An integrative approach is valuable in omics

studies; single omics-approaches often carry misinterpretations and ineffective or partial explanations (Zapalska-Sozoniuk, 2019). Proteomics can be measured in either a targeted or untargeted fashion. Targeted proteomics is focused on measuring the presence and/or amount of a specific protein in a sample, which is based on prior knowledge of that protein. Technological advancements pairing liquid chromatography coupled with high-resolution mass spectrometry have allowed for the analysis of proteins in a high-throughput manner (Liang, 2020). This technology has enabled untargeted proteomics, which is an exploratory technique focused on the qualitative aspect of proteins in a sample (Liang, 2020). Untargeted proteomics is a valuable technique to discover biomarkers for toxicants of concern, indicating the presence or absence of a particular chemical (Liang, 2020).

1.3.3 Non-lethal sampling for omics

The EcoToxChip is traditionally designed for analyzing liver tissue, the primary focus tissue in toxicology research (Basu et al., 2019). However, non-lethal animal sampling is becoming increasingly popular in toxicogenomic studies as it presents an opportunity to repeatedly sample an individual organism, providing insight into temporal trends. The non-lethal nature of sampling also avoids harming population densities, and facilitates sampling endangered species, or species at risk. Furthermore, non-lethal endpoints coincide with the 3R's philosophy by Russel and Bursch (1959) to reduce, refine, and replace animals in research activities. Minimally invasive sampling also facilitates investigation of long-term impacts and recovery following exposure to a stressor (Jeffries et al., 2021). As omics techniques continue to advance, non-lethal methods to study the cellular response in fish have expanded to include blood, scales/skin, gills, muscle tissues, fins, and mucus (Jeffries et al., 2021).

Fish epidermal mucus is a unique biological matrix consisting of a complex mixture of molecules predominantly composed of glycosylated proteins known as mucins, but also containing a suite of other molecules serving diverse functions (Reverter et al., 2018). Mucus plays a role in many processes including communication, enhancing immunity, pheromone transmission to support reproduction, and osmotic regulation (Ren et al., 2015). Secreted by goblet cells, sacciform cells, and club cells, mucus is highly viscous and therefore serves as the first line of defense, sequestering pathogens prior to entering the fish body (Brinchmann, 2016; Reverter et al., 2018). Furthermore, mucus carries antimicrobial, antifungal, and potentially antiparasitic functions, with the excretion of lysozymes, immunoglobulins, lectins, and antimicrobial peptides (Brinchmann, 2016; Reverter et al., 2018). Under stressful conditions the composition and rate at which mucus is produced can be altered. For example, lower production of immune molecules, which can then affect the health and defence capabilities of the fish (Reverter et al., 2018). In Atlantic salmon (*Salmo salar*), genes associated with mucin production in the gill increased in expression following handling stress, yet decreased epidermally (Sveen et al., 2017). Epidermal mucus production increased with chronic intensive rearing conditions, therefore it appears that transcriptional regulations depend on the type and exposure time of the stressor (Sveen et al., 2017).

Fish are constantly producing and secreting mucus, making it an ideal tissue to sample even in small-bodied fish such as the fathead minnow (See et al., 2022). The analysis of mucus presents many opportunities to examine the health and stress response of fishes. For example, mucus can be used to identify biomarkers for oxidative stress (Bulloch et al., 2020). Previous studies have also successfully measured protein, DNA, and RNA in mucus (U. Pajankar, MSc Thesis, 2022; Le Vin et al., 2011; Ren et al., 2015). Epidermal mucus is the host for a plethora of

proteins used to protect the fish from pathogens, and other biotic and abiotic factors, which has allowed for the identification and characterization of immune relevant proteins for many teleost fish (Brinchmann, 2016). Unlike other non-lethal approaches, mucus tissues present a unique opportunity to sample fish without causing any external harm to the animal, likely increasing the chance of survival post-sample, and affording an opportunity to assess the effects of chronic toxicant exposure over multiple time points. Epidermal mucus has the potential to monitor the effects of environmental contaminants, such as wastewater or ammonia toxicity, on fish populations, detecting early signs of disease or infections (Al-Zaidan et al, 2013; Reverter et al., 2018).

1.4 Model organism: Fathead minnow

Fathead minnows (FHM) are an ideal organism to investigate the potential toxicity of aquaculture wastewater due to their established use in toxicogenomic and environmental monitoring research and known toxicity resulting from ammonia exposure (Ankley and Villeneuve, 2006; Armstrong et al., 2012; Vidal-Dorsch et al. 2013). FHMs are a small-bodied teleost fish widely distributed across North America (Ankley and Villeneuve, 2006). The FHM has a well characterized life-history including reproductive behaviour and development, making it an ideal model organism for laboratory and field studies (Ankley and Villeneuve, 2006). Traditional toxicity tests focus on apical endpoints such as body morphology, survival, behaviour, and development, while molecular techniques have become well established in recent years (Moreton et al., 2020). Analyses of biological molecules including measuring both gene and protein expression may identify the mechanism of action of a toxicant, creating a better understanding of its toxicological risks to an ecosystem (Moreton et al., 2020; Zapalska-Sozoniuk, 2019).

1.5 Ecological risks in fish – rice co-culture systems

With the demand for fish and wild rice products increasing globally, implementing a co-culture practice, and minimizing the quantity of contaminants in wastewater outputs presents a sustainable and viable option to feed the world's growing population. Yet, introducing uncharacterized wastewater to an ecosystem presents ecological risks. The accumulation of ammonia, which is toxic at low concentrations, and phosphorus loadings, which can lead to eutrophication and consequently oxygen depletion, are examples of such consequences. Furthermore, a series of contaminants of emerging concern are present in aquaculture wastewater effluents which pose an additional risk to receiving systems. Therefore, it is important that these risks are assessed before commencing a co-culture practice.

Long-term monitoring of fish inhabiting lakes adjacent and downstream of flooded paddies of wild rice is required to assess chronic impacts. Therefore, it is important that an effective tool is available which can screen resident fish for the onset of stress, disease, or infection. Furthermore, repeated sampling of important fish populations is necessary to assess chronic impacts of paddy effluents. Thus, the development of minimally invasive methods such as using epidermal mucus tissues to monitor potential stress responses is ideal. It is imperative to assess the health and stress response of fish populations in a sustainable manner following exposure to aquaculture wastewater effluent.

1.6 Objectives and hypotheses

This research aims to understand the ecotoxicological effects of chronic nutrient and aquaculture wastewater exposure to a small-bodied fish. The primary objective of these studies is to assess the effects of exposure to excess nutrients on cellular stress response of fathead

minnows using mucus as a non-lethal and minimally invasive monitoring tissue. This objective is set in the context of a larger project that is examining the ecotoxicological risks of adding aquaculture wastewater to fertilize wild rice in a co-cultured production scenario. I hypothesize that:

H_1: Exposure to increasing concentrations of aquaculture wastewater will affect RNA expression indicative of a cellular stress response in fathead minnow liver tissues.

H_2: Exposure to excess nutrients will affect protein expression indicative of a cellular stress response in fathead minnow mucus and liver tissues.

H_3: Mucus protein patterns will reflect the cellular stress response observed in liver proteins of fathead minnows exposed to excess nutrients.

Predictions based on these hypotheses include:

P_1: Exposure to aquaculture wastewater and high nutrient levels will impact transcripts and proteins indicative of oxidative stress such as an upregulation/increase in abundance of catalase, glutathione peroxidase, superoxide dismutase, and glutathione-S-transferase in fathead minnow mucus and liver tissues.

P_2: Exposure to aquaculture wastewater and high nutrients will impact transcript and proteins related to ammonia metabolism and toxicity including upregulation/increase in abundance of glutamate dehydrogenase, glutamine synthetase, and subunits of NMDA-receptors.

Chapter 2: Cellular stress responses of adult fathead minnows (*Pimephales promelas*) chronically exposed to aquaculture wastewater effluent.

Preface

This chapter is being prepared for publication. The following outlines the author contributions, formatted according to the Contributor Roles Taxonomy (CRediT):

Krista Robertson (University of Manitoba): Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; visualization; writing–original draft. **Leah Dickenson** (University of Manitoba): Conceptualization; investigation; methodology; writing–review and editing. **Nicholas C. Blandford** (University of Manitoba): Conceptualization; investigation; methodology; writing–review and editing. **Kenneth Jeffries** (University of Manitoba): Conceptualization; methodology; resources; supervision; writing–review and editing. **Markus Hecker** (University of Saskatchewan): formal analysis; methodology; resources. **Bruce Hardy** (Myera Group): Conceptualization; funding acquisitions; resources. **Vince Palace** (International Institute for Sustainable Development – Experimental Lakes Area): Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing–review and editing.

2.1 Introduction

With exponential population growth and an increasing demand for low fat nutritious foods, food production sectors, including aquaculture, are growing (FAO 2024). Aquaculture is defined as the farming of aquatic organisms (e.g., finfish, shellfish, and algae) occurring in either freshwater or saltwater environments (FAO, 2024). A decline in wild-caught fish has necessitated aquaculture finfish production to become the leading sector in food production

worldwide (Osterblom, 2016). Among aquaculture operations, land-based aquaculture now accounts for 62.5% (51.3 million tonnes) of global fish production (FAO, 2024). The wastewater discharged from land-based aquaculture systems pose critical environmental concerns. Finfish aquaculture feed contains substantial quantities of protein, leading to wastewater effluents with high levels of ammonia and phosphorus. The ammonia in aquaculture wastewater (AWW) is the primary residue resulting from protein metabolism in fish, while phosphorus results from uneaten feed and decaying fish bodies (Dauda et al., 2019, Bernardi et al., 2018). The intensification of aquaculture has led to increasing amounts of wastewater effluents, with land-based AWW in China now surpassing municipal sewage discharge by seven times, and 85,000 L of AWW produced in North America per year (Kashem et al., 2023). With intensified fish production, chemical usage has increased to raise yields and prevent the proliferation of nuisance species and diseases (Ahmad et al., 2021). This has resulted in several contaminants of emerging concern (CECs) becoming a concern for aquaculture operations, including antibiotics, antifoulants, disinfectants, and metals (Ahmad et al., 2021; Hoga et al., 2018). Importantly, the discharged effluents from inland aquaculture facilities can pollute recipient environments, where around 10% of these facilities discharge effluent without any treatment in Canada (Lalonde et al., 2012). Meeting global demands for finfish while maintaining sustainable practices remains an on-going challenge, requiring innovative methods to support continued intensification of this industry (Laktuta et al., 2023).

Wetland plants like northern wild rice (*Zizana palustris*) can help remediate ecosystems polluted with wastewater effluents (Tepe and Temel, 2018; Abed et al., 2017). Wild rice crops require large quantities of nitrogen and phosphorus to ensure high yield (Sims et al., 2012). Furthermore, ammonia-based nitrogen fertilizers are ideal for wild rice paddy fields due to their

stability in flooded soils and ability to prevent N_2 loss to the atmosphere via denitrification (Oelke et al., 1982). As wild rice acts as a nitrogen and phosphorus sink, and thrives with ammonia-based fertilizers, AWW presents an ideal sustainable alternative to replace conventional fertilizers. There exists a potential to couple land-based finfish aquaculture with wild rice production on flooded paddy fields, which can effectively remediate wastewater effluents prior to discharge into natural systems (Ahmed et al., 2020; Qi et al., 2020). While repurposing AWW as a fertilizer for wild rice presents an innovative and sustainable mechanism to combat the intensification of the aquaculture industry, draining the flooded paddy field at the end of the growing seasons presents substantial ecological concerns for neighbouring waterbodies because of the potential to introduce ammonia waste, CECs and nutrients.

Ammonia is a particularly toxic contaminant to aquatic animals. Ammonia occurs in its ionized (NH_4^+) and unionized form (NH_3), with the total of both referred to as total ammonia nitrogen (TAN). Ammonia is toxic at low concentrations, especially in its unionized form, with levels as low as 0.06 mg L^{-1} causing reproductive toxicity in adult fathead minnows (*Pimephales promelas*; Armstrong et al., 2012). Ammonia has been studied in detail to determine toxic endpoints and the molecular mechanisms behind biological responses in fish under chronic ammonia stress are becoming clearer (Connon et al., 2011). Exposure to ammonia has been shown to elevate concentrations of reactive oxygen species (ROS) in fish, resulting in oxidative stress and tissue damage mediated by free oxygen radicals (Hegazi et al. 2010; Sun et al. 2014; Maltez et al. 2017). Nitrates, an oxidized product of ammonia, in combination with phosphates, can contribute to eutrophication, threatening water quality and dissolved oxygen levels (Schindler et al., 2016; Dauda et al., 2019). Furthermore, the abundance of CECs found in AWW is particularly concerning due to the lack of regulations and characterization of chemical

additives and metals (Ahmad et al., 2022). Monitoring for potential downstream environmental consequences of AWW in receiving systems is typically accomplished using a sentinel fish species. Fathead minnows (FHM) are an ideal organism to investigate the toxicity of AWW due to their wide-distribution across North America, well-characterized life-history, and their use in standard toxicity testing involving toxicogenomic and environmental monitoring research since the 1960s (Environment Canada, 2014). Furthermore, because of the FHM's relevance to toxicology, the FHM EcoToxChip was developed to investigate stress responses at the cellular level using a qPCR array, with 375 genes chosen based on biological and toxicological pathways, known biomarkers, and relevance to regulatory users (Crump et al., 2023).

The purpose of this study was to determine the effects of chronic AWW exposure on FHMs. The study was conducted in field-based wild rice wetland mesocosms to assess wastewater loadings that were relevant to commercial fertilizer applications. Effects were determined by assessing fathead minnow mortality, apical endpoints (i.e., Fulton's condition factor, gonadosomatic, and hepatosomatic indices), and cellular stress response (CSR) using the FHM EcoToxChip over a chronic exposure period of 60 days. We hypothesized that apical indices and CSR in FHMs would be negatively impacted with increasing exposures of AWW. In addition to providing the first study to inspect chronic exposure of AWW to fish in a fertilized wild rice field, this study provides an assessment of apical and molecular endpoints of FHMs in a field-based system.

2.2 Methods

2.2.1 Study design and aquaculture wastewater additions

2.2.1.1 Study site and simulated wetland mesocosms

This study was conducted at a commercial farm near Sperling, Manitoba, Canada between June, and September 2022. The experimental setup consisted of 16 circular polyethylene tubs with a capacity of 1,500 L (n = 13) or 3,000 L (n = 3), and diameter of 2.20 m or 2.46 m, respectively (Figure 2.1). The tubs were amended with 20 cm of soil, sourced from local agricultural fields, and 30 cm of water from an adjacent pond, both were analyzed for nutrient content (Appendix A; STable 1 and 2). Due to high nutrient levels present in the pond, all subsequent water additions to mesocosms were accomplished using fresh water from the municipal water system of Carmen, Manitoba, as it possessed a lower nutrient content (Appendix A; STable 3). On June 11, 2022 (day -19), each mesocosm was seeded with 300 g (80 g m^{-2}) of a commercial strain of Northern wild rice (*Zizania palustris*) obtained from the North Central Research and Outreach Center (NCROC) wild rice research program at the University of Minnesota, Grand Rapids, Minnesota. The seed germinated on June 16th, 2022 (day -14). One mesocosm was randomly assigned to remain unplanted and with no soil, but with 30 cm of water added, to serve as a reference for determining atmospheric nutrient inputs to the water column. On June 27th, 2022 (day -3), the successful wild rice plants were culled in each mesocosm to achieve a uniform density.

2.2.1.2 Aquaculture wastewater collections

On May 31st, 2022, wastewater was collected from a commercial inland Arctic charr (*Salvelinus alpinus*) aquaculture facility located 45 km Southeast of Winnipeg, Manitoba (Ridgeland Aquafarms, https://ridgelandaquafarms.ca/). Aquaculture wastewater was obtained from the solids settling tank using a trash pump with a two-inch diameter unscreened intake that allowed both liquids and solids to be collected. The effluent was stored in a ~1000 L plastic tank and transported to the University of Manitoba, where it was kept outside but shaded from direct sun. Effluents were sampled and submitted to a commercial laboratory (Farmers Edge Laboratories, https://www.farmersedge.ca/) to determine nutrient concentrations prior to additions to mesocosms (Appendix A; STable 4). This primary source of wastewater was renewed on August 11th when wastewater was collected from the same source and in the same manner into a second plastic water tank, which was transported to the mesocosm site in Sperling, MB. The new wastewater effluents were again, sampled and submitted to a commercial laboratory (Farmers Edge Laboratories, https://www.farmersedge.ca/) to determine nutrient concentrations (Appendix A; STable 4 and 5).

2.2.1.3 Aquaculture wastewater additions

Wastewater was added to the mesocosms following a logarithmic regression design, with the midpoint of the regression targeting recommended nutrient supplementation that is typically used in commercial wild rice farming (Kaiser, 2018 Appendix A; STable 6). Due to the high density of plants, AWW nominal biweekly addition volumes were doubled for the last two additions on day 42 and day 60 (Appendix A; STable 6). Aquaculture wastewater nutrient loadings were determined by multiplying determined nutrient concentrations by the nominal volume of wastewater added to each mesocosm. The total nutrient loading described was

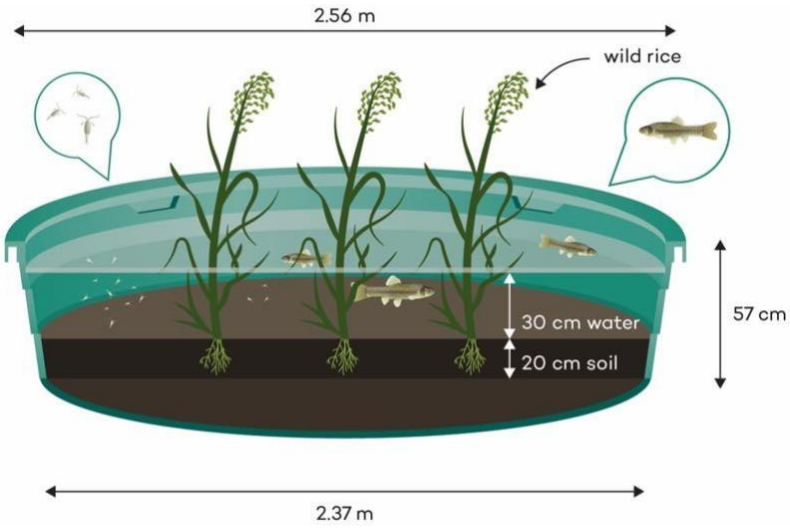
calculated as the sum of each mass of each nutrient parameter added during AWW additions throughout the duration of the study. Total phosphorus is also described as mg/wetland m^2 which is total phosphorus normalized for the surface area of each mesocosm. Minnesota wild rice fertilizer recommendations is 11.3 kg of P_2O_5 per 4047 m^2, equalling around 1200 mg PO_4-P/wetland m^2 (Kaiser, 2018). Mesocosms were randomly assigned a given volume of wastewater to be added biweekly over the growing season (10 weeks) with total nutrient loadings ranging from 1364.52 to 20467.84 mg PO_4-P and 1537.08 to 23056.16 mg NH_4-N (Table 1). On day 0 (June 30, 2022), wastewater was added to treatment mesocosms with five mesocosms remaining as references, and one reference remaining unplanted.

Table 2.1 Total aquaculture wastewater volumes (L) and nutrient loadings (mg) in wild rice wetland mesocosms (n = 10) following a logarithmic regression. Total PO_4 – P (mg)/wetland m^2 was calculated for reference to commercial wild rice fertilizing recommendations.

Total volume of wastewater applied (L)	**Total PO_4 – P Loading (mg)/wetland m^2**	**Total PO_4 – P loading (mg)**	**Total NH_4 – N Loading (mg)**
7	399.7	1364.52	1537.08
9.8	559.6	1910.33	2151.91
12.6	719.5	2456.14	2766.74
17.5	792.0	3411.31	3842.7
23.1	1319.0	4502.92	5072.36
31.5	1798.6	6140.35	6916.85
42.7	2438.2	8323.59	9376.17
57.5	3277.5	11208.58	12625.99
77.7	4436.7	15146.20	17061.56
105	5995.5	20467.84	23056.16

Five wastewater additions were performed biweekly over the course of the study. Prior to decanting, wastewater was homogenized in the holding tank using a handheld drill with a paddle mixer. After mixing, wastewater was decanted using a spigot at the bottom of the tank and measured prior to additions. For additions, the measured wastewater was slowly poured uniformly around the periphery of each mesocosm. A sub-sample of wastewater was obtained for nutrient analysis (Farmers Edge Laboratories, https://www.farmersedge.ca/) at each wastewater addition timepoint (Appendix A; STable 4). Sub-samples of wastewater were selected from three different time points (June 22, July 28, August 29, 2022) for total metal analysis (ALS Laboratory Group, https://www.alsglobal.com/en/). Briefly, wastewater samples were digested with nitric and hydrochloric acids and analyzed by Collision/Reaction Cell ICPMS (method reference: EPA 200.2/6020B; ALS Laboratory Group). The approximate total metal loadings accumulated from the five wastewater additions were calculated based on wastewater measurements at three separate time points; one prior to additions, at the midpoint, and the final wastewater addition (Appendix A; STable 7).

A.



B.

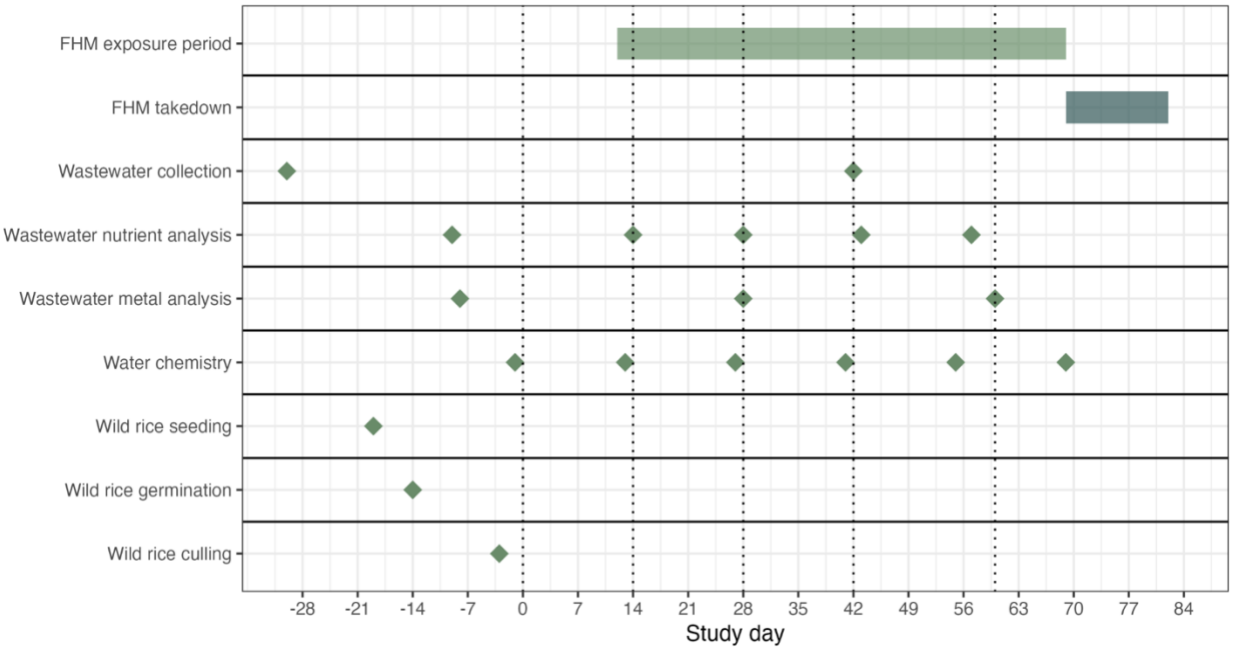


Figure 2.1. A. Schematic of a large simulated wild rice wetland mesocosm (n = 3), used in a study to inspect the toxicity of aquaculture wastewater on the survival and cellular stress response of adult fathead minnows (FHM). **B**. Study timeline detailing sampling days, with day 0 designated as the start of the experiment when aquaculture wastewater was first added. Vertical dashed lines indicate wastewater additions.

2.2.2 Adult fathead minnows

Fish were collected and handled according to a Scientific Collection Permit number 41189175 (SCP 05-2022), and Live Fish Handling (IT) Permit number: 41191149 (LFH-ITP 06-2022), administered by the province of Manitoba and an Animal Use Protocol (F22-006) approved by the Animal Care Committee at the University of Manitoba.

The fathead minnows used for this study were obtained from Fort Whyte Alive (Winnipeg, MB), a local protected wetland and environmental education center (https://www.fortwhyte.org/). Fathead minnows were captured in baited gee-type minnow traps set overnight in the littoral zone of Muir Lake (49.816, -97.224), and transported to each field site in a cooler, aerated using an oxygen gas cylinder. The fish were brought to the mesocosm in the morning, when the water temperature of the mesocosms were within 1°C of the water at the source location. In July 2022, on study day 12, twelve fathead minnows were added to each of the 15 mesocosms. Fathead minnows were sexed based on secondary sexual characteristics, and a mix of male and female identified adults were targeted to populate each mesocosm.

Before addition, each fish was individually anesthetized in water from Fort Whyte Alive containing pH buffered (pH=7.0) tricaine methanesulfonate (MS – 222; 0.1 g L^{-1}) until gill movement ceased. Fish were then measured to the nearest millimeter, weighed, and allowed to recover in fresh water. Once they had regained equilibrium, fish were added to the appropriate mesocosm. Mesocosms were inspected daily for mortalities.

Beginning on study day 69, three unbaited gee-type minnow traps were set in each mesocosm the morning of sampling to recapture fish from the mesocosms. Traps were checked after a minimum of two hours, and if unsuccessful they were inspected every 30 minutes to one hour subsequently. If an insufficient number of fish were caught, traps were left overnight, and sampling continued the following day. This effort was maintained for 13 days, until each mesocosm was deemed “fished out”, where mesocosms required three consecutive days with no catch, or all twelve fish had been removed.

Upon retrieval, fish were anesthetized in reference tub water containing pH buffered MS – 222 (0.1 g L^{-1}) until gill movement ceased, weighed, and measured as previously described to obtain a metric for Fulton's condition factor (K).

$$K = \frac{body\ mass}{fork\ length^3} \times 100$$

Each fish was then euthanized using an overdose of anesthetic (MS-222, 0.4 g L^{-1}, pH = 7) followed by pithing of the brain and severing the spinal cord. Liver and gonad tissues were then dissected from the body cavity, weighed to calculate hepatosomatic (HSI) and gonadosomatic indices (GSI), and the liver was preserved on dry ice and later stored at -80°C until it could be analyzed.

$$HSI = \frac{liver\ mass}{body\ mass} \times 100$$

$$GSI = \frac{gonad\ mass}{body\ mass} \times 100$$

2.2.3 Water quality

Water quality (temperature (°C), pH, dissolved oxygen (mg L^{-1}), and specific conductivity (μS cm^{-1})) was assessed in each mesocosm at least once per week using a Yellow Stone Instrument (YSI) Portable Multiparameter Water Quality Meter (606962-01**,** Xylem, Yellow Springs, OH). The YSI was calibrated biweekly for pH and specific conductivity, while dissolved oxygen was calibrated in the field prior to each use. Generally, water quality measurements were taken in the late morning (10:00 - 12:00).

Biweekly water sub-samples were taken from each mesocosm 1 - 24 hours prior to wastewater addition for analysis of nutrients on study days -1, 13, 27, 41, 55, and 69, with the exclusion of day 55 in which water samples were taken 5 days prior to AWW addition (Figure 2.1). Water was sampled using 500-mL Nalgene™ bottles (Thermo Scientific™ 20060016) previously rinsed three times in hot tap water, and three times in distilled water. Water was collected by submerging two bottles six inches below water surface, rinsing twice with mesocosm water prior to sample collection. A mesh screen (0.6 mm) was placed over the top of each bottle to prevent the entrance of larger debris or algae. Water samples were put on ice packs in a cooler and transported to the University of Manitoba for filtration and analysis within two hours of collection.

Total nutrients were assessed on unfiltered water, while dissolved nutrients were analyzed as the fraction filtered through a Whatman grade GF/C glass microfiber filter (1.2 µm effective pore size; Cytiva; Marlborough, MA, USA). Filters for chlorophyll a were folded and stored in aluminum foil preserved at -20°C. Total phosphorus, total dissolved phosphorus, total nitrogen, total dissolved nitrogen, and total alkalinity were measured spectrophotometrically with HACH kits (London, Ontario, Canada) at the University of Manitoba. Soluble reactive phosphorus (SRP), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), and ammoniacal nitrogen (NH_4-N) were measured using a flow injection analyzer (Lachat QuickChem 8500, HACH) following standard methods (Rice et al., 2012). Water was analyzed for chlorophyll a and dissolved organic carbon (DOC) following standard methodology outlined in Stainton et al., (1977). The expected concentrations of unionized ammonia in each of the treatments was predicted using total ammonia nitrogen results and water quality data collected from the YSI. Calculations outlined by Armstrong et al (2012) were applied (Delos and Erickson, 1999).

$$Total\ ammonia = TAN * \left(\frac{17}{14}\right)$$

$$NH_3 = \frac{Total\ ammonia}{1+10^{\left(0.09018+\left(\frac{2729.92}{Temp°C+273.15}\right)\right)-pH}}$$

2.2.4 Soil

Baseline soil samples were taken preceding the initial AWW additions on day 0. Additional soil samples were collected again on day 92, a few weeks following the final addition of AWW. Soil samples were accomplished through combining grab subsamples taken at 10 evenly spaced points across the mesocosm's surface area. Soil samples were collected from each mesocosm excluding the unplanted reference. The samples were freeze dried for 48 hours prior to submission at a commercial laboratory for nutrient and metal analysis (Farmers Edge Laboratories).

2.2.5 Mesocosm Water Volume and Corrections

Water depths in each mesocosm was attempted to be maintained at 30 cm throughout the study period. However, to account for water volume changes due to evaporation and wild rice uptake/evapotranspiration, water volumes were estimated using a cylindrical volume calculation ($V = \pi r^2 h$). Reference water depths were estimated by taking the mean water depth from 25 randomly selected points across the surface of each mesocosm prior to the commencement of the study. Following measurements, a reference water depth location was allocated which denoted a calculated water volume for each mesocosm, allowing changes in water depth to be directly

related to changes in water volume of each mesocosm for the duration of the experiment. Therefore, the difference in water depth from the reference point was measured biweekly prior to water sampling for the duration of the study period to understand the volume of water in each mesocosm at each measurement of nutrients in the water column. The upper and lower diameter of each mesocosm was recorded to address potential variations in mesocosm size, and the upper diameter was used to calculate the surface area of wild rice wetland occupied using the area of a circle ($A = \pi r^2$).

2.2.6 Molecular Analyses

A transcriptomic analysis was conducted using male fathead minnow livers and V1.0 EcoToxChips (https://www.ecotoxchip.ca/). Males from only four of the 10 treatments were solely chosen due to the number of fish that were recovered at the conclusion of the study, and to control for variation with several endocrine genes influenced by sex. These treatments included: 77.7 L (n = 6), 42.7 L (n = 4), 23.1 L (n = 5), and 9.8 L (n = 3) mesocosms, and two control mesocosms were selected for references (n =6). These mesocosms were selected to capture the potential effects of aquaculture wastewater exposure above and below the environmentally-relevant treatment (23.1-L).

2.2.6.1 RNA extractions

Total RNA extractions were completed using RNeasy® Mini Prep Kits (Qiagen, Cat. No. 73404), according to the RNeasy® Plus Universal Handbook (Qiagen, 2021). Approximately 20 mg of male fathead minnow liver tissue was excised into a 2-ml lid-locking microcentrifuge tube with a 5 mm stainless steel bead (Omni International, SKU. 277005), where 900 µL of QIAzol® Lysis Reagent (Qiagen, Cat. No. 79306) was added. Tissues were homogenized using the

TissueLyser II® (Qiagen, Cat. No. 85300) for two minutes at 30 Hz, rotating plates at one minute for even homogenization. Samples were incubated on the benchtop for five minutes before 100 µL of gDNA Eliminator Solution (Qiagen, Cat. No. 73404) was added and the tubes were vigorously shaken for 15 seconds. 180 µL of chloroform was then added, and tubes were vigorously shaken for an additional 15 seconds. Samples were incubated at room temperature for three minutes, followed by centrifuging for 15 mins at 4°C at 12,000 x g. Approximately 500 µL of the upper aqueous phase was pipetted into a new microcentrifuge tube. Samples were then loaded into the QIAcube® (Qiagen, Cat. No.) for fully automated sample preparation. The QIAcube® was loaded with filter-tips, 200 µL, 1000 µL, 30 mL reagent bottles containing, buffer RPE, buffer RWT, RNAse-free water, and 70% ethanol (Qiagen, Cat. No. 990395). Here, 600 µL of 70% ethanol was pipetted into the aqueous phase of each sample, and then filtered through RNeasy mini spin-columns. The spin column was then washed with 700 µL of buffer RWT and centrifuged for 15 seconds at 8,000 x g to wash the membrane. 500 µL of buffer RPE was added to the spin column twice, first spun for 15 seconds at 8,000 x g to wash the membrane and second for 2 minutes at 8,000 x g to wash the membrane and ensure ethanol was discarded. Finally, a new collection tube was placed under the spin column, and the spin column was eluted with 50 µL of RNAse-free water and centrifuged for one minute at 8,000 x g. Sample RNA concentrations were determined using the QIAxpert® (Qiagen, Cat. No. 9002340), a UV-spectrophotometer where 2 µL of RNA was transferred to the well of a QIAxpert® Slide-40 (Qiagen, Cat. No. 990700). The QIAxpert® also determines nucleic acid concentration and impurities, using absorbance readings (A260 and A280).

2.2.6.2 cDNA Synthesis and real-time PCR using the EcoToxChip

The measured RNA concentrations from QIAxpert® readings were used to prepare the genomic DNA elimination mix for each sample, consisting of 1 µg of total RNA, 2 µL of Buffer GE from the RT^2 First Strand Kit (Qiagen, Cat. No. 330404), and RNase-free water to reach a total volume of 10 µL per sample. Once the components were added to a 0.2-ml PCR tube, the gDNA elimination mix was mixed using a pipette to draw the sample in and out followed by brief centrifugation. The gDNA elimination mix for each sample was then incubated for 5 minutes at 42°C using the SimpliAmp™ Thermal Cycler (Applied Biosystems™ Cat. No. A24811) and held at 4°C for one minute. The reverse-transcriptase mix was then prepared on ice using the RT^2 First Strand Kit (Qiagen, Cat. No. 330404), containing 4 µL of 5x Buffer BC3, 1 µL of RE3 Reverse Transcriptase Mix, 1 µL of Control P2, and 3 µL of Nuclease-Free Water for each reaction. 10 µL of the reverse transcriptase mix was added to each sample. Samples were incubated for 15 minutes at 42°C, five minutes at 95°C, and cooled to 4°C. When the incubation was complete, 91 µL of RNase-free water was added to each sample. Finally, samples were placed in the -20°C freezer until real-time PCR analysis.

A master mix was prepared in a 5 mL sterile tube on ice, containing 2100 µL of SYBR green (Qiagen, RT^2 SYBR Green qPCR MasterMix, Cat. No. 330502), 1998-uL of RNAse-free water, and 102 µL of cDNA from an individual sample. The mixture was vortexed briefly, and added to a 10 mL reservoir, where 10 µL was pipetted into each well of the FHM v1.0 EcoToxChip custom RT^2 Profiler PCR Array (Qiagen CAPU14227E). An optical adhesive film was placed on top of the plate and centrifuged for 60 seconds at 1,000 x g at room temperature until bubbles were removed. The EcoToxChip was inserted into the QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems™ Cat. No. 4485697) and the cycling conditions were:

cycle once for 10 minutes at 95°C, and cycle between 15 seconds at 95°C and 1 minute at 60°C for 40 cycles.

2.2.7 Statistical Analyses

2.2.7.1 Apical Endpoints

Linear mixed effects models were constructed in RStudio (R version 4.3.3) using the *lme4* package to investigate the effects of wastewater applications on condition factor, LSI, and GSI of both male and female fathead minnows retrieved at the conclusion of the study, as well as water quality parameters including dissolved oxygen, pH, specific conductivity, and temperature (R Core Team, 2024; Bates et al., 2015). Growth indices were analysed using the equation y ~ treatment + sex + (treatment x sex) + (1 | mesocosm). The fixed effects are treatment and sex, the interaction effect is treatment x sex, and the random effect is (1 | mesocosm) to account for pseudoreplication from each mesocosm. For YSI water quality parameters, the model incorporated repeated measures and therefore the equation for the model is y ~ treatment + time + treatment x time + (1 | mesocosm). The fixed effects are treatment and time, the interaction effect is treatment x time, and the random effect is (1 | mesocosm) to account for repeated measures over time. Models were assessed to make sure the data met the assumptions for the analysis (linearity, independence, homoscedasticity, and normally distributed errors) using diagnostic plots and the *performance* package (Lüdecke et al., 2021). A time-weighted average (TWA) concentration was calculated to estimate the exposure of fathead minnows to total ammonia nitrogen (TAN; mg L^{-1}). The TWA_{TAN} was calculated using TAN measurements from biweekly water samples (C), and fish exposure time (T), in days.

$$TWA = \frac{[C_1T_1] + [C_2T_2] + \cdots + [C_nT_n]}{T_1 + T_2 + \cdots + T_n}$$

2.2.7.2 Data Processing on EcoToxXplorer

Transcriptomic analysis was conducted in EcoToxXplorer (www.ecotoxxplorer.ca), where data files containing well position and raw CT values were added to the *P. promelas* Version 1.0, 384-well module. Because several samples failed the EcoToxChip's genomic DNA control (GDC) quality check, the raw melt curve data was plotted in RStudio to inspect for multiple amplified products in each well of each sample. Wells with two products of different size amplified were removed from the analysis by assigning them as non-detect. Additional quality checks included a Ct cut-off of 35.0 to remove unreliable low or no expression reads (Ct greater than 35.0 was considered non-detect), and reverse transcription control (RTC) – positive PCR (PPC) control < 6. The RTC reads an artificial RNA control template, and the PPC monitors each sample for PCR inhibitors by adding external DNA in known amounts to ensure Ct values are met under optimal PCR conditions (Crump et al., 2023). Both are added during cDNA preparation, and the difference between the two is used to monitor the reverse transcription efficiency and screen for technical variability within and between samples (Crump et al., 2023). Data were normalized by calculating delta Ct values from a set of reference genes which were chosen based on their stability, displaying p-values ~0.9, and a low fold change standard deviation (Ctrl, 9.8, 23.1, 77.7: *actb1*, *il6*, *neu3.3*, *nr5a2*, *stat3*, and *thraa*; Ctrl vs 42.7L: *gapdh*, *dusp3a*, *slco1c1*, and *nr2f2*; Appendix A; SFigure 19 and 20).

Principal component analysis displaying 95% confidence intervals, as well as heatmaps were used to identify outlier samples. Two samples were identified as outliers and removed from the analysis prior to the differential expression analysis. Two independent differential expression

analyses were conducted using EcoToxXplorer. An ANOVA was conducted between the control group and the 77.7 L, 23.1 L, and 9.8 L groups to assess statistical differences (Figure 2.2). A separate differential expression analysis using a t-test was conducted between the control group and the 42.7 L treatment group (Figure 2.2B). This treatment exhibited a notably higher TWA concentration of TAN in comparison to the other treatments (Table 2.2), and exhibited a different, more severe response in comparison to the other treatments assessed. A non-adjusted p-value < 0.05 was considered statistically significant. EcoToxXplorer computes a summary statistic using all genes, which are each grouped into one of five broad categories (endocrine, immune, metabolism, signalling, and cellular processes), deemed EcoToxProcess. Each EcoToxProcess houses a set of biological processes called EcoToxModules chosen based on the EcoToxChip gene-set and relevance to ecotoxicology. A total of 21 EcoToxModule gene-sets exist, effectively describing molecular responses to environmental stressors (Ewald et al. 2020).

Normalized data was exported from EcoToxXplorer, and RStudio was used to create figures. Principal component analysis was conducted using the *factoextra* package and plotted using *ggplot2* (Kassambara and Mundt, 2020; Wickham 2016). Heatmaps were constructed using the *ComplexHeatmap* package (Gu, 2022).

2.3 Results

2.3.1 Water Chemistry

Wastewater loadings did not affect temperature, specific conductivity ($\mu s\ cm^{-1}$), dissolved oxygen ($mg\ L^{-1}$), or pH (Appendix A; SFigure 1- SFigure 4; STable 8). A time

weighted average for total ammonia nitrogen (TAN) was calculated (Table 2.2) due to the significant spike in TAN observed in the 42.7-L treatment (Appendix A; SFigure 5 and 6).

Table 2.2. Time weighted average exposure of total ammonia nitrogen as N (TAN; mg L^{-1}) to adult fathead minnows (*Pimephales promelas*) from experiment day 12 to day 70.

Treatment	TAN
Control one	0.029
Control two	0.060
Control three	0.889
Control four	1.451
Control five	0.035
7 L	0.049
9.8 L	0.916
12.6 L	0.050
17.5 L	0.029
23.1 L	2.406
31.5 L	0.036
42.7 L	9.815
57.5 L	0.058
77.7 L	1.257
105 L	0.042

The total metal loadings amassed from five wastewater additions were estimated for the 10-week exposure period (Table 2.3).

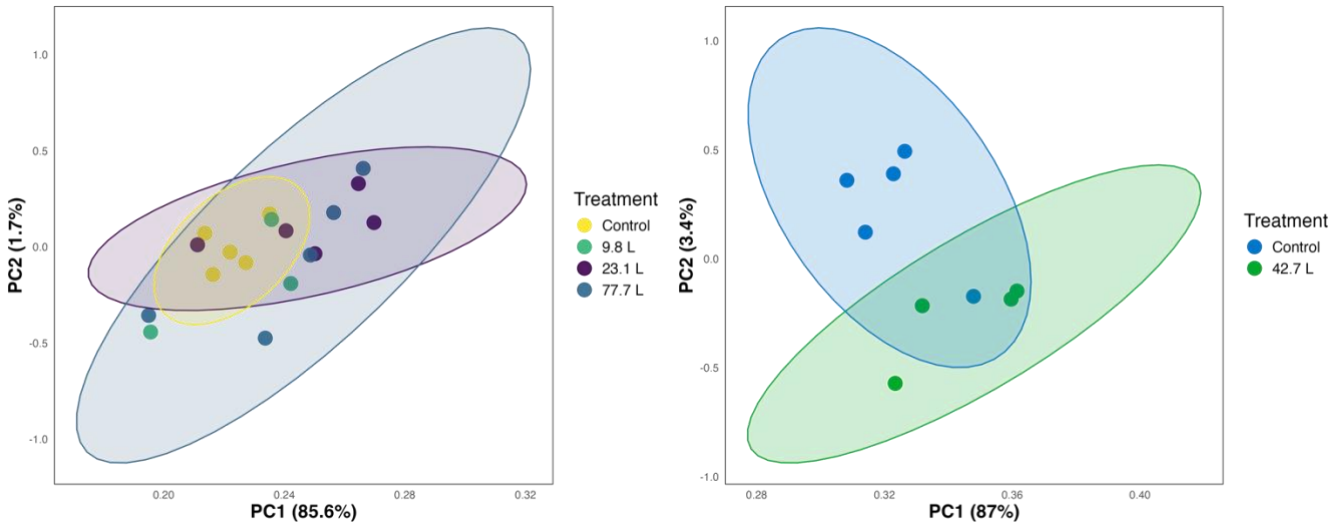
Table 2.3. Approximate total metal loadings from five wastewater additions, including copper, zinc, cadmium, lead, and nickel (mg).

Treatment	Copper	Zinc	Cadmium	Lead	Nickel
7 L	0.43	2.86	0.00	0.01	0.06
9.8 L	0.61	4.00	0.00	0.01	0.08
12.6 L	0.78	5.15	0.00	0.02	0.10
17.5 L	1.09	7.15	0.00	0.03	0.14
23.1 L	1.43	9.43	0.00	0.03	0.19
31.5 L	1.96	12.86	0.01	0.05	0.26
42.7 L	2.65	17.44	0.01	0.06	0.35
57.5 L	3.56	23.44	0.01	0.08	0.47
77.7 L	4.82	31.73	0.01	0.11	0.64
105 L	6.52	42.88	0.02	0.15	0.87

2.3.2 Adult Fathead Minnow Apical Endpoints

There were no mortalities of adult male and female fathead minnows related to aquaculture wastewater throughout the ~70-day exposure. No differences were observed among LSI , GSI, or K (Appendix A SFigure 16 - SFigure 18, STable 8).

2.3.3 Male Fathead Minnow EcoToxChip



A. **B.**

Figure 2.2. Principal component analysis displaying mRNA transcript abundance from male fathead minnow (*Pimephales promelas*) liver tissues. Each symbol represents a sample. **A.** Samples belong to one of four experimental groups, control (n = 5), 9.8-L (n = 3), 23.1-L (n = 5), or 77.7-L (n = 5) total aquaculture wastewater exposure (L) applied to wetland mesocosms over a 70-day study period. **B.** Four additional samples were investigated in the 42.7-L treatment.

2.3.3.1 Control, 9.8-L, 23.1-L, and 77.7-L

Of the 375 analyzed genes, 25 were upregulated and 7 were downregulated in the 77.7-L treatment compared to the control. Whereas in the environmentally relevant, 23.1-L treatment, 15 genes were upregulated and 17 downregulated compared to the control. In the 9.8-L treatment 21 genes were upregulated and 11 were downregulated (Figure 2.3A). Two adverse outcome pathways (AOPs) were upregulated in each of the treatments including mitochondrial dysfunction in nigrostriatal neurons, involving both *gpx1a* (p = 1.02 e-4; fold- change$_{9.8\text{-L, }23.1\text{-L,}}$

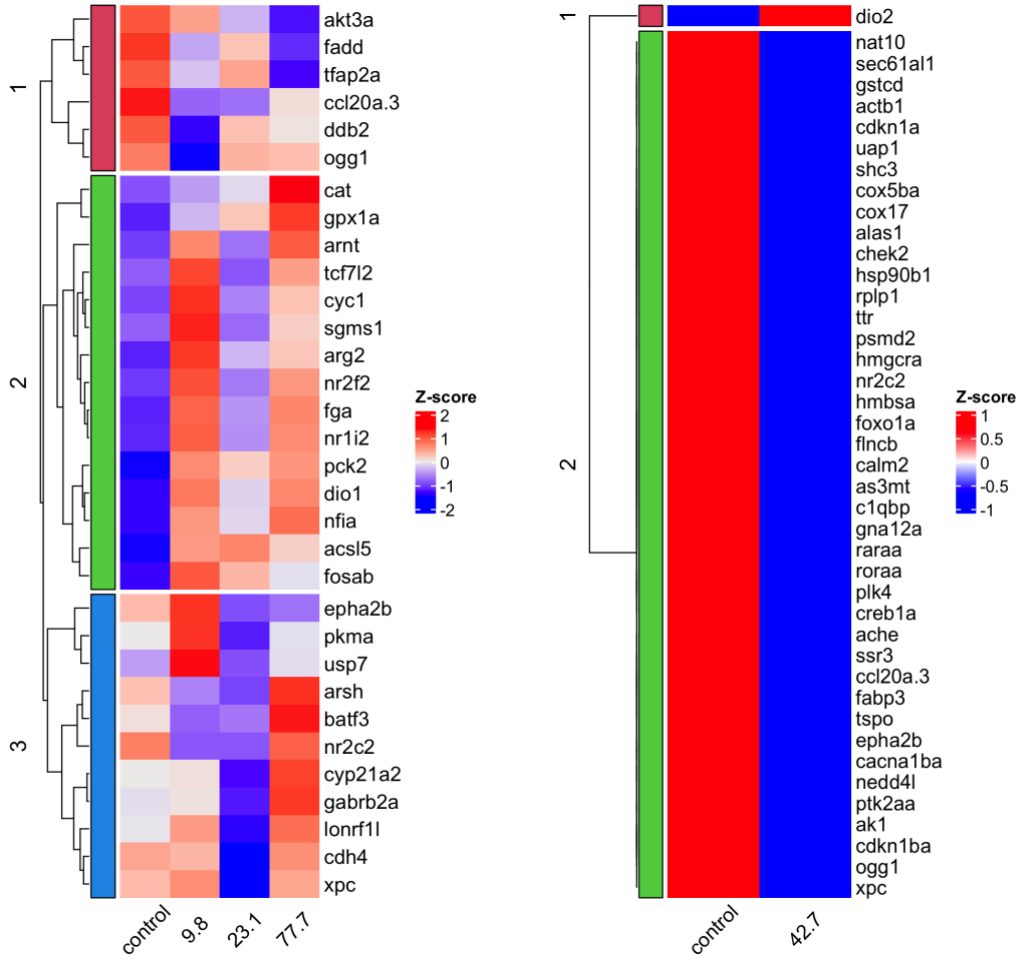
$_{77.7\text{-L}}$ = 1.36- 2.317) and *cat* (p = 0.0386; fold change = 1.08- 1.90). As well, Ahr activation was significantly upregulated, involving *arnt* (p = 0.02912; fold-change$_{9.8\text{L, }23.1\text{L, }77.7\text{L}}$ = 1.04- 1.49).

The most enriched EcoToxModules ordered by median absolute Log2FC in the lowest treatment, 9.8-L total wastewater exposure, resulted in the metabolism process as the most enriched EcoToxModule (|Log2FC| =0.79; interquartile range (IQR) = 0.36 – 1.40), followed by replication and repair in cellular processes (|Log2FC| =0.77; IQR = 0.38 – 1.26). The fold changes of dysregulated genes in the 9.8-L exposure fluctuated greatly in downregulated and upregulated genes but in general overall fold changes were low (upregulated between Log2FC = 0.04 and 6.27, downregulated between Log2FC = -9.43 and -0.15).

Exposure to the environmentally relevant treatment (23.1-L total wastewater), resulted in corticosteroid module in the endocrine process to be the most enriched (|Log2FC| = 0.83; IQR = 0.18 – 1.75), followed by metabolism (|Log2FC| =0.59; IQR = 0.25 – 0.81), and reproduction in the endocrine process (|Log2FC| =0.48; IQR = 0.23 – 1.09). The effects on the fold changes in the 23.1-L exposure were similar in upregulated genes and downregulated genes, with overall fold change in significant genes being low (upregulated between Log2FC = 0.014 and 4.83, downregulated between Log2FC = -0.16 and -5.47).

Exposure to the 77.7-L total wastewater treatment resulted in dysregulation of several biological processes. The most enriched EcoToxModules includes innate immunity (|Log2FC| = 0.68; IQR: 0.32 – 1.06), immunity (|Log2FC| = 0.68; 0.28– 1.04), corticosteroid endocrine processes, (|Log2FC| = 0.67; 0.54– 1.06) PPAR endocrine processes (|Log2FC| = 0.65; 0.41 – 1.08), and xenobiotic and ROS metabolism (|Log2FC| = 0.65; 0.32 – 0.81). The overall effects on fold changes in the 77.7-L exposure fluctuated greater in downregulated genes than

upregulated genes (upregulated between Log2FC = 0.04 and 3.93, downregulated between Log2FC = -6.36 and -0.02).



A. **B.**

Figure 2.3. Heatmaps of top differentially expressed genes, measured using EcoToxChip, in male fathead minnow (*Pimephales promelas*) liver tissue among the control, 9.8-L, 23.1-L, and 77.7-L treatment (A; $p < 0.05$), and the control and 42.7-L treatment (B; $p < 0.02$). The treatment groups denote the amount of total aquaculture wastewater (L) applied to wetland mesocosms over a 70-day study period. The normalized deltaCt values are represented by Z-scores, and the cluster blocks represent hierarchical clustering using Euclidean distances.

2.3.3.2 Chronic Ammonia Exposure

The four samples from the 42.7-L treatment were analyzed with the FHM EcoToxChip separately. Compared to the control, three genes were upregulated, and 77 downregulated in the 42.7-L treatment (Figure 2.3B). The most enriched EcoToxModules according to the median absolute Log2FC include corticosteroid in the endocrine process (|Log2FC| = 1.27; IQR = 0.60 – 1.93), metabolism (|Log2FC| = 1.26; IQR = 0.73 – 1.69), endocrine (|Log2FC| = 1.04; IQR = 0.63 = 1.91) and signalling in the nervous system process (|Log2FC| = 1.01; IQR = 0.57 – 1.84; Figure 2.4). The overall effects of fold-change were much greater in downregulated genes, with only three upregulated genes (upregulated between Log2FC = 0.95 and 4.79, downregulated between Log2FC = -6.59 and -0.45).

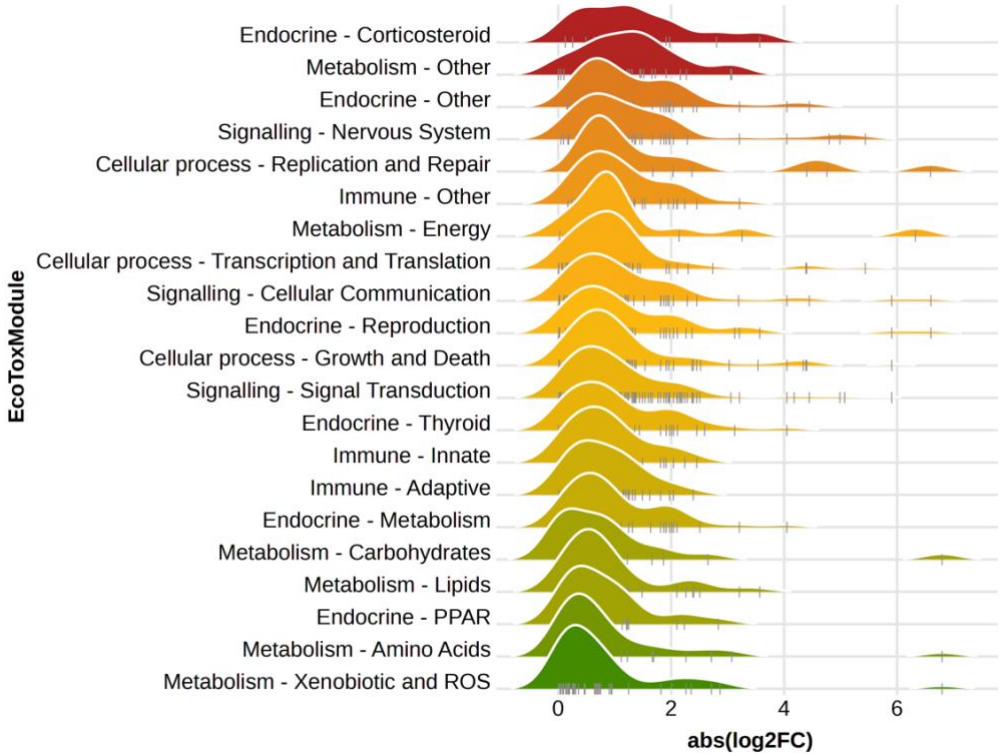


Figure 2.4. EcoToxModule ridge plot denoting the most enriched pathways from male fathead minnow (*Pimephales promelas*) liver tissues (n = 4) in a 42.7-L total aquaculture wastewater treatment over a study period of 70 days. Enriched EcoToxModule pathways were calculated based on mRNA transcript abundance and are ordered according to the median absolute Log2 fold change (abs(Log2FC)).

2.4 Discussion

2.4.1 Exposure to AWW elicits a cellular stress response in male fathead minnows.

The objective of this study was to investigate the chronic toxicity of AWW effluents to FHM using apical and molecular endpoints in a field-based, bioremediation setting. Most previous research has been related to increasing sustainability of aquaculture systems, or new techniques for remediating AWW (Laktuka et al. 2023; Iber et al. 2023; Kashem et al., 2023). Relatively little work has investigated the toxicity of land based AWW effluents. Because toxic components can be found in AWW, we hypothesized that apical indices and/or CSR would be impacted with increasing wastewater loadings. We found that chronic exposure to AWW beyond an environmentally-relevant concentration did not affect mortality, or apical indices of FHMs. However, even the lowest AWW treatment (9.8-L) resulted in a CSR. The most extreme response was observed in the treatment below the highest, the 42.7-L exposure, which experienced a spike in TAN that appeared large in comparison to the other treatments around experiment day 13 (Table 2.2).

2.4.2 Chronic AWW exposure effects oxidative stress genes

2.4.2.1 Ammonia

Aquaculture wastewater is extremely variable, depending on the geographical location and regulations in place, species of fish farmed, the feed used, and the chemical and pharmaceutical additives used. Therefore, it is useful to look at the toxicity of its components (Dauda et al., 2019; Ahmad et al., 2021). Ammonia is a consistent factor in all finfish AWW effluents as is the main nitrogenous metabolite in fishes. In response to environmental stressors like ammonia that increase ROS production, fish mitigate oxidative damage by enhancing antioxidant defenses (Kim et al., 2015). This includes upregulating the transcription of genes leading to key antioxidant enzymes such as catalase (*cat*), glutathione peroxidase (*gpx*), and glutathione S-transferase (*gst*; Hegazi et al., 2010). Several genes related to ammonia and general stress in fish were impacted in the 42.7-L treatment, including downregulation of glutathione S-transferase, C-terminal domain containing (*gstcd*), heat shock protein 90 beta 1 (*hsp90b1*), heat shock protein family A (HSP70) (*hspa9*; Cheng et al., 2015; Hegazi et al., 2010). These genes are consistently used to measure molecular responses to ammonia stress in aquatic animals (Cheng et al., 2015; Zhang et al., 2020; Hegazi et al., 2010; Cao et al., 2023). Heat shock proteins are used to measure CSRs due to their role in maintaining cellular homeostasis, particularly acting as protein chaperones to protect from conformational changes to proteins that can affect function (Gupta et al. 2010). *Hsp90* and *hsp70* decreased following chronic exposure of ammonia to *P. trituberculatus* (Lu et al. 2022). Glutathione S-transferase protects from oxidative damage through the detoxification of electrophilic xenobiotics; therefore, expression of *gst* can be a useful biomarker of aquatic contamination due to its sensitivity to chemical stressors (Habashy et al. 2019; Hegazi et al. 2010). Only the 42.7-L treatment had reduced levels of *gstcd*,

the expression of *gst* has been found to initially increase, followed by a trend of decreasing expression upon both acute and chronic ammonia exposure in the common carp (*Cyprinus carpio*) and hybrid grouper respectively *(Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*; Kim et al. 2020; Li and Qi. 2019). This decline may be attributed to depletion of glutathione (GSH), or due to excessive ROS production, where the enzyme may be unable to carry out functions under high oxidative stress (Habashy et al. 2019; Xu et al. 2021).

Catalase and glutathione peroxidase are essential antioxidant enzymes responsible for metabolizing hydrogen peroxide (Habashy et al. 2019). In several instances, *cat* has been observed to decrease with increasing ammonia exposure in aquatic animals, and eventually return to normal levels, or upregulate during recovery (Sun et al., 2014; Lu et al., 2022; Zhang et al., 2020). This is believed to occur because of inhibition of *cat*, where generated ROS persist and are not efficiently removed (Xu et al. 2021). Interestingly, the 42.7 L treatment which experienced a spike in TAN, did not have differential expression of *cat* or *gpx1a*, yet the other treatments assessed, which had lower TAN, upregulated *cat* and *gpx1a*. The lack of DE of *cat* and *gpx1a* in the 42.7-L treatment may be due to severe oxidative stress with an overwhelming quantity of ROS, which more closely resembles the trend of ammonia exposure previously described (Xu et al. 2021). *Gpx1a* showed a dose response upregulation in the 9.8-L, 23.1-L, and 77.7-L treatments, where previous studies investigating ammonia toxicity have found *gpx* to show dose response effects in liver tissues of several fish species in a 96-hour, and a 30-day chronic exposure, but not during an acute 48-hour exposure (Li and Qi et al. 2019; Guo et al. 2020; Li et al., 2020). Further, *gpx* expression was upregulated at different exposure times and ammonia concentrations under acute stress, but a dose-response trend was observed after exposure day 7 in Swimming crabs (*Portunus trituberculatus*; Lu et al. 2022). Therefore, the

dose-response upregulation of *gpx1a* observed in the present study may be due to the chronic exposure period. Although a p-value adjustment was not used in this study, the overall trend of oxidative stress genes impacted, and the significant dysregulation in the xenobiotic and ROS EcoToxModule gives some confidence that this pattern is a true effect of AWW exposure.

2.4.2.2 Immunity

Exposure to chronic stress can elicit suppressive effects on immunity in fish (Tort, 2011). The top enriched genes identified by the EcoToxModule from the 77.7-L exposure included innate immunity, and general immunity. However, immunity genes were not impacted at lower treatment levels suggesting that the 77.7-L treatment may be a threshold for immune system effects. Elevated ammonia levels can induce immune suppression in fish, which may be another driver of the CSR that we observed (Li et al. 2014; Kim et al. 2015). Immunosuppressive factors include reduced phagocytes, and total lymphocyte count (TLC) following prolonged exposure to ammonia (Xu et al. 2021). Ammonia influences immunity through excessive ROS production, leading to a cytokine response including interleukins (IL) and tumor necrosis factors (TNFs), which are common biomarkers used to assess stimulation or suppression of immunity in fish (Cheng et al. 2015; Li et al. 2020; Xu et al. 2021). In the 42.7-L treatment, TNF superfamily member 13b (*tnfsf13b*) was significantly downregulated compared to the control, indicating immunosuppression. ILs were not impacted, however, chemokine (C-C motif) ligand 20a, duplicate 3 (*ccl20a.3*), a small cytokine, was downregulated across all treatments. Several other genes involved in immunity were impacted including upregulation of fibrinogen alpha chain (*fga*),which increased in pigs exposed to ammonia (Zhang et al. 2021). Downregulation of immune related gene, Fas (tnfrsf6)-associated via death domain (*fadd*), was also observed, which is known to be influenced by ammonium chloride, copper, and zinc acetate (Bodega et al. 2006;

Li et al. 2019; Lecane et al. 2005). Therefore, exposure to high doses of AWW resulted in an immunosuppressive response likely owing to the higher concentrations of ammonia present, as demonstrated by the effects being observed in the 42.7-L and 77.7-L treatments.

2.4.2.3 Zinc and copper

Among CEC in aquaculture practices, biocides are commonly used as antifoulants to prevent the proliferation of nuisance organisms that ultimately cause destruction of equipment and impair fish productivity (Ahmad et al., 2021). Copper (Cu) and zinc (Zn) are two commonly used biocides in aquaculture systems, however, the persistence of both metals in wastewater presents an ecological concern (Miller et al., 2020). Both metals are acutely toxic to aquatic animals, and can accumulate in sediment, periphyton, invertebrates, and fish (Miller et al., 1992; Meylan et al., 2004). Zn and Cu exposure in fish can increase ROS production leading to oxidative stress and dysregulation of genes including several genes affected in this study such as *cat*, *gst*, *hsp70*, and *gpx* (Rajkumar et al. 2022; Sielska et al. 2024). At day 70 in the 77.7-L treatment, the Cu loadings were 6.52 mg, equating an estimated maximum concentration of 8.15 µg L^{-1}, and Zn loadings were 31.73 mg for an estimated maximum concentration of 39.7 µg L^{-1} (Table 2.3). Chronic Cu exposure has been found to affect growth and reproduction of FHMs between 11-33 µg L^{-1}, while chronic Zn exposure displayed reproductive toxicity in FHM at a concentration of 0.18 mg L^{-1} (Mount, 1968; Brungs, 1969). With maximum concentrations observed in mesocosms being low in comparison to known toxic concentrations described, the CSR observed may be due to the sensitivity of transcriptomic responses in comparison to growth and reproductive endpoints. Multiple metals can also have an additive effect on CSR. For example, Cu and Zn were found to have a more than additive effect on FHMs when in

combination (Lynch et al., 2016). Water chemistry has been found to greatly affect the bioavailability of Cu and Zn; the toxicity of these metals is influenced by pH, water hardness, calcium, dissolved organic carbon (DOC) and dissolved organic matter (DOM; Erickson et al., 1996; Bringolf et al., 2006; Li et al., 2019). The toxicity of copper and zinc increases as hardness decreases and reductions in DOC can also increase Cu toxicity (Ebrahimpour et al., 2010; Sciera et al., 2009; Bringolf et a., 2006). In the current study, at day 12 when FHM were added, specific conductivity measurement was high, varying between 650-950 μs cm^{-1}, but by day 70, when FHM were lethally sampled, the specific conductivity had declined to between ~400-625 μs cm^{-1} (Appendix A SFigure 4). At day 13, DOC was ~100 mg L^{-1} in most treatments, but by day 55, it had decreased and persisted at ~10 mg L^{-1} in the 77.7-L treatment. The collective effects of decreased water hardness and DOC, and the greater than additive effects of Cu and Zn may therefore contribute to the oxidative stress response observed in the transcriptomic results.

2.4.3 Endocrine response

Fish cope with disturbances and stress by enabling an adaptive response to preserve homeostasis (Schreck and Tort, 2016). When a disturbance exceeds the capacity of the fish to maintain homeostasis, the physiological response can be maladaptive, and even damaging to health and survival (Schreck and Tort, 2016). An endocrine stress response is a primary response to stress, with stimulation of the hypothalamic-pituitary-interrenal (HPI) axis, resulting in the release of the corticosteroid cortisol in fishes (Barton, 2002). The most consistently impacted EcoToxModule was corticosteroid in the endocrine process, potentially suggesting a CSR resulting from chronic exposure to AWW. This response was highest in the 23.1-L and 42.7-L

treatment, followed by 77.7-L treatment. Chronic environmental stressors can impact the cortisol response via negative feedback resulting in down regulation of the HPI axis (Barton, 2002). When introducing secondary stressors in fish already under chronic stress, such as additional contaminants, or poor water chemistry parameters like low DO or critical temperatures, it can result in a significant reduction in the fish's corticosteroid response (Barton, 2002). Effects consistent with this response were observed in the 42.7-L treatment, where a global downregulation of most genes, and main effects on the corticosteroid endocrine process, were observed (Figure 2.3B). A caveat to this line of reasoning is the lack of differential expression of glucocorticoid and mineralocorticoid receptors (*nr3c1* and *nr3c2*) which are mediators of the cortisol stress response (Kloet and Joëls, 2020). Yet, measuring chronic stress responses in fish is complex, where cortisol levels of fish recovering from an acute stressor may resolve in a few hours, whilst systemic responses such as immune response can take at least a week to recover, suggesting fish have a dynamic response to stress (Schreck and Tort, 2016). Furthermore, a study measuring cortisol levels in fish experiencing chronic crowding-stress found cortisol to lack concordance with the severity of the stressor, which was explained by the increase in cortisol clearance rates in chronically stressed fish (Schreck and Tort, 2016). For example, hypoxia stress has been consistently shown to lack differences in measured cortisol between stressed and control fish following a chronic exposure period (two-, three-, and 4-week exposure periods; Carbajal et al., 2019; Hosoya et al., 2007), which has been owed to desensitization resulting from increased cortisol catabolism (Cámara-Ruiz et al., 2022). Therefore, although corticosteroid receptors were not affected implying a lack of cortisol difference between treatments, the enriched corticosteroid response observed could still be an explanation for the global downregulation of genes observed in the 42.7-L treatment. The FHMs in the present study were

also exposed to pulsed exposures of a stressor because AWW was added biweekly. Several fish species have demonstrated a cumulative response to multiple and repeated stressors that can reduce a fish's capacity to deal with the disturbance and lower its tolerance to further stressors such as decreasing DO over time (Schreck and Tort, 2016; Barton, 2002; Appendix A; SFigure 1).

2.4.4 Outcome of Stress

When the concentration of ROS exceeds an organism's capacity to detoxify them with antioxidant defenses, free oxygen radicals can damage cellular components including DNA hydroxylation, protein denaturing, lipid peroxidation, and apoptosis (Hoseinifar et al. 2020). Lipid peroxidation is the oxidative degradation of lipids within membrane lipid bilayers, where ROS remove electrons from lipids producing reactive intermediates which elicit further damage (Su et al. 2019). Peroxisome proliferator-activated receptors (PPARs) are involved in maintaining lipid homeostasis, and other key cellular processes including lipid metabolism and antioxidant defense (Ayala et al. 2014). PPAR was a significantly dysregulated EcoToxModule in the 77.7-L group. Acyl-CoA synthetase long chain family membrane 5 (*acsl5*), encodes an enzyme involved in lipid metabolism, and its overexpression is tied to increased mitochondrial oxidative stress in humans (Luo et al. 2023). *Acsl5* was upregulated in the lowest treatment, 9.8-L (Log2FC = 5.99), with the 23.1-L treatment (Log2FC = 4.83), and the 77.7-L treatment (Log2FC = 3.93) following suit.

Cellular damage due to oxidative stress can also be manifested as DNA hydroxylation, eliciting DNA damage responses which are pathways involved in maintaining genomic integrity (Lu et al., 2022). Replication and repair in the cellular process was a top enriched EcoToxModule in the 9.8-L and 42.7-L treatments, with a series of genes significantly impacted.

Guanine is the most studied base in oxidative DNA damage due to its high susceptibility to oxidation (Poetsch, 2020). Excision of hydroxylated guanine by 8-oxoguanine DNA glycosylase (*ogg1*) is the first step in repair (Poetsch, 2020). *Ogg1* was significantly downregulated in the 9.8-L treatment and was one of the top down-regulated genes in the 42.7-L treatment, ordered by p-value. Downregulation of *ogg1* is known to make cells resistant to DNA methylation caused by oxidative stress (Zhou et al., 2016). Further, the enzyme *ogg1* can become inactivated during excessive oxidative stress (Poetsch, 2020). Damage specific DNA binding protein 2 (*ddb2*), was another top differentially expressed gene across all treatments, but especially in the 9.8-L treatment (Log2FC = -9.43), as well as xeroderma pigmentosum, complementation group C (*xpc*), the top DE gene in 42.7-L treatment. Together, *ddb2* and *xpc* proteins bind to DNA lesions and guide DNA repair processes. *Ddb2* is a key gene involved in genomic repair required due to environmental stressors, which is known to be induced by several genotoxic agents (Zhao et al. 2021). Therefore, the differential expression of transcripts related to cellular repair may provide insight into the damage occurring due to chronic AWW exposure.

2.5 Conclusion, Limitations, Future Directions

Unadjusted p-values were considered in this study for the purpose of discussing overall patterns of biological processes in the dataset. FDR adjusted p-values were observed to be between 0.09-0.22 in the 42.7-L analysis, and between 0.04-0.54 in the 9.9-L, 23.1-L, and 77.7-L analysis. Therefore, it is important to be careful when drawing conclusions from individual gene response results and to consider the potential for false positives. Conclusions were therefore derived from both EcoToxModule enrichment and evidence of gene dysregulation to improve confidence in the patterns being biologically important.

Measured concentrations of ammonia remained low throughout the study, however, it is critical to acknowledge that measurements were taken 13 days following wastewater additions, allowing sufficient time for nitrification and assimilation by wild rice, or loss as volatilized N_2. If estimated concentrations based on AWW nutrient measurements are considered, the 77.7-L treatment on average would be exposed to 3.15 mg L^{-1} TAN biweekly, which is between the no observable effect and the 10% effect concentration detected in previous studies (2.19 – 5.0 mg L^{-1} TAN; Armstrong et al. 2012). In addition, the metal analysis was conducted only on wastewater samples, and was not measured in water from the mesocosms. Therefore, the actual exposure concentrations can only be extrapolated.

We hypothesized that FHMs would exhibit CSR with increasing exposure to AWW effluents. Our data suggests that CSR can be observed from relatively low amounts of AWW effluents applied, and do not necessarily follow a dose-response trend. AWW are extremely variable and inconsistent, generating CSR that are occasionally more severe in lower treatments. Furthermore, the presence of multiple stressors in AWW have the potential to cause additive effects, eliciting a pronounced CSR which impair cellular functions. Overall, exposure to AWW resulted in a stress response, including an oxidative and corticosteroid stress response that ultimately impacted immunity, lipid peroxidation, and DNA-damage and repair. If AWW is applied to reach nitrogen and phosphorus fertilization recommendations (23.1-L treatment), it would be important to monitor the health of fish experiencing long-term repeated exposures, as well as water quality due to the exacerbating effects of multiple contaminants. Future research should be aimed at characterizing non-nutrient parameters in raw and treated AWW effluents, including antifoulants, antibiotics, disinfectants, and metals, as well as monitoring wild fish populations exposed to AWW effluents.

Chapter 3: Proteomic evaluation of epidermal mucus and liver tissues from fathead minnows (*Pimephales promelas*) exposed to environmentally-relevant nutrient loadings.

Preface

This chapter is being prepared for publication. The following outlines the author contributions, formatted according to the Contributor Roles Taxonomy (CRediT):

Krista Robertson (University of Manitoba): Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; visualization; writing–original draft. **Denina Simmons** (Ontario Tech University): Conceptualization; formal analysis; methodology; resources. **Kenneth Jeffries** (University of Manitoba): Formal analysis; methodology; resources; supervision; writing–review and editing. **Bruce Hardy** (Myera Group): Conceptualization; funding acquisitions; resources. **Vince Palace** (International Institute for Sustainable Development – Experimental Lakes Area): Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing–review and editing.

3.1 Introduction

Nutrient pollution is a growing concern in freshwater ecosystems due to excess inputs from anthropogenic activities (Environment and Climate Change Canada, 2017). Globally, densely populated regions have experienced nitrogen and phosphorus pollution that has plagued freshwater environments, causing detrimental and lasting damage to aquatic habitats (Grizzetti et al., 2021; Camargo et and Alonso, 2006). Major contributions to observed nutrient pollution include agricultural, wastewater, and industrial activities (Selman and Greenhalch, 2009). Nutrient pollution occurs through ground and surface water run-off, breakdown of nitrogen-rich

mineral deposits, atmospheric deposition, and biodegradation of organic matter (Camargo et al., 2005). Rapid urbanization in many regions of Canada with growing populations and industrialization indicate the likelihood for a similar trend of pollution (Shindler et al., 2006). Aquaculture, the farming of aquatic plants and animals, produces large volumes of nutrient rich wastewater, and has become a growing problem as a source of nutrient pollution in Canada as the demand for fish products increase (Kashem et al., 2023; Lalonde et al., 2015). Land-based aquaculture is of particular concern in Atlantic Canada, where aquaculture wastewater (AWW) is discharged, treated or untreated, into receiving aquatic systems as a point-source of pollution (Lalonde et al., 2015).

Highly toxic to aquatic animals at low concentrations, ammonia is the utmost concern for nutrient pollution in aquatic ecosystems and is a main pollutant resulting from fish farming as it is the primary nitrogenous metabolite in fishes (Dauda et al., 2019). Ammonia is present in an ionized (NH_4^+) and unionized (NH_3) form existing in equilibrium in water, with the total of both equalling total ammonia nitrogen (TAN). Unionized ammonia is inherently more toxic to fish due to its capacity to penetrate gill epithelia, whereas the larger charged ammonium cation cannot diffuse across epithelial membranes (Thurston, 1981; Allen and Collinson, 2020). Ammonia is known to be chronically toxic at concentrations as low as 5.0 mg L^{-1} TAN to fathead minnows (FHM; *Pimephales promelas*; Armstrong et al., 2012). Chemoautotrophic bacteria can oxidize ammonia into nitrite (NO_2^-) and eventually nitrate (NO_3^-; Camargo et al., 2005). Nitrite is acutely toxic to FHM fry at concentrations less than 3 mg L^{-1} (96-hour LC_{50} = 2.3 – 3.0 mg L^{-1} NO_2-N; Russo and Thurston, 1977). Both nitrite and nitrate oxidize hemoglobin to a non-oxygen binding alternative called methemoglobin, leaving aquatic animals susceptible to anoxia (Lewis and Morris, 1986). Phosphorus on the other hand is not inherently toxic to

aquatic animals; however, it is the limiting factor in primary production, and therefore is the main contributor to eutrophication and oxygen depleted ecosystems (Schindler, 1975; Chislock et al., 2013).

Proteomics techniques are increasingly employed in environmental toxicology, where protein biomarkers can be identified to uncover sublethal toxicity patterns in aquatic animals (Alderman et al., 2017; Simmons et al., 2015). Liver tissue analysis is often viewed as the gold standard in fish toxicology as it is the site of detoxification, revealing the impact of pollutants on the cellular and tissue level (Topić Popvić et al., 2023). However, non-lethal techniques are gaining momentum due to efforts to reduce, refine, and replace animal use in research (Russel and Bursch, 1959). Sampling techniques for "omics" have expanded to include non-lethally collected tissues such as blood serum, scales and skin, muscle, and mucus tissues (Jeffries et al., 2021). Non-lethal sampling gives researchers the opportunity to monitor species at risk, repeatedly sample populations, and to sample the same fish over multiple time points to investigate the temporal nature of chronic exposures. Secreted mainly by goblet cells, fish epidermal mucus is the first line of defense, capturing pathogens and carrying antimicrobial, and antifungal properties with a suite of enzymes and molecules with capacities to protect the host (Brinchmann, 2016; Reverter et al., 2018). Protein, DNA, and RNA have been successfully measured in mucus of several fish species (Andrzejczyk et al., 2022; Pajankar, U., MSc Thesis, 2022; Le Vin et al., 2011; Ren et al., 2015). The analysis of mucus presents an opportunity to capture the health and stress response of fish exposed to environmental contaminants and detect early signs of disease or infection (Bulloch et al., 2020; Al-Zaidan et al, 2013; Reverter et al., 2018).

Aquaculture wastewater is proposed to be a suitable fertilizer for aquatic crops, in flooded agricultural fields or wetlands (Ahmed et al., 2020). However, these systems are ultimately drained into receiving freshwater environments so there is a need to monitor potential impacts from excess nutrients and other contaminants. Fathead minnows (FHMs) are an ideal organism to investigate the sub-lethal toxicity of excess nutrients, and the efficacy of mucus as a method of proteomic evaluations due to their wide distribution across North America and established use in toxicogenomics and environmental monitoring since the 1960s (Environment Canada, 2014). We assessed the toxicological effects of ammonium chloride and phosphorus at concentrations similar to those found in AWW effluent discharges by measuring proteins in mucus of adult male and female FHMs chronically exposed to these nutrients for 30 days in a laboratory setting. The exposure concentrations were selected to be comparable with a preceding field study which determined the appropriate nutrient loadings to fertilize flooded wild rice paddy systems with aquaculture wastewater effluent (N. C. Blandford, MSc thesis 2024, University of Manitoba).

The purpose of this study was to conduct a non-targeted proteomic evaluation of FHMs chronically exposed to environmentally-relevant concentrations of nutrients. We aimed to advance minimally invasive techniques to evaluate protein expression patterns in fish using epidermal mucus, while validating the efficacy of mucus as a non-lethal alternative to liver tissues for proteomic analysis. We hypothesized that no fish mortality would be observed from the excess nutrients, but apical endpoints and protein abundance would be impacted with increasing nutrient exposure and time. Additionally, we hypothesized that patterns observed in mucus proteins will reflect the cellular stress response observed in liver proteins when compared at the conclusion of the study. We predicted that protein abundances measured in mucus would

become increasingly dysregulated as exposure time increased and mucus and liver protein abundances would reflect patterns of a general stress response. By assessing mucus as a minimally invasive alternative for proteomics data, we hope to identify protein biomarkers that can be sampled non-lethally to reduce lethal sampling requirements for both laboratory and wild fish studies.

3.2 Methods

3.2.1 Fish Housing

Experiments and fish handling were conducted in accordance with the Animal Use Protocol (AUP) #15880 administered by the Animal Care Committee at Ontario Tech University (Oshawa, ON). Eight-month-old fathead minnows were reared in-lab at Ontario Tech University, and seven male and eight female fathead minnows were obtained from rearing tanks for each of the nine experimental tanks, for a total of 15 fish selected $tank^{-1}$. Nine 70 L glass tanks were arranged side by side with an opaque barrier between tanks and at the rear. The front side was left unobstructed. Each tank was topped with a transparent lid to allow light penetration, an air stone for continuous oxygen supply, and two habitat enrichments consisting of a 10 cm diameter black PVC pipe cut horizontally to create a 15 cm long half pipe main shelter, and a small 5 cm diameter PVC pipe 10 cm long acting as a secondary shelter.

Fish were placed in experiment tanks with water temperatures gradually decreased from 25ºC to 22.1ºC and acclimated for 10 days prior to beginning the experiment. Water was obtained from the Region of Durham's municipal water system, which is sourced from Lake Ontario and Lake Simcoe. Tap water was filtered and dechlorinated prior to filling experimental tanks (Appendix B; STable9). The experiment maintained a constant 16h light and 8h dark

photoperiod, and each tank of 15 fish was fed ~10 mL of thawed adult brine shrimp daily, excluding experiment day 29 where fish were fasted prior to dissections. Seven fish mortalities occurred following baseline mucus sampling prior to day 0. These fish were replaced from laboratory stocks before the commencement of the study.

3.2.2 Experimental Design

The experimental design consisted of three experimental groups in triplicate, for a total of nine experimental tanks. Nutrient concentrations were selected for this experiment based on concentrations reflecting environmentally-relevant exposures to AWW applied to experimental wetlands (N. C. Blandford, MSc thesis 2024, University of Manitoba). The target low concentration was chosen to reflect a eutrophic system (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4 – P), where total nitrogen (TN) concentrations > 1.5 mg L^{-1} and TP > 0.075 mg L^{-1} are indicative of eutrophic conditions (Savic et al., 2022; Dodds and Smith, 2017). The target concentration for the high experimental treatment was 4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4 – P. This concentration reflects the highest exposure in the field study signifying environmental relevance and simulates a point source of aquaculture wastewater pollution frequently observed in nature (Lalonde et al., 2015). Ammonium phosphate dibasic (Sigma-Aldrich: 215996) was primarily used to achieve exposure concentrations, with supplement from ammonium chloride (Sigma-Aldrich: 213330) to accomplish a higher TAN concentration while limiting chloride additions. Exposure solutions were prepared by dissolving weighed salts in pre-measured dechlorinated tap water and mixing thoroughly until dissolved prior to daily water change.

The commencement of the study was staggered across three days for each experimental replicate such that each treatment replicate would start on the consecutive day in an effort to

stagger the sampling days. This was accomplished by staggering day zero across three days such that a control, a low, and a high treatment tank would start on the same day, and thus repeated across the three days until each of the nine tanks was sampled.

3.2.3 Water Changes and Nutrient Measurements

A daily 50% static renewal water change was selected to reduce ammonia build-up in each experimental tank arising from fish excretion, while limiting stress of reduced water levels. The water changes began during the acclimation period and continued throughout the entire 30-day exposure. Feces and uneaten food were siphoned prior to removing water for the water changes. The volume for half of the tank was determined by measuring the length, width and height of the experimental tank and calculating volume.

$$Volume = l\ x\ w\ x\ h$$

$$Volume\ (half\ the\ tank) = \frac{(71\ cm)(30.5\ cm)(15\ cm)}{1000\ cm^3}$$

$$Volume\ (half\ the\ tank) = 32.48\ L$$

Each day, 32.5-L of water was removed from each experimental tank and replenished with the same volume of 22.1ºC dechlorinated and filtered tap water. To achieve the target concentrations for the high and low treatments the quantity of $(NH_4)_2HPO_4$ and NH_4Cl was determined by calculating the moles of each compound required based on the target concentrations and the volume of the tanks (Appendix B). At day zero, pre-weighed ammonium phosphate dibasic and ammonium chloride salts corresponding to each treatment were dissolved in the fresh 32.5-L of lab water by mixing thoroughly, and water was replenished for the corresponding tank. This process was repeated daily during water changes for each experimental tank.

3.2.4 Water Quality and Chemistry

Water parameters (temperature (°C), pH, dissolved oxygen (mg L^{-1}), and specific conductivity (μS cm^{-1})) were assessed in each experimental tank every day following feeding and prior to water changes using a Yellow Stone Instrument (YSI) Portable Multiparameter Water Quality Meter (606962-01, Xylem, Yellow Springs, OH). Water samples from each of the nine experimental tanks were taken at four time points throughout the study for a complete nutrient analysis at York-Durham Regional Environmental Lab (REL; Duffin Creek Water Pollution Control Plant, 901 McKay Road, Pickering). Samples were taken prior to exposure for baseline sampling (day -1 through day -3), after week one (day 7 – 9), between week two and three (day 16 – 18), and during week four (day 25 – 27). Water was collected from each tank by taking a grab sample using a Thermo Scientific™ Nalgene™ Polypropylene Griffin Low-Form Plastic Beaker, and filling two pre-labelled sample bottles retrieved from the REL. The beaker was rinsed three times with hot water and three times with distilled water between each tank. Each sample was put directly in a cooler of ice and delivered to York-Durham Regional Environmental Lab within 1.5 hours for analysis. The parameters analyzed included ammonia & ammonium as N, nitrate as N, phosphate as P, nitrate & nitrite as N, nitrite as N, total Kjeldahl nitrogen, total phosphorus as P. The expected concentrations of unionized ammonia in each of the treatments was predicted using total ammonia nitrogen results and water quality data collected from the YSI. Calculations outlined by Armstrong et al (2012) were applied (Delos and Erickson, 1999).

$$Total\ ammonia = TAN * \left(\frac{17}{14}\right)$$

$$NH_3 = \frac{Total\ ammonia}{1+10^{\left(0.09018+\left(\frac{2729.92}{Temp°C+273.15}\right)\right)-pH}}$$

Ammonia water quality tests were taken every 2 – 3 days using the Ammonia Low-Range Checker® (Hanna Checker, SKU: HI700), PO_4 was tested every 5 – 7 days using the Phosphate Low-Range Checker® (Hanna Checker, SKU: HI713), and chloride was assessed weekly using the Total Chlorine Ultra Low-Range Checker® (Hanna Checker, SKU: HI761).

3.2.5 Fathead Minnow Sampling

Mucus was sampled on experiment days -5 (five days prior to exposure), 5, 15 and 30. As per the rotating schedule described previously, one tank from each treatment was sampled per day (i.e., a total of three tanks per day) for three consecutive days. FHMs were selected in an attempt to achieve a uniform sex ratio across samples, caught using an aquarium net, and placed into a bath containing the respective tank water and pH buffered tricaine methanesulfonate (MS – 222; 0.15 g L^{-1}). Once opercular movements had slowed, the fish lost equilibrium, and pressure on the caudal peduncle elicited no response, the fish was deemed to be in the appropriate plane of anesthesia. At that time a 2 x 2 cm pre-cut kimwipe (Kimberly-Clark Professional™ Kimwipes™ Delicate Task Wipers, 34256EMD) was placed over the surface of the fish, and using tweezers, the kimwipe was gently pulled laterally down the body of the fish. A second kimwipe was then used to repeat this process, collecting any additional mucus missed during the first swab. The saturated kimwipes were placed into a Spin-X centrifuge tube filter (Corning® Costar® Spin-X® centrifuge tube filters (Sigma: CLS8160)), pre-rinsed with 0.1N NaOH and Milli-Q® water and put on ice. The fish was then measured for fork length and weighed before returning to a recovery bath containing the corresponding treatment water. This process was repeated for each fish, until all 15 fish from a tank had been sampled. Preliminary trials determined that mucus from five fish was required to obtain a suitable concentration of proteins.

With a primary interest in overall response to nutrients independent of sex related differences, mucus samples from five fish were pooled with near equal sex ratios for each pooled sample. Afterward, 250 μL of 200 mM ammonium bicarbonate buffer was pipetted into Spin-X filter tubes and vortex mixed for 30 seconds followed by centrifuging at 700 g for 20 minutes at 4°C to pull the mucus from the kimwipe sitting in the upper portion through the filter and into the bottom of the tube. This process was repeated to ensure all mucus was separated from kimwipe swabs. Each isolated pooled sample was transferred to a 0.5 mL Amicon filter with a 10kDa molecular weight cut-off (Amicon Ultra-0.5 Centrifugal Filter tube (Sigma: UFC5010)) pre-rinsed with 0.1N NaOH and Milli-Q® water and spun at 14,000 g for 30 minutes. To recover the concentrated sample, Amicon filters were then placed upside down into a clean microcentrifuge tube and spun at 1,000 g for 10 minutes. The retentate was flash frozen in liquid nitrogen, and stored at -80°C.

At the conclusion of the study (day 30), following mucus sampling and measurements, the FHMs were individually euthanized in an overdose of anesthetic (0.2 g L^{-1} MS-222, pH = 7) followed by pithing of the brain. Due to its importance in the protein analysis, the liver tissue was removed first and placed in a pre-tared 2.0 mL microcentrifuge tube, where it was quickly weighed for hepatosomatic index (HSI) calculation, flash frozen in liquid nitrogen, and stored at -80°C. Three livers were pooled from a given treatment to obtain sufficient tissue mass for protein analysis, with sexes pooled near equal for each pooled sample. The gonad tissues were removed to verify sex and to calculate gonadosomatic index (GSI) measurements.

3.2.6 Protein Preparation via Chemical Digestion

Mucus retentate and liver tissues were thawed completely on ice for 20 minutes and vortexed to achieve a uniform mixture prior to the addition of five volumes of 200 mM ammonium bicarbonate buffer. Homogenization of liver tissues was accomplished using a handheld Pellet Pestle® Motor, with an attached Pellet Pestles™ Tissue Grinder, for approximately 30 seconds until the tissue was uniform in a liquified state. The sample tubes were centrifuged for 15 minutes at 14,000 x g at 4°C, and the supernatant was pipetted into a clean microcentrifuge tube.

Average protein concentrations were determined with a Traycell and Varian Cary® 50 UV-Vis Spectrophotometer using 2 µL of 200 mM ammonium bicarbonate buffer to zero absorbance. The buffer was wiped away using a cleaning swab, and 2 µL of liver protein homogenate or concentrated mucus protein was added to the Traycell and analyzed. To determine the concentration of protein, the following equation was used:

$$Protein\ Concentration\ (\frac{mg}{ml}) = \frac{Abs\ (280\ nm)}{Path\ length\ (cm)}$$

The protein concentration determined from each sample was used to normalize the volume of homogenate for protein processing. One mg of protein was pipetted into a clean microcentrifuge tube, where 2.65 µL of 100mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 200mM ammonium bicarbonate buffer was added to reduce the proteins. Each tube was capped, and gently tapped to ensure the reducing agent reached the homogenate. The tubes were placed in a Thermo Scientific™ Digital Vortex Mixer at 500 rpm for ten seconds to mix and incubated at room temperature for 45 min. At the end of the incubation period, the proteins

were alkylated by adding 2.8 µL of 200 mM Iodoacetamide (IAA) in 200 mM ammonium bicarbonate buffer into each tube, and vortexed gently and incubated for 45 min in the dark at room temperature. Proteins were then digested by adding 50 µL of 20% formic acid to each sample. The tubes were given a lid-lock guard, vortexed gently and incubated at 115°C for 30 min in a Thermo Scientific™ VWR® 96 place heating block. At the end of the heated incubation, the samples were removed from the heating block and left to cool for one min. Samples were spun in a Thermo Scientific™ Sorvall Legend Micro21R Centrifuge at 500 rpm for five seconds to remove condensation. To prepare the samples for high performance liquid chromatography (HPLC) analysis, the samples were evaporated using a Savant SpeedVac SPD1030 Integrated Vacuum Concentrator until 20 µL of sample remained.

To reconstitute the dried samples for HPLC analysis, 20 µL of HPLC buffer containing 0.1% formic acid, 5% acetonitrile, and 95% water was added. The samples were then vortexed gently (500 rpm) until particulates had returned into solution uniformly. Once a constant mixture was obtained, the samples were centrifuged for 10 minutes at 14,000 x g to precipitate remaining debris. The supernatant of the sample was transferred to 2 mL screw thread HPLC vial (Chromatographic Specialties, 12 x 32 mm, S425: C779100) containing 250 µL polypropylene bottom spring inserts (Canadian Life Sciences, 6 x 29 mm: 1025PBS-629). The vials were capped with 9 mm blue PTFE/silicone/PTFE screw caps (Chromatographic Specialties: C396-09SB) and stored at 4°C until analysis.

3.2.7 Reverse-Phase Liquid Chromatography Mass Spectrometry Analysis

The prepared HPLC vials were placed into a temperature regulated autosampler in a randomized order, where 2 μL of the peptide solution was injected and then separated by a Zorbax 300SB-C18, 1.0 × 50 mm 3.5 μm reversed-phase high performance liquid chromatography column (Agilent Technologies Canada Inc., Mississauga, ON) using an Agilent 1260 Infinity Binary liquid chromatogram. The separation was accomplished using two solvents including buffer A1, consisting of 95% HPLC grade water (Fisher Scientific; W5-4), 5% HPLC optima grade acetonitrile (Fisher Scientific; A), and 0.1% formic acid (Sigma Aldrich: 06440). As well as buffer B1, consisting of 95% HPLC optima grade acetonitrile, 5% HPLC grade water, and 0.1% formic acid. For detection, the Agilent 6545 Accurate-Mass Quadrupole Time of-Flight (Q-TOF) was used in tandem to the Agilent 1200 series liquid chromatography system. All analytical runs included a solvent blank and a BSA digest standard (Agilent Technologies Canada Inc., Mississauga, ON) injection every 10 samples to monitor baseline, carry-over, drift, and sensitivity during runs. All mucus and liver samples were randomized and analyzed in the same analytical run. Further information on instrumental methods is detailed in the appendix.

3.2.8 Protein identification

Spectral data obtained for each sample were analyzed using Spectrum Mill Software (Version B.04.01.141) to identify peptides. A database containing a reference proteome was created using the NCBI reference proteome for the fathead minnow (NCBI Taxonomy ID: 90988), containing 96,616 identified protein sequences (downloaded March 2023). In Spectrum Mill, detected peptides were searched against the reference proteome and auto-validated and accepted when at least one peptide had a peptide score (raw match quality between the observed

spectrum and the theoretical database spectrum) greater than 6 and a scored peak intensity (SPI; the percentage of the spectral peak-detected ion current explained by the search interpretation) of greater than 70% as recommended by the manufacturer. After peptides were identified at the MS/MS level, peptides were uploaded to Skyline 20.2 (MacCoss Lab Software) and quantified at the first spectrometer (MS1) level. This was performed with the DDA workflow with a score of 0.9, retention time window of five min, and five missed cleavages with transition settings for time of flight (TOF; Pino et al., 2020).

The NCBI accession numbers from the identified proteins were mapped to human orthologous accession numbers by manually taking top sequences matched in a protein query in Basic Local Alignment Search Tool (BLAST). Human orthologous accession numbers were converted to gene symbols in RStudio using the *org.Hs.eg.db* package in Bioconductor (Carlson, 2019). Redundancies in gene symbols were consolidated in Microsoft Excel by choosing the gene symbol with the maximum value.

3.2.9 Statistical analyses

3.2.9.1 Protein analysis

The reconstitution step previously described was accomplished in several batches for mucus proteins. Due to a technical repair on the LC-QTOF-MS, the first batch of samples that were reconstituted were degraded in storage and showed a clear batch effect (Appendix B; SFigure 45). This first batch contained samples randomly selected from experiment days 5 and 15, and ultimately were removed from the analysis to prevent inaccurate results. The remainder of the mucus protein samples were included in the analysis.

The statistical analyses were completed with RStudio (R version 4.3.3) using the *limma* (Linear Models for Microarray Data) Bioconductor package for protein differential expression analyses (R Core Team, 2024; Ritchie et al., 2015). Data preparation for *limma* analysis consisted of normalizing between and within samples. Raw protein data normalizations within samples consisted of a log2 transformation, scaling, and centered using 'pareto_scale' with the *IMIFA* package which scaled protein intensities by the square root of the column-wise standard deviations (Murphy et al., 2020). For between sample normalization 'normalizeBetweenArrays' was implemented using the method Cyclic Loess, which ensures intensities have similar distributions across samples (Ritchie et al., 2015). A design model was created using 'model.matrix' which created conditions for all pairwise comparisons to be conducted (Ritchie et al., 2015). Finally, to estimate quality weights for each sample 'arrayWeights' was used which estimates the reliability of each replicate by calculating how it follows the linear model, reducing the influence of poor-quality samples (Ritchie et al., 2015). Normalized data, weights, and model design were then inputted into the function 'lmFit', which fits a linear model for each protein across all samples by weighted least squares (Ritchie et al., 2015). A contrast matrix was designed using 'makeContrasts' which specifies differential expression comparisons to be conducted for individual treatment and time effects, as well as interaction effects, and is then fit into 'contrasts.fit' which re-orientates the model from 'lmFit' to the contrast matrix defined (Ritchie et al., 2015). The fitted model is inputted into 'eBayes' which ranks genes in order of validation of differential expression, and computes t-statistics. Data was adjusted for multiple testing using Benjamini-Hochberg method to control for false discovery rates (FDR), with a correction of FDR < 0.20 considered significant (McDonald, 2014).

Enrichment analyses were conducted using the *limma* package and the functions 'goana' and 'topGO' to investigate the most dysregulated gene ontology (GO) biological processes (Ritchie et al., 2015). The adjusted p-value cut off was set to $p < 0.2$, denoting the significance required to be included in the enrichment analysis. Each GO term required a minimum of three significant protein hits to be included.

Principal component analysis was conducted using the *factoextra* package and plotted using *ggplot2* (Kassambara and Mundt, 2020; Wickham 2016). Heatmaps were constructed using the *ComplexHeatmap* package (Gu, 2022).

3.2.9.2 Apical Endpoints

Fathead minnow K, HSI, and GSI were calculated with the following equations using measurements collected at the conclusion of the study (day 30).

$$K = \frac{body\ weight}{fork\ length^3} \times 100$$

$$HSI = \frac{liver\ weight}{body\ weight} \times 100$$

$$GSI = \frac{gonad\ weight}{body\ weight} \times 100$$

An analysis of variance (ANOVA) was conducted separately for both sexes for each endpoint. Models were assessed prior, ensuring the data met the assumptions for the analysis. To avoid pseudoreplication, male and female FHM measurements were averaged separately in each experimental replicate prior to the analysis.

3.3 Results

3.3.1 Water Chemistry

Nutrient concentrations remained similar between control and low experimental groups, with a notable increase observed only in the high exposure group (Table 3.1). Despite daily 50% water changes, NH_3 and TAN levels in the controls were above zero and similar to the low treatment at several time points (Table 3.1; Appendix B; SFigure 35). Nitrite levels were greater in the high treatments, whereas nitrate was higher in the low treatment except for measurement day 6 – 8 (Table 3.1; Appendix B: SFigure 33 and 34).

Table 3.1. Water chemistry from four sampling periods throughout a 30-day nutrient exposure. Measurements for total phosphorus as P (TP - P), total ammonia nitrogen as N (TAN - N), expected ammonia as N (NH_3 - N), nitrite as N (NO_2^-- N) and nitrate as N (NO_3^-- N) are displayed the mean (± SD) of each treatment group (n = 3) in mg L^{-1}.

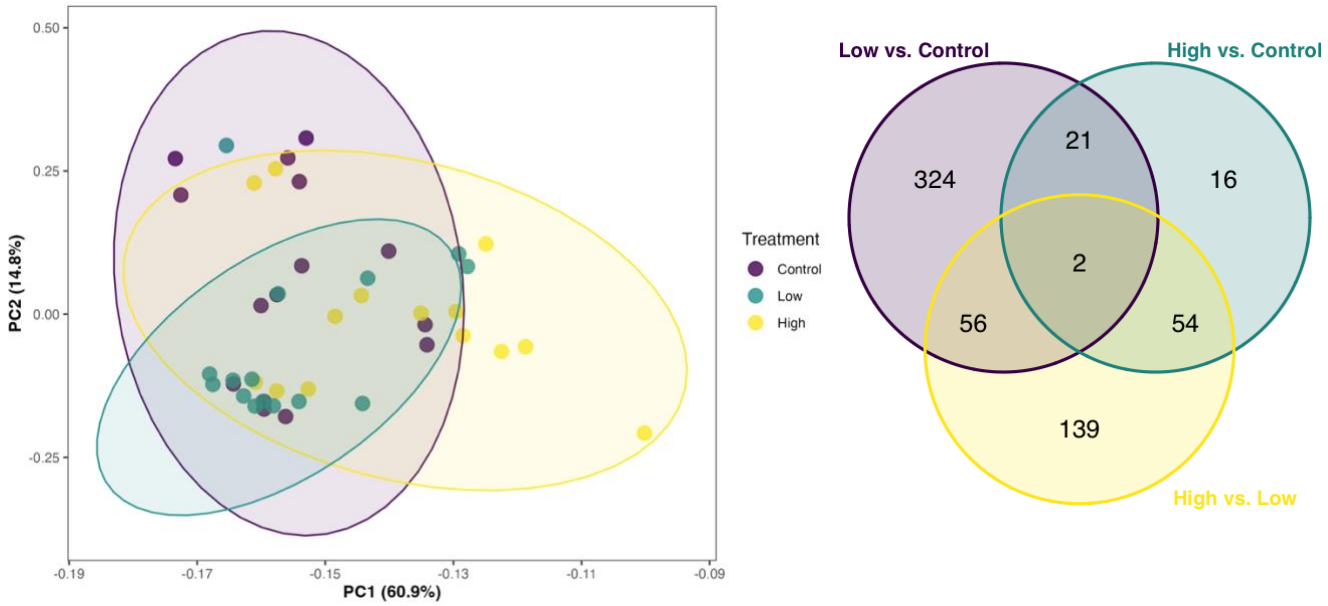
	Treatment (n =3)	*TP*	*TKN*	*NH_3*	*TAN*	*NO_2^-*	*NO_3^-*
Pre-Exposure	Control	0.079(0.004)	0.597(0.195)	0.020(0.008)	0.273(0.085)	0.346(0.156)	0.543(0.162)
	Low	0.085(0.013)	0.513(0.061)	0.016(0.003)	0.227(0.035)	0.521(0.022)	0.507(0.068)
	High	0.079(0.013)	0.607(0.230)	0.022(0.015)	0.293(0.121)	0.430(0.187)	0.533(0.179)
Day 6 – 8	Control	0.047(0.004)	0.360(0.044)	0.020(0.012)	0.243(0.179)	0.191(0.158)	0.497(0.176)
	Low	0.156(0.011)	0.393(0.049)	0.016(0.004)	0.203(0.059)	0.291(0.266)	0.667(0.315)
	High	1.180(0.020)	1.370(1.013)	0.031(0.025)	0.930(0.921)	2.530(0.868)	0.833(0.499)
Day 15 - 17	Control	0.035(0.004)	0.333(0.038)	0.000	0.000	0.168(0.142)	0.473(0.197)
	Low	0.131(0.003)	0.283(0.015)	0.013(0.011)	0.173(0.151)	0.17(0.269)	0.687(0.339)
	High	1.160(0)	1.473(0.720)	0.051(0.054)	0.793(0.657)	2.463(0.513)	0.330(0.572)
Day 25 - 27	Control	0.051(0.026)	0.357(0.021)	0.008(0.001)	0.107(0.006)	0.123(0.129)	0.613(0.096)
	Low	0.139(0.009)	0.343(0.050)	0.009(0.004)	0.153(0.085)	0.162(0.272)	0.863(0.315)
	High	1.213(0.006)	0.977(0.400)	0.021(0.019)	0.617(0.341)	2.830(0.580)	0.520(0.901)

3.3.2 Apical Endpoints

There were no mortalities of adult male and female FHMs related to nutrient exposure throughout the 30-day experiment. No differences were observed among LSI, GSI, or K (Appendix B; SFigure 42 - 44).

3.3.3 Liver Protein Results

A total of 3064 unique proteins were identified in liver tissues from 44 samples among the three treatments obtained on day 30 of the exposure (Figure 3.1). Between all pairwise comparisons of treatments, a total of 612 proteins differed in abundance ($p < 0.05$, FDR < 0.2), 403 in the low treatment compared to the control, 93 in the high treatment compared to the control, and 251 in the high treatment compared to the low treatment (Figure 3.1B). Of the 403 proteins that were significantly different between the low treatment and the control group ($p < 0.05$, FDR < 0.2), 243 proteins were higher in abundance and 160 were lower in abundance. Of the 93 proteins that significantly differed in abundance ($p < 0.05$, FDR < 0.2) between the high treatment and the control, 62 of those were lower in abundance, and 31 were higher. Of the 251 proteins that changed in abundance between the high treatment compared to the low treatment ($p < 0.05$, FDR < 0.2), 135 proteins were higher in abundance, and 116 were lower in abundance in the high treatment relative to the low treatment.



A. **B.**

Figure 3.1. A summary of treatment effects on liver protein abundances following chronic exposure of adult male and female fathead minnows (*Pimephales promelas*) to excess nutrients. **A**. Principal component analysis of liver proteins, highlighting experimental treatments, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P). Each data point is one sample (n = 15 per control and high treatment, n=14 for low treatment) containing liver tissues randomly pooled from three fish residing in each respective treatment tank. **B**. Venn diagram of proteins that significantly changed in abundance between the low treatment and the control (left), the high treatment and the control (right), and the high treatment and the low treatment (bottom), as well as shared proteins that were significantly altered between treatments (center). The Benjamini-Hochberg FDR value of 0.2 was used to generate lists of significantly impacted proteins, with a total of 612 proteins changing in abundance between treatments.

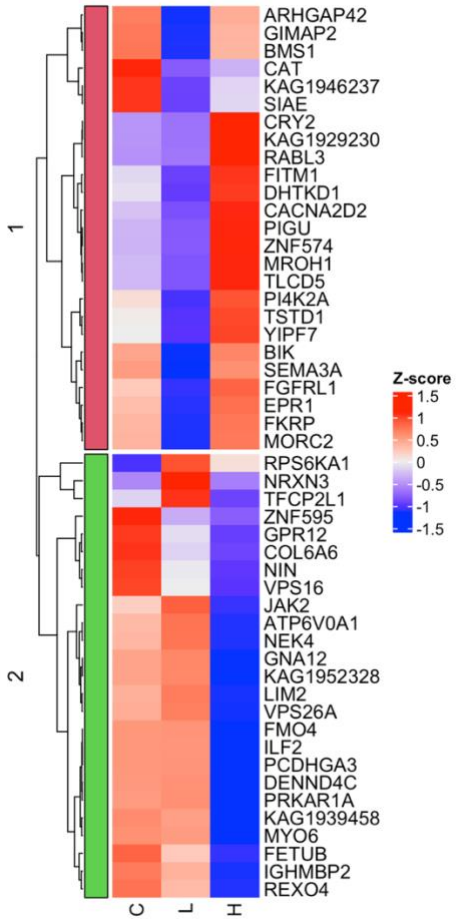


Figure 3.2. A heatmap of the top 50 differentially expressed proteins in the liver tissues of male and female adult fathead minnows (*Pimephales promelas)*, derived from a Limma differential expression analysis between the control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) treatments. The protein abundances are represented by z-scores, and the cluster blocks represent hierarchical clustering using Euclidean distances.

3.3.4 Mucus Protein Results

A total of 4428 unique proteins were identified in mucus during this experiment from a total of 108 samples across three sampling time points (Figure 3.3).

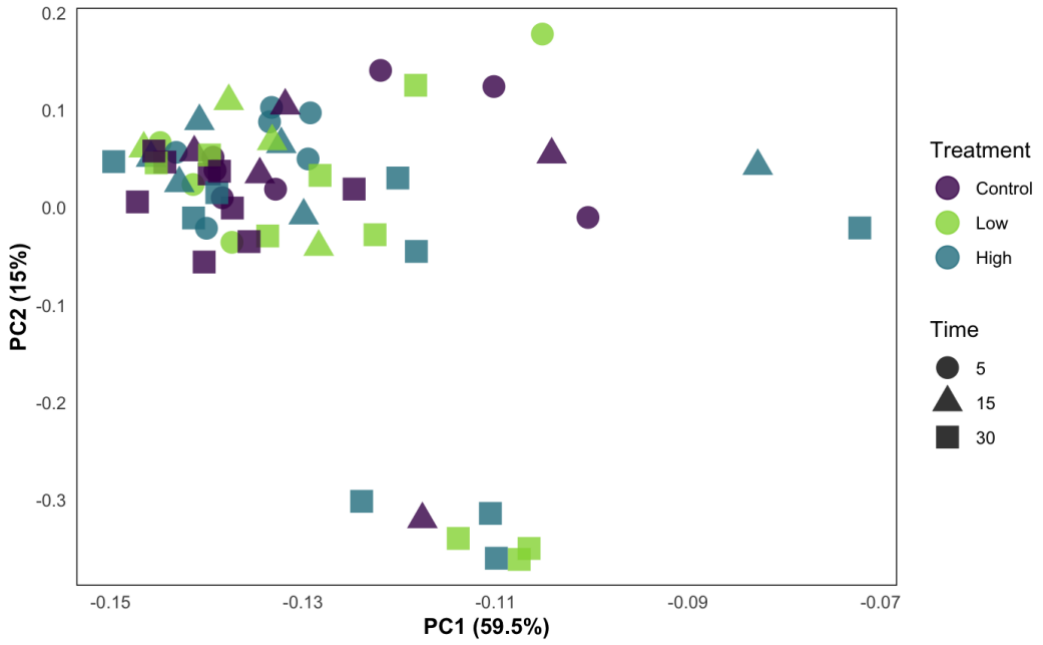


Figure 3.3. Principal component analysis of mucus proteins highlighting experimental treatments, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P), as well as sampling time points, day 5, day 15, and day 30. Each data point is one sample containing dermal mucus tissues randomly pooled from five fathead minnows (*Pimephales promelas*), a mix of male and female, residing in each respective treatment tank.

3.3.4.1 Treatment Effects

Few treatment-specific changes were observed in mucus protein abundances with a total of seven ($p < 0.05$, FDR < 0.2) between each treatment on each sampling day. Five of those occurred on sampling day 5, where four proteins changed in abundance between the high treatment and the low treatment, and one between the low treatment and the control ($p < 0.05$, FDR < 0.2). On day 30, two proteins significantly changed in abundance between the high treatment and the low (FDR adjusted $p = 0.03$).

3.3.4.2 Temporal Effects

The majority of the differences in mucus protein abundance were owed to temporal effects. Between day 30 and day 5, 234 proteins changed in abundance in the high treatment, 108 proteins in the low treatment, and 34 proteins in the control ($p < 0.05$, FDR < 0.2; Appendix B; SFigure 46). Whereas, between day 30 and day 15, 223 proteins changed in abundance in the high treatment, 16 in the low treatment, and 10 in the control ($p < 0.05$, FDR < 0.2; SFigure 46). No mucus proteins were observed to change in abundance between day 15 and day 5 between any of the treatments.

3.3.4.3 Interaction Effects

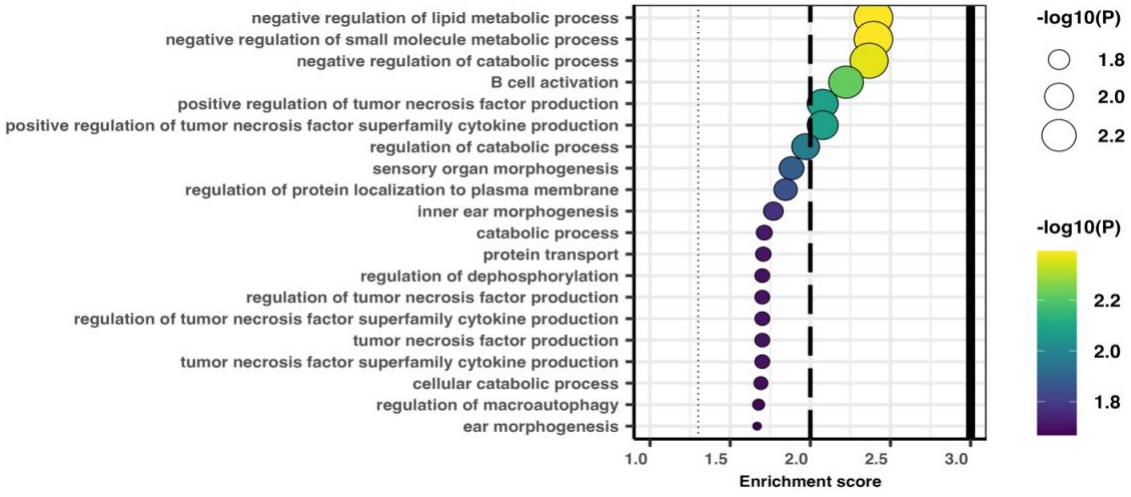
Few proteins changed in abundance owing to the combined effects of treatment and time. The only significant changes in abundances occurred between the high treatment and the control on day 15 compared to day 5, where three proteins changed in abundance, day 30 compared to day 5 between the low and the control where two proteins changed, and between the high and the low treatment where one protein changed. Finally, one additional protein changed between high treatment and the low treatment on day 30 vs day 15. A total of seven proteins changed in

abundance owing to the effects of treatment and exposure time ($p < 0.05$, FDR < 0.2; Appendix B; SFigure 46).

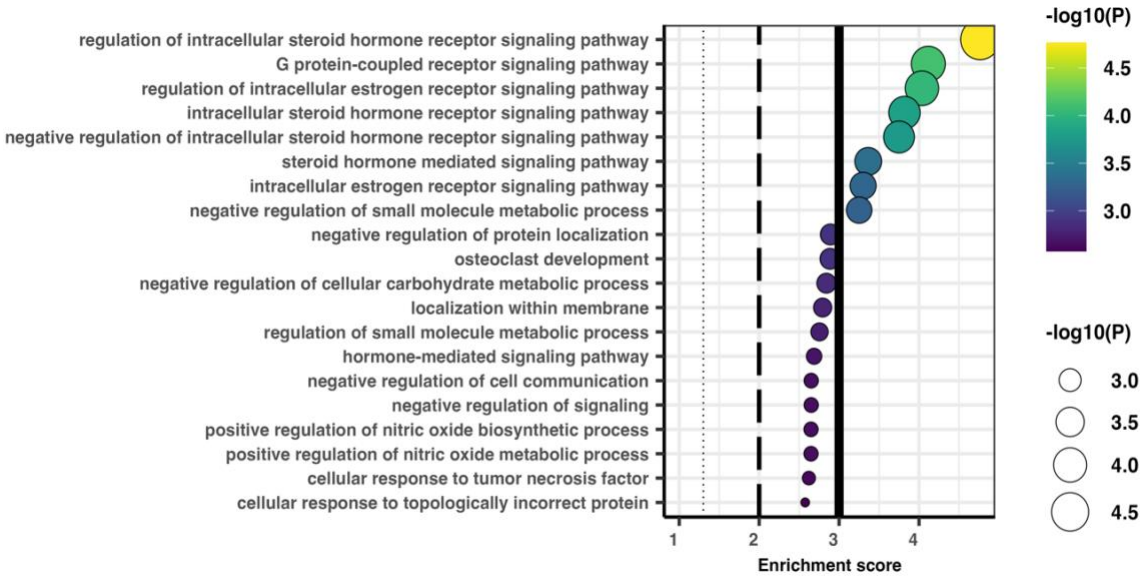
3.3.5 Protein Enrichment Analysis

3.3.5.1 Liver Enrichment

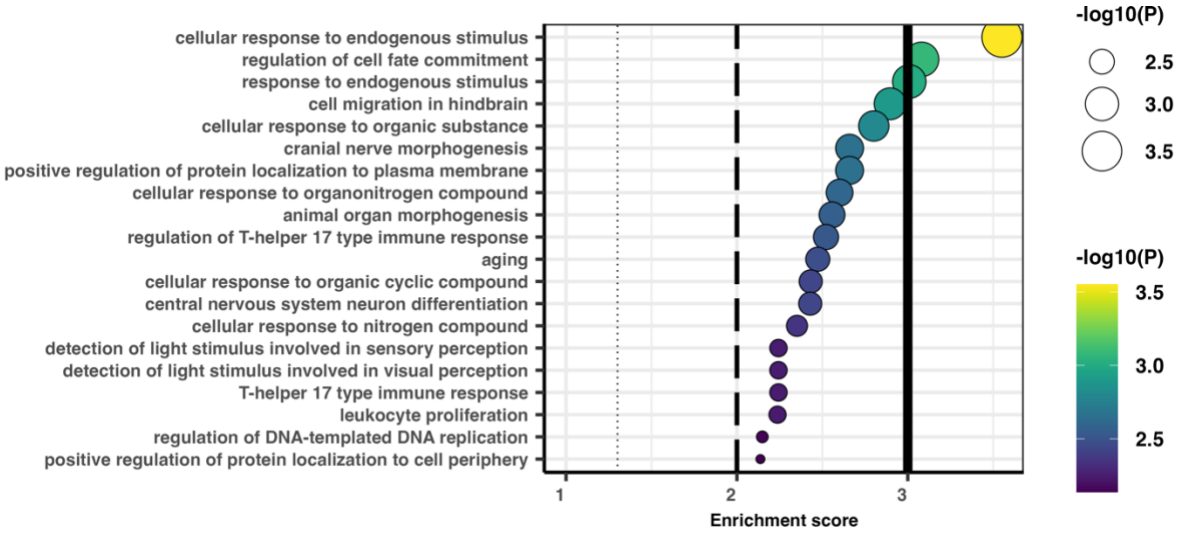
The top 20 enriched GO biological process terms of liver proteins were found for each treatment comparison in FHM liver proteins (Appendix B; STable 10 - 12). Enrichment patterns are shown for each treatment comparison including high, low, and control (Figure 3.4). Metabolic processes and immune responses emerge at the forefront of the high treatment enriched pathways in both comparisons, where responses to endogenous stimuli and nitrogen compounds are mainly visible in the low treatment enrichment compared to the control (Figure 3.4).



A.


regulation of intracellular steroid hormone receptor signaling pathway
G protein-coupled receptor signaling pathway
regulation of intracellular estrogen receptor signaling pathway
intracellular steroid hormone receptor signaling pathway
negative regulation of intracellular steroid hormone receptor signaling pathway
steroid hormone mediated signaling pathway
intracellular estrogen receptor signaling pathway
negative regulation of small molecule metabolic process
negative regulation of protein localization
osteoclast development
negative regulation of cellular carbohydrate metabolic process
localization within membrane
regulation of small molecule metabolic process
hormone-mediated signaling pathway
negative regulation of cell communication
negative regulation of signaling
positive regulation of nitric oxide biosynthetic process
positive regulation of nitric oxide metabolic process
cellular response to tumor necrosis factor
cellular response to topologically incorrect protein
1
2
3
4
Enrichment score
-log10(P)
4.5
4.0
3.5
3.0
-log10(P)
3.0
3.5
4.0
4.5

B.


cellular response to endogenous stimulus
regulation of cell fate commitment
response to endogenous stimulus
cell migration in hindbrain
cellular response to organic substance
cranial nerve morphogenesis
positive regulation of protein localization to plasma membrane
cellular response to organonitrogen compound
animal organ morphogenesis
regulation of T-helper 17 type immune response
aging
cellular response to organic cyclic compound
central nervous system neuron differentiation
cellular response to nitrogen compound
detection of light stimulus involved in sensory perception
detection of light stimulus involved in visual perception
T-helper 17 type immune response
leukocyte proliferation
regulation of DNA-templated DNA replication
positive regulation of protein localization to cell periphery
1
2
3
Enrichment score
-log10(P)
2.5
3.0
3.5
-log10(P)
3.5
3.0
2.5

C.

Figure 3.4. The enrichment score (-log10(P-value)) of the top 20 biological process GO terms with at least three significant protein hits per term from liver tissues of fathead minnows (*Pimephales promelas*) belonging to one of three experimental groups: control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), or high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P). Each plot contains GO terms derived from a differential expression analysis between experimental groups including high nutrient exposure compared to the control (A), high nutrient exposure compared to the low (B), and low nutrient exposure compared to the control (C). Cut-off lines drawn at equivalents of p=0.05 (dotted), p=0.01 (dashed), p=0.001 (solid).

3.3.5.2 Mucus Enrichment

Few proteins were differentially expressed in mucus owing to treatment effects and the combined effects of treatment and exposure time. Therefore, only temporal effects were investigated for enrichment. Temporal effects were observed only in the high treatment and the low treatment between experiment day 30 and day 5 (Appendix B; STable 13 and 14). Whereas all three treatments saw differential expression of proteins between day 30 and day 15 (Appendix B; STable 15 – 17).

3.4 Discussion

3.4.1 Exposure to sublethal concentrations of nutrients effects liver protein abundances

The proteome can describe the state of a cell, showing sensitivity to environmental influences and time, making it a useful tool in toxicogenomic research with potential future applications in environmental monitoring and chemical risk assessments (Gouveia et al., 2019). The objective of this experiment was to conduct a non-targeted proteomic evaluation of liver and epidermal mucus tissues from FHMs exposed to sublethal, and environmentally-relevant concentrations of nutrients. We aimed to validate epidermal mucus as a non-lethal alternative for proteomic evaluation of cellular stress responses. There is a growing body of research analyzing mucus proteins in a variety of fishes (Fæste et al., 2020; Raposo de Magalhães et al., 2023). Yet, targeted proteomic evaluations of vitellogenin remain the only research conducted for epidermal mucus tissues in FHMs, with no prior research having investigated the entire mucus proteome (See et al., 2022). Due to the sublethal nature of our exposures to elevated nutrients, we hypothesized that mortalities would not be observed, but that apical indices and cellular stress response would be impacted, with a relationship between mucus and liver protein patterns on day 30. However, we found that chronic exposure to environmentally-relevant concentrations of nutrients did not cause mortality or affect apical indices in male and female adult FHMs (Appendix B; SFigure 42 - 44). Chronic nutrient exposure elicited a cellular stress response observed in liver tissues, but not mucus tissues (Figure 3.2; Appendix B; SFigure 46). Mucus protein abundances remained almost completely unaffected due to treatment effects and the combined effect of treatment and exposure time, yet mucus proteins showed temporal effects in each treatment (Appendix; SFigure 46). Due to the lack of differences in mucus protein

abundance owing to treatment effects on day 30, we found limited evidence for the suitability of the mucus proteome for quantifying stress due to chronic nutrient exposure in fathead minnows.

3.4.2 Nutrient water chemistry

The combined effect of TAN build-up in control tanks, and the suboptimal concentrations of TAN in treatment groups may explain the lack of protein level changes observable in mucus tissues. Despite a daily water volume change of 50% of the experimental tank, TAN in the control groups were above zero during experiment day 6 – 8 and day 25 – 27 (Table 3.1; Appendix B; SFigure 35). Additionally, the low and high experimental groups did not reach targeted nutrient concentrations during the 30-day exposure. For example, the high treatment had exposure concentrations ranging from 0.24 – 1.98 mg L^{-1} TAN throughout the study, where target concentrations were 4.17 mg L^{-1} TAN (Appendix B; SFigure 35). This may be partially attributed to a shift to alkaline water conditions which are favorable for the switch of NH_4^+ to NH_3. Unionized ammonia can readily undergo volatilisation, allowing NH_3 to escape from the tank water into the atmosphere (White and Reddy, 2009). This event can occur quickly if water conditions shift, where NH_3 gas can increase from 0.005% to 31.3% when pH increases from 5 to 8.9 (She et al., 2018). Ammonia can be retained in the water column through denitrification, in which ammonia is oxidized to nitrite, and then nitrate but even here nitrogen can eventually be lost as atmospheric nitrogen (N_2; White and Reddy, 2009). Despite lower concentration of TAN across treatment tanks, several trends that are consistent with ammonia exposure in fish were detected in liver tissues. These responses include immunological effects and alterations in metabolism (Figure 5; Zhang et al., 2021; Wang et al., 2021). Liver is the primary site of detoxification and metabolism of xenobiotics making it a sensitive tissue to examine cellular

changes resulting from ammonia stress (Topić Popović et al., 2023). Whereas epidermal mucus, although acting as a physical and biochemical barrier to contaminants, is not known to play a role in detoxification of environment pollutants and therefore the differing exposure pathways and tissue specific functions may explain the lack of consistency between protein responses in the two tissues (Reverter et al., 2018). Mucus is constantly being secreted, aiding in its acclimatization to the surrounding environment to reach homeostasis, making the chronic exposure period likely sufficient to allow acclimatization to occur and prevent responses from being visible between treatments. Obtaining mucus following an acute exposure, such as 48-hours post dosage, would be a useful period to measure and would provide insight into mucus' acute response to nutrients. While mucus may be sensitive to certain types of chronic stressors, such as handling stress (Cámara-Ruiz et al., 2022), the suboptimal TAN concentrations were likely insufficient to see a response. Furthermore, a maximum fold change of only 3.16 was observed (MROH1) among the high treatment of liver proteins, which may also be explained by the lower than anticipated environmental ammonia conditions.

3.4.3 Immune response

Alterations in fish immune responses have been used as an indicator of toxic effects for exposure to ammonia due to its role as an immunotoxic agent (Xu et al., 2021). Ammonia influences immune responses in fish, particularly in cytokines, lymphocytes, inflammation, and apoptosis (Xu et al., 2021). Cytokines are a group of signalling proteins secreted by peripheral immune cells and play an important role in controlling the activity of the immune system by inducing inflammation (Yu et al., 2020). Two important pro-inflammatory cytokines include tumour-necrosis factors (TNFs) and interleukins (ILs), which are commonly used as markers of macrophage inflammatory responses (He et al., 2021). In the present study, GO biological

processes enrichment analysis of liver proteins showed an inflammatory response in the high treatment. This includes several GO terms related to positive regulation and regulation of TNF superfamily cytokine production, TNF production, B cell activation, and regulation of macroautophagy in the high treatment compared to the control. As well, fish from the high treatment showed enrichment of cellular response to TNF compared to fish from the low exposure group. Upregulation of genes for inflammatory cytokines have been observed in several fish species exposed to ammonia including the hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), pufferfish (*Tetraodontidae*), and yellow catfish (*Pylodictis olivaris*; Yan et al., 2021; Cheng et al., 2015; Li et al., 2020). Interleukin-1, IL-2, and IL-12 are commonly assessed cytokines to detect immune responses following ammonia exposure in fishes (Zhang et al., 2021; Li et al., 2020; Yu et al., 2020). Interleukin enhancing binding factor 2 (ILF2), a transcription factor required for interleukin 2 gene, was significantly lower in the high exposure treatment compared to the low exposure group and the controls. Further in the low treatment, IL1RAP, a protein that forms the interleukin-1 receptor complex, was found to be lower, while IL-12B was found to be higher compared to the control. Zhang et al. (2021) found serum inflammatory cytokines to initially increase, followed by reduction in large-scale loach (*Paramisgurnus dabryanus*) exposed to ammonia, which could explain the inconsistency in IL expression patterns shown between the treatment groups.

Genes involved in the TNF and cytokine regulation GO terms include a cluster of differentiation 2 (CD2) and 84 (CD84), janus kinase 2 (JAK2), and Ephrin type-B receptor 2 (EPHB2). In the high treatment, CD2 and JAK2 proteins were significantly lower compared to the low and control treatments. JAK2 is a part of the janus kinase family of tyrosine kinases that

contribute to cytokine-mediated inflammatory responses, while CD2 plays an important role in T cell activation and T-cell mediated immune processes (Xie et al., 2023; Binder et al., 2020). T-cells, primarily T helper cells, are a key producer for cytokines, where increased ammonia levels have been found to decrease T-cell proliferation, and in turn several cytokines including IL-2 in human models (Bell et al., 2023). JAK2 is essential for cytokine signalling, and chronic ammonia exposure may have initiated the suppressor of cytokine signalling (SOCS) family, which are cytokine-induced and can negatively feedback to inhibit cytokine receptor signalling pathways, specifically by inactivating JAKs, which could be the reason for the observed decreased abundance (Gonçalves et al., 2017). The lower abundance of two key proteins observed which serve immunological functions suggest immunosuppression due to ammonia. Immune suppression is a common outcome observed during high ammonia exposure in fishes (Guo et al., 2022; Gonçalves et al., 2012; Zhang et al., 2021). Furthermore, both CD2 and JAK2 mRNA expression are known to be altered by ammonium chloride exposure in rat astroglial cells (Bodega et al., 2006). On the other hand, CD84, a member of CD2 immunoglobulin superfamily known to play a large role in immune interactions, was higher in the high treatment compared to the control (Cuenca et al., 2019). This protein is a part of the signalling lymphocyte activation molecule family which aids in immune signalling pathways, and acts as an essential piece to both the innate and adaptive immune responses (Cuenca et al., 2019). The higher protein abundance may be a compensatory mechanism to maintain immune cell signalling and communication during high environmental ammonia when other areas of the immune response are being activated or suppressed. Ephrin type-B receptor 2, a receptor tyrosine kinase, partakes in several immune responses involved with cell adhesion and trafficking, as well it plays a role in T-cell activation and regulation, where interactions between EPHB2 and its ligands can induce pro-

inflammatory cytokine secretion (Butler and Schmidt, 2015; Darling and Lamb, 2019). EPHB2 was significantly higher in both the high and low treatment compared to the control, and EPHB1 was higher solely in the low treatment. Not only are EPHBs known to play a role in immunity, but they also assist in a wide array of signalling pathways including neurological physiology and diseases (Darling and Lamb, 2019). Acute ammonia toxicity leads to activation of NMDA receptors in the brain, where excessive activation during high ammonia exposures lead to death (Monfort et al., 2002). EPHB1/EPHB2 are tied closely with NMDA receptor function, acting as key regulators of NMDA receptor synaptic localization and regulation (Nolt et al., 2011). Therefore, the response of EPHB proteins may be a result of ammonia induced activation of immunity or activation to assist in NMDA receptor function during neurological ammonia toxicity.

B cells are important regulatory components of the immune system with capacity to contribute to inflammatory pathways directly, and through the production of key members of an immune response (Althwaiqeb and Bordoni, 2023). B cell activation GO biological process was significantly higher in the high treatment compared to the control, and leukocyte proliferation was observed in the low treatment. B cell activation is associated with several signalling pathways that are mediated by phosphatidylinositol 3-kinase (PI3K). PIK3CG, the catalytic subunit of the enzyme PI3K, was significantly higher in the low treatment compared to the control. The formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) through PI3K helps to form a signalosome containing the enzyme phospholipase-C (PLC; Fruman and Limon, 2012). Phospholipase-C then hydrolyzes phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2) leading to an eventual proliferation of B cells (Fruman and Limon, 2012). The protein phosphatidylinositol 4-phosphate 5-kinase type-1 beta (PIP5K1B), responsible for producing PI-4,5-P2, as well as

protein PLCL2, involved in B-cell signalling, were both significantly higher in the low treatment compared to the control. Alternatively, PI3K can act in the AKT pathway downstream of B cell activation, where PIP3 recruits AKT ultimately playing a significant role in B cell regulation (Limon and Fruman, 2012). In the present study, AKT1 was significantly downregulated in the high treatment relative to the low, but it was not affected in the high treatment compared to the control. The transcription levels of *akt* mRNA have been found to be significantly reduced in the head kidney of Wuchang bream (*Megalobrama amblycephala*) upon chronic ammonia exposure (Guo et al., 2022). Additionally, AKT protein levels were significantly reduced in pig spleen exposed to ammonia (Zhou et al., 2022). Alternatively, Il-4 can also act to help stimulate B cells when PI3K-AKT signalling is inhibited. Interleukin 4 receptor (IL4R) was upregulated in the low treatment compared to the control, potentially acting as a compensatory mechanism when ammonia decreased abundance of AKT protein. Interestingly the protein changes were only observed in the low treatment, and not the high treatment, despite the GO term enrichment of B cell activation.

Following B cell activation, B lymphocytes differentiate into plasma cells which secrete antibodies, or immunoglobulins (Althwaiqeb and Bordoni, 2023). Immunoglobulin mu DNA binding protein 2 (IGHMBP2) was significantly downregulated in the high compared to the low treatment and control. Ammonia stress that persisted longer than 48 hours has resulted in decreased immunoglobulin (IgM) in the liver of hybrid grouper. Furthermore, ammonium chloride is known to affect the expression of *ighmbp2* mRNA in rat astroglial cells (Yan et al., 2021; Bodega et al., 2006).

Nitric oxide (NO) is a diverse, gaseous, signalling molecule which functions in several physiological processes including immunity, metabolism, and vasodilation (Lundberg and

Weitzberg, 2022). Nitric oxide also plays a role in responding to different stressors in fish, including high environmental ammonia (Kumari et al., 2018). Positive regulation of nitric oxide biosynthetic and metabolic processes was significantly enriched in the high treatment compared to the low treatment. Nitrite levels were elevated in the high treatment, which provides a substrate for NO synthesis, especially with slightly more acidic conditions in the high treatment (Table 1; Appendix B; Lundberg and Weitzberg, 2022). Nitric oxide synthase (NOS) is an immune related enzyme that has been found to be impacted by ammonia exposure in fish species including the amur minnow (*Rhynchocypris lagowskii*; Yu et al., 2020). More specifically, NOS is involved in neuronal ammonia toxicity, where over activation of NMDA receptors due to high ammonia causes influxes of Ca^{2+}, resulting in the binding of calmodulin, and subsequent activation of NOS (Monfort et al., 2002). NOS enzymes are found to be upregulated in response to stress and inflammatory cytokines, as observed in the present study exhibiting several related enriched GO terms and proteins (Gadek-Michalska et al., 2013).

As a whole, the immune responses of FHMs were affected by sub-lethal chronic exposure to ammonia, leaving fish with altered or reduced immune function. This response may leave FHMs susceptible to infections or disease, therefore chronic ammonia exposure even at lower environmental concentrations may be deleterious to the health and long-term survival of small-bodied fish.

3.4.4 Excess nitrogen effects metabolic processes

Hepatotoxicity due to ammonia exposure is often accompanied by disruptions in metabolic processes (Dong et al., 2020). High environmental ammonia can impair excretion and result in accumulation of endogenous ammonia, thereby disrupting the homeostatic mechanisms it normally helps to regulate (Edwards et al., 2024). The accumulation of ammonia may have

affected nitrogen metabolism in the low treatment, where the enrichment of liver proteins was observed as the GO terms cellular response to organonitrogen compound, cellular response to nitrogen compound, and cellular response to endogenous stimulus (Figure 3.4). However, this effect was not observed in the high treatment, perhaps due to other metabolic processes that showed a stronger response. For example, in the high treatment compared to the control, several other metabolic processes were enriched including down regulation of lipid and small molecule metabolic processes, and negative regulation of catabolic processes. When environmental conditions are poor, fish can reduce the rate of ammonia produced from protein catabolism to slow endogenous ammonia build up resulting in decreased ammonia excretion (Ip and Chew, 2010). Ammonia toxicity in fish can be observed as mitochondrial dysfunction in the liver where oxidative phosphorylation can be uncoupled, reducing energy production with a shift to the less efficient anaerobic metabolism (Ip and Chew, 2010). Ammonia toxicity can result in the stimulation of glycolysis, and interference with metabolism by disrupting the mitochondrial tricarboxylic acid cycle (Ip and Chew, 2010). An upregulation of pyruvate dehydrogenase kinase 3 (PDK3) was observed in the high treatment compared to the low. Through phosphorylation, PDK3 can inhibit pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA prior to entering the TCA, therefore promoting anaerobic metabolism by preventing glucose metabolism in the TCA (Wang X. et al., 2021). The increase in PDK3 abundance may further indicate a shift to anaerobic metabolism as a protective mechanism against ammonia toxicity. This phenomenon was observed in Chinese mitten crabs (*Eriocheir sinensis*) exposed to ammonia, which showed a downregulation of the tricarboxylic acid cycle process (Wang T. et al., 2021). On the other hand, this may not be a protective mechanism against toxicity but rather an outcome of ammonia accumulation. NH_4^+ can dissociate readily to NH_3 and H^+ under alkaline

conditions present in the mitochondrial matrix and therefore NH_3 can move freely through membranes or aquaporin channels into the intermembrane space, where it couples back to NH_4^+ under slightly more acidic conditions, binding hydrogen ions that have been pumped to form a proton gradient for ATP synthesis (Ip and Chew, 2010). This permeation to the intermembrane space can disrupt the proton gradient and therefore uncouple oxidative phosphorylation (Ip and Chew, 2010; Figure 6). These phenomena may together explain the downregulation of catabolic processes observed in the high treatment. Specifically, cellular responses of FHMs appear to be ameliorating ammonia accumulation to reduce an already compromised metabolic state.

Shifting metabolism to anaerobic respiration would limit the accumulation of reactive oxygen species (ROS) produced by oxidative phosphorylation that are common by-products of ammonia exposure in fish (Guo et al., 2023). Ammonia is shown to increase ROS production in a dose-dependent manner in rat astrocytes (Murthy et al., 2001). Fish exposed to high environmental ammonia consistently show dysregulation of oxidative stress genes such as catalase and glutathione S-transferase, due to the increased production of free oxygen radicals (Cheng et al., 2015; Hegazi et al., 2010). This could potentially explain the dysregulation of catalase and glutathione S-transferase P1 in fish from the low treatment. While there was no effect on oxidative stress genes observed among fish from the high treatment, the enriched GO biological processes which suggest a shift to anaerobic metabolism in these fish signify a reduction in ROS production by uncoupling the electron transport chain, which may potentially affect the abundance of antioxidant enzymes. Whereas the low treatment did not display enrichment of GO terms indicative of anaerobic metabolism. Furthermore, down regulation of lipid metabolism was also observed in the high treatment, which may indicate another mechanism for liver cells to reduce the production of ROS. Lipid metabolism in the liver can

generate a significant amount of ROS, especially during fatty acid oxidation (Endale et al., 2023). Decreased abundance of acyl-CoA dehydrogenase (very long chain (ACADVL)) among fish from the high treatment supports the notion that oxidative lipid metabolism was reduced. ACADVL handles β-oxidation of fatty acids to generate acetyl-CoA (Uniprot, 2024), therefore a decreased abundance implies a shift towards glycolytic metabolism to reduce ROS production. Among FHMs from the high exposure, several metabolic processes were inhibited that were not affected among FHM from the low treatment, which exhibited only downregulation of small molecule and cellular carbohydrate metabolic processes. This suggests a potential dose-dependent response in metabolism, in which the higher concentrations of ammonia can result in inhibited metabolism, while lower exposures affect fewer metabolic pathways.

3.4.5 Mucus protein abundances

While few mucus proteins responded to ammonia exposure, temporal effects, baseline ammonia levels in controls, and lowered sample sizes in day 5 and 15 may have obscured treatment-related responses in mucus, which revealed distinctions in tissue sensitivity to chronic ammonia exposure. Due to the batch effects observed, 22 samples from experiment day 5 and 15 were removed from the analysis (Appendix B; SFigure 45). The lower sample size on day 5 and 15 may have prevented treatment level effects from being visible on these days. Only two mucus proteins, RPS27A and POSTN, significantly differed in abundance between the high and low exposures on day 30, while neither protein differed in abundance in liver tissues. Additionally, mucus tissue results highlight that exposure time influenced mucus protein abundances, with a clear trend of more dysregulated protein enrichment as treatment severity increased on day 30 compared to day 5 (Appendix B, SFigure 46). Interestingly, the GO term ‘TCA cycle’ was also enriched in the high treatment of mucus proteins, with three proteins having lower abundance in

day 30 compared to day 15 (Appendix B; SFigure 48). Furthermore, several immune-related biological processes were also enriched in this group including lymphocyte differentiation and activation (Appendix B; STable 48). These temporal-level mucus responses coincide with the patterns described in metabolic and immunological differences in liver proteins. Mucus may not provide the same level of sensitivity as liver tissues to chronic ammonia exposure, but it is important to note that the fish from the control treatments in this experiment were also exposed to measurable concentrations of ammonia, TAN, nitrite and nitrate values over the course of the experiment (Table 3.1). These exposures likely arose as residual ammonia built up prior to each static renewal of water changes in the exposure tank. Therefore, it is possible that the sublethal exposure concentrations were not sufficient, especially as targets were not met, or the presence of low ammonia in the control exposures masked a treatment-level response in mucus tissues.

Mucus proteins in control FHMs had few differences in abundance between day 30 and day 15 but differences in abundance were observed between day 30 and 5, indicating that the effects of the external ammonia build-up may have caused a response beginning around day 15 and continuing to the end of the experiment. Therefore, the differences in abundance in control mucus proteins presents an additional explanation as to why mucus proteins did not pick up on treatment-level effects. The control had above zero nutrient values and possessed differences in measured protein abundances between experiment days, which could be sufficient to mask treatment level effects in the low and high treatments, which already did not reach optimal nutrient exposure concentrations. For example, a protein of interest in ammonia detoxification, GLUD1, or glutamate dehydrogenase, responsible for converting α-ketoglutarate and ammonium to glutamate, was greater in mucus proteins in the high treatment on day 30 compared to day 5 (FDR = 0.08). However, this result was not observed when compared with the control,

indicating that GLUD1 also increased in the control between day 30 and day 5 to some extent, likely due to ammonia build up where GLUD1 was required to detoxify ammonia to glutamate in control tanks. Therefore, the lack of a true control makes it difficult to uncover treatment-level effects, especially under lower than desired exposure conditions, which also showed a weak response in liver proteins, a more sensitive tissue, with 3.16 being the highest fold change observed. Despite the absence of comparable results between tissues, FHM epidermal mucus may still provide important information regarding the cellular stress response to excess nutrients. Future studies should focus on investigating acute effects, ensuring exposure nutrient concentrations meet targets, and preventing ammonia build up in the controls, to uncover reliable markers of stress in mucus proteins.

3.5 Limitations, conclusions, and future directions

We hypothesized that chronic exposure to excess nutrients would not cause mortality in adult fathead minnows, but that apical endpoints, and protein-level stress responses would be impacted in both liver and mucus tissues with increasing nutrient concentration and exposure time. Chronic exposure to low environmental ammonia in fact did not affect apical endpoints but was sufficient to produce immunological and metabolic stress responses in the proteomes of liver tissues of male and female FHMs. Further, we hypothesized that mucus protein patterns would be representative of cellular stress patterns observed in liver tissues, specifically on day 30 when both tissues were sampled at the same time point. Epidermal mucus proteins did not exhibit a treatment-level response, whereas liver tissues appeared to be a more sensitive tissue to ammonia stress. However, increased exposure time to ammonia did elicit a progressively greater temporal response in mucus tissues. It is important to recognize that an FDR adjustment of 0.20 was

examined in this study for exploratory purposes, and for the purpose of discussing overall patterns of biological processes in the dataset in combination with enriched protein sets. Therefore, these conclusions were derived from both evidence of protein-level changes and GO term enrichment to improve confidence in the observed patterns being biologically important. Furthermore, our results suggest that FHM mucus proteins can be effectively sequestered using Kimwipe™ and ultrafiltration methods. Replication of this study should focus on reducing residual ammonia in the control exposures and using a higher sublethal concentration to achieve target environmentally-relevant values. This could potentially increase the ability to resolve mucus protein changes as a result of ammonia exposure. Finally, it would be useful to investigate mucus proteins during the acute (i.e. 48-hour or 96-hour) and chronic phases of ammonia exposure to compare protein level responses of mucus tissues to the large body of literature focused on acute effects of ammonia on cellular responses in a variety of aquatic animals.

Chapter 4: Synthesis and suggestions for further research

4.1 Laboratory versus wild-caught fish models

A common discussion in biological and toxicological research is the use of laboratory reared versus wild fish models. More specifically, is research conducted using laboratory reared fish relevant to wild fish populations? This was an important consideration when conducting both the field study in chapter two and the laboratory study in chapter three. Each study was performed using fish from different populations with different life histories. The laboratory study utilized first generation progeny of fathead minnows (FHMs; *Pimephales promelas*) sourced from two different laboratory populations. The field study utilized a population of FHMs sourced from Lake Muir, Manitoba, situated in a local wildlife preserve. The wild population had generations to adapt to a system dealing with diurnal cycles of temperatures resulting in considerable fluctuations, and to develop compensatory mechanisms for reduced dissolved oxygen while living in a eutrophic system. For example, FHMs residing in Lake Muir the year prior, during May 2021, experienced dissolved oxygen levels ranging from 0.35 mg L^{-1} at 6m depth – 12.49 mg L^{-1} at 1m depth, while in October 2021, levels ranged from 0.02 mg L^{-1} at 7m to 3 mg L^{-1} at 1m depth (Appendix A; SFigure 23). The dissolved oxygen levels in Lake Muir have followed a similar trend since its first recording in 2013 (Appendix A: SFigure 28). Meanwhile, total phosphorus began spiking above 0.4 mg L^{-1} in September of 2017, and continued to increase to maximum levels of 0.69 mg L^{-1} in years following (Appendix A; SFigure 27). The water quality conditions in Lake Muir may have impacted the early life stage and development of FHMs, where fish are reliant on environmental cues such as dissolved oxygen, temperature, and dissolved solids for development and ontogeny (Ali et al., 2017). The exact time FHMs are believed to have occupied Lake Muir following excavation in 1920 is

unknown, however the earliest report of FHMs occupying the lake is 1982 (R. A. Ratynski, MSc Thesis, University of Manitoba). This population had experienced stressful fluctuations in water quality for several years, and therefore, may have developed genetic adaptations to deal with these stressors. This was apparent in Chapter two, where FHMs experienced severe dissolved oxygen levels and high temperatures, yet survived throughout the ~70-day study period (Appendix A; SFigure 1 and 2). Therefore, the opportunity for developmental plasticity and adaptation owing to exposure to poor water quality over generations may have been advantageous for this population of fathead minnows during the aquaculture wastewater (AWW) exposure period. In contrast, the lab-reared population experienced constant temperature, light patterns, and dissolved oxygen for generations. Laboratory reared fish can in turn acclimatize to their consistent environment, where a series of alterations have been noted from the domestication of zebrafish (*Danio rerio*), including behavioural, morphological, physiological, and genetic adaptations (Robinson and Rowland, 2005). This is an especially important consideration for a study focused on investigating the effects of excess nitrogen and phosphorus. Again, the wild population would have experienced fluctuating levels of nitrogen and phosphorus prior to the study period, but the laboratory reared fish would have been reared in unchanging water quality conditions. Consequently, developmental plasticity and genetic adaptations of the two FHM populations used in this thesis are an important consideration when synthesizing conclusions from both experiments.

Both in-situ and ex-situ research have their respective places in toxicology to determine the true effect of a contaminant on target endpoints. Laboratory-based studies have the advantage of controlling environmental parameters including temperature, dissolved oxygen, light-dark cycles, diet, and habitat, to exclusively investigate the effect of a contaminant on your study

organism. However, these results are limited in their application to actual ecosystems which are not controlled for the above parameters and may possess additional external variables such as dissolved oxygen and temperature fluctuations, which would impact the molecular response observed. Therefore, *in-situ* research performed in miniature ecosystems called 'mesocosms', as in Chapter two which aim to mimic aquatic environments, are a valuable tool for assessing the impact a toxicant will have on a wild fish population in conditions closer to its true habitat or one similar. Mesocosms have the advantage of being pragmatic, mirroring what an organism would truly experience. However, with additional factors at work such as fluctuating temperatures and dissolved oxygen, inconsistent diet, increased risk of disease or infection, it can be difficult to determine the true effect of the contaminant. In this way, it is useful to conduct both in and ex – situ experiments to gain a full understanding of a stressor in diverse ecosystems.

While exposing the organism in a realistic manner using mesocosm or field-based studies has ecological implications, it would be incorrect to assume that wild fish populations share an identical ecosystem and experience the same external stressors, and therefore it cannot be presumed that each wild population would respond in the same manner. For this reason, replication of field studies using fish populations sourced from diverse ecosystems would be important when drawing conclusions on the effect of a contaminant on wild fish species due to the impact of adaptation.

4.2 Recommendations for future research

4.2.1 Considerations for transcriptomic and proteomic analyses of fathead minnow epidermal mucus tissues

One of the main objectives of this thesis was to conduct minimally invasive sampling using epidermal mucus tissues of small-bodied fish. Here, tissues would be analyzed using well-developed and widely used molecular techniques to identify underlying cellular stress responses while minimizing harm to the fish. The two techniques investigated include targeted transcriptomics using qPCR discussed in chapter two, and untargeted proteomics using LC-MS/MS protein quantification discussed in chapter three. While experiments in chapter three were largely successful in identifying and quantifying mucus proteins, this method would benefit from further modifications. Pooling 4 – 5 FHMs was required to obtain adequate protein content for a single sample. The amount of FHMs required depended on the size of the fish, in which larger fish reached adequate protein levels with fewer pooled fish. For example, trials conducted for mucus proteins from field experimented FHMs, which were considerably larger (5 – 7 cm) compared to lab reared minnows (3 – 5 cm), reached more than adequate protein concentrations at only 4 pooled fish per sample. The FHMs in chapter three's laboratory study were 8-months old and yet to reach full size of maturity. Therefore, the amount of mucus sampled is highly size-dependent in FHMs and presents limitations of environmental realism when attempting to capture molecular effects in mucus tissues that reflect a population which consists of a wide range of ages and sizes. Additionally, future research should consider pooling FHM male and female fish separately. In this experiment, pooling male and female fish was completed because of insufficient numbers of each sex in experimental tanks (8 females, 7 males), where five pooled fish was determined to reach adequate protein levels for one sample. Therefore,

combining sexes was required to reach pooling numbers, and have three replicates within each experimental treatment replicate. Consequently, this limited results in chapter three with an inability to investigate sex-specific effects. Therefore, future research should ensure adequate numbers of each sex are present in each experimental group to prevent pooling sexes, and thus losing sex-specific variation.

In chapter two, epidermal mucus tissues were attempted to be analyzed on the EcoToxChip in addition to liver tissues. Mucus was sampled from FHMs by running a sterile CultureSwab™ (BD BBL™ 220115) laterally across the fish's body until the swab appeared saturated. The swab was stored in a 5-mL cryogenic vial (Corning®, CLS431417) and frozen immediately at -78.5°C on dry ice for transportation to the laboratory where it was stored at -80°C until analyzed. Several methods were attempted for RNA extraction, each using the RNeasy® Mini Kit (Qiagen, Cat. No. 73404) as described in chapter two. Here, each swab was placed into a 2-ml lid-locking microcentrifuge tube with a 5 mm stainless steel bead (Omni International, SKU. 277005), where 900 µL of QIAzol® Lysis Reagent (Qiagen, Cat. No. 79306) was added, the remainder was followed according to methods previously described in chapter two. The result of mucus RNA extractions was an inadequate concentration of RNA and a high contamination rate including impurities (A260/A280 < 2). Therefore, this method was repeated with several variations including fractioning the 900 µL of QIAzol® Lysis Reagent such that 450 uL were used for extraction, and 450 µL was used to rinse the swab when removed from solution following homogenization. Trials also consisted of leaving the swab in solution until the aqueous phase was removed, however this resulted in higher contamination rates. An additional clean-up step was also implemented using the RNeasy® MinElute Cleanup Kit (Qiagen, Cat. No. 74204) which aided in purifying mucus RNA; however, the amount of mucus

RNA extracted was still insufficient for an EcoToxChip analysis. A potential solution may be using alternative RNA extraction methods, such as using Trizol, which recovered the highest yield and purity of total RNA in sputum compared to RNeasy Mini kit and acid guanidinium thiocyanate–phenol–chloroform extraction (Xiang et al., 2001).

To successfully capture mucus RNA from FHMs, future methodologies should focus on the mucus collection stage. Using appropriate swabbing materials and ensuring fish are lightly dried prior to swabbing would help ensure sufficient mucus is collected and prevent swab saturation by residual water. To effectively capture adequate mucus RNA in small-bodied fish, I would recommend that the kimwipe™ mucus swab technique followed by ultrafiltration described in chapter three is implemented. Trials using this method would be important to understand if pooling fish would be required to obtain adequate quantities of RNA for analysis on EcoToxChips. This method has the benefit of combining multiple swabs per fish, with a greater swab surface area for collection. Furthermore, the second swab would collect mucus that may have been missed due to the saturation of the first swab by residual water remaining on the fish.

4.2.2 Considerations for experimental design

The nutrient exposure study in Chapter three utilized chemical salts including ammonium phosphate dibasic and ammonium chloride to achieve nutrient levels that mirrored exposure concentrations of AWW in Chapter two. As this research was aimed to understand the effects of nutrient exposures due to AWW effluents on FHMs, using an alternative to AWW worked, but it was not ideal. Aquaculture wastewater is a diverse effluent which varies depending on several factors including the organism being farmed, and the regulations in place in the region which determine chemical additives used. These additives are referred to as contaminants of emerging

concern (CEC) and include antibiotics, antifoulants, disinfectants, and metals (Ahmad et al., 2022). Unfortunately, it was not practical to use raw effluent in a laboratory setting, with additional logistical concerns with transporting the wastewater to conduct the study. Consequently, this left a set of potential contaminants that were unaccounted for between the two studies and my exposures did not represent the true complexity of the AWW. Therefore, future studies investigating the effect of AWW on small-bodied fish should consider conducting a laboratory-based study with the AWW effluent. Furthermore, repeated studies should utilize AWW sourced from diverse aquaculture farms throughout Canada to account for facility, organismal, and provincial differences. Additionally, only nutrients and metals were analyzed in the AWW effluent from chapter two. This introduced a problem in the study design, where it is unclear which portion of the wastewater was responsible for the cellular stress response observed in the FHMs. As a result, conclusions regarding the cellular stress response due to AWW effluent exposure can only be drawn from measured nutrients and metals. Therefore, future research should consider characterizing AWW to uncover each contaminant present, and how it may affect small-bodied fish in receiving systems.

While utilizing chemical salts was a good alternative to raw AWW, the exposure concentrations in the laboratory study described in Chapter three did not reach target levels. For example, the high treatment had a target concentration of 4.615 mg L^{-1} total ammonia nitrogen as N (TAN) and 3.45 mg L^{-1} PO_4 – P. These concentrations were chosen due to their environmental relevance, where they mimicked biweekly dosing of the highest treatment in Chapter two, the 105-L treatment. However, sub-optimal levels of TAN were measured in water chemistry where the high treatment had TAN concentrations ranging between 0.617 – 0.930 mg L^{-1} across each replicate throughout the experiment. The goal of this treatment in the laboratory study was to

achieve concentrations that reflect the initial dosing with AWW, to simulate chronic point source of pollution. As these concentrations were not met, future studies should consider dosing with higher quantities of nutrients to achieve these target concentrations. It is recommended that dosing trials are conducted prior to the experiment to ensure that concentrations can be met over time.

4.3 Connections between chapter two and three

While target nutrient levels were not achieved in chapter three, the measured nutrient water concentrations are comparable enough to find relationships to the field study. Furthermore, the comparable water chemistry between the two studies may explain why similar gene set enrichment patterns were observed. Both studies in this thesis underline cellular stress responses owing to chronic ammonia exposure in adult fathead minnows. Although the stressors are inherently different, one being AWW and the other chiefly ammonium phosphate and ammonium chloride, ammonia is the common placeholder between the two contaminants. The complexity of AWW makes it difficult to distinguish which component of the effluent is driving the response patterns observed. Therefore, conducting an additional laboratory-based study with an isolated component of the wastewater with a comparable concentration allows general conclusions to be drawn. Both the field and laboratory-based study identified significant immunological, metabolic, and steroid hormone consequences of ammonia exposure in liver tissues. Specifically, both studies found that ammonia elicited immunosuppressive qualities, where cytokines were ultimately impacted, a common result of ammonia induced immunosuppression in a variety of fishes (Li et al., 2020; Cheng et al., 2015). Metabolic processes were affected in each AWW exposure assessed, but primarily the 42.7-L, 23.1-L, and 9.8-L treatments, which are more comparable to the suboptimal TAN concentrations that were

observed in the laboratory study. Additionally, the corticosteroid biological process was the most dysregulated biological process in the 42.7-L and 23.1-L treatments and was also affected in the 77.7-L treatment. Several GO terms related to steroid-hormones were significantly affected in the proteins of the high treatment compared to the low in Chapter 3. Therefore, environmentally-relevant concentrations of ammonia were sufficient to affect metabolic, immunological, and steroid hormone pathways in fathead minnows, although the fold change was not large. Additionally, oxidative stress was a clear outcome of fathead minnows exposed to a higher concentration of AWW, the 77.7-L treatment, whereas this result was not as clear in the laboratory-based setting. The protein catalase, an antioxidant enzyme, was the top affected protein by adjusted p-value in the low treatment compared to the control in the laboratory study, where it was found to decrease in abundance upon ammonia exposure. However, in the high treatment, the same trend of decreased abundance was observed, however it was not statistically significant. Whereas in the field setting, it was found that all treatments possessed an upregulation of catalase mRNA, except the 42.7-L treatment which saw a significant spike in TAN. It is clear from several ammonia exposure studies on aquatic animals that the regulation of antioxidant genes and proteins is sensitive to shifts in ammonia exposure concentrations and exposure time. In several studies, catalase has been found to downregulate upon increased ammonia exposure, only to rebound and even increase in recovery (Sun et al., 2014; Lu et al., 2022; Zhang et al., 2020). Therefore, a common conclusion can be drawn from both experiments in which varying sub-lethal concentrations of ammonia can influence antioxidant activity in fathead minnows over chronic exposure periods.

4.4 Conclusion and broader implications

Although the attempts to use epidermal mucus as a method of assessing stress responses in this thesis were only partially successful, epidermal mucus tissue analyses remain a valuable opportunity to measure cellular changes in small-bodied fish. Alternative swabbing techniques and method developments will be required to ensure the effectiveness of this tool in FHMs. Advancing mucus omics techniques may aid in the knowledge and preservation of economically, socially, and environmentally important fishes in Canada. As mucus provides a non-invasive method to assess fish health, there lies great opportunity to advance this technique for numerous fish species across Canada, especially those that are of conservation concern, in which lethal sampling must be prevented. Epidermal mucus collection holds the benefit of repeatedly sampling not only the same population of fish, but the same individual over time to investigate temporal changes in several environmental risk factors such as climate change, novel toxicant exposures, or anthropogenic activities. Furthermore, there is significant potential for omics analyses of epidermal mucus tissues to become key in environmental monitoring practices. Once mucus collection, processing, and data analyses are streamlined and standardized, mucus may become an ideal medium to sample for freshwater fish.

The exposure of FHMs to varying concentrations of AWW and nutrients in the two levels outlined provide insight into acceptable environmental AWW dosing concentrations. In chapter two, it is described that AWW fertilization is environmentally-relevant at 23.1-L of total wastewater throughout the growing season. Although this concentration was sufficient to elicit a cellular response in liver tissues of male FHMs consisting of oxidative stress, impacts on innate immunity, lipid peroxidation and DNA damage and repair, it was not severe. Furthermore, environmentally-relevant total ammonia nitrogen concentrations reflecting AWW loadings were

also sufficient to elicit a protein-level response in liver tissues. However, the fold-change levels in both studies were consistently quite low (<2), and a combination of transcript-level/protein-level responses and transcript or protein-set enrichment was required to uncover overall trends. Therefore, fertilization of flooded wild-rice paddy fields at this concentration would likely not be great enough to impair the health and survival of FHMs. These results highlight the potentially important role AWW may have as a fertilizer for wild rice without the concern of downstream negative implications on wild fish populations. Even so, consistent monitoring would be important to ensure that long-term, repeated exposure of AWW does not exacerbate the response observed in this study. Furthermore, water quality should be a top priority for monitoring affected water bodies, including the characterization of non-nutrient parameters, specifically metal accumulation. The experiments detailed in this thesis highlight the potential of non-lethal omic techniques and its application in ecotoxicology, while acknowledging the current limitations in small-bodied fish. With continued method development and refinement, the approaches outlined possess the potential for more sustainable and innovative research practices for fish biologists and ecotoxicologists.

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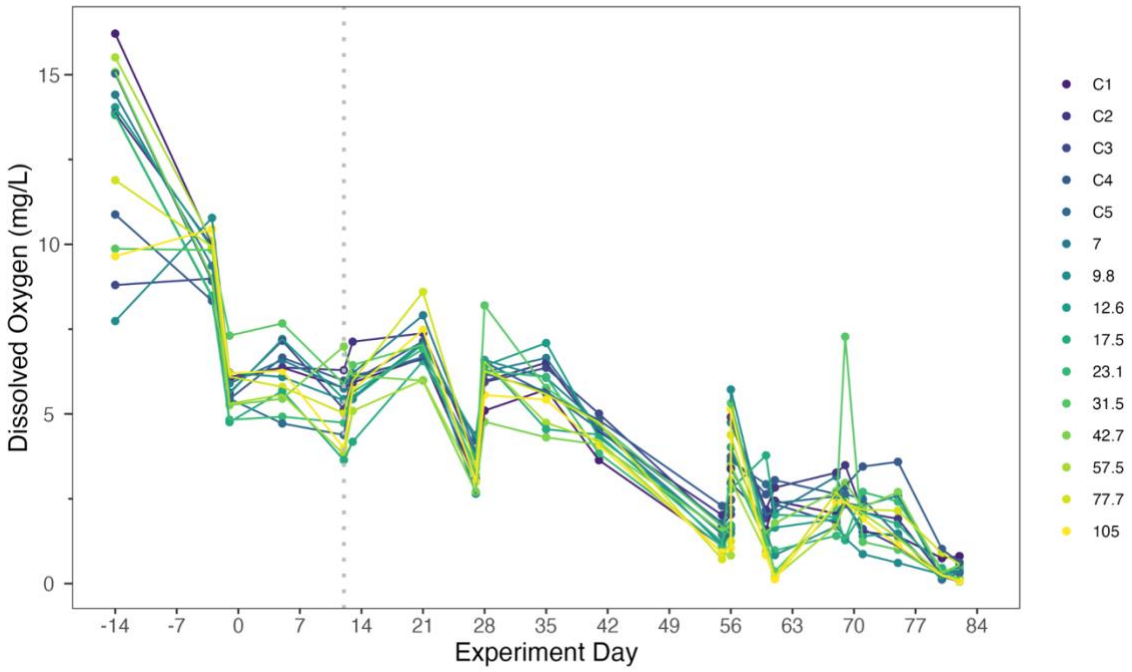
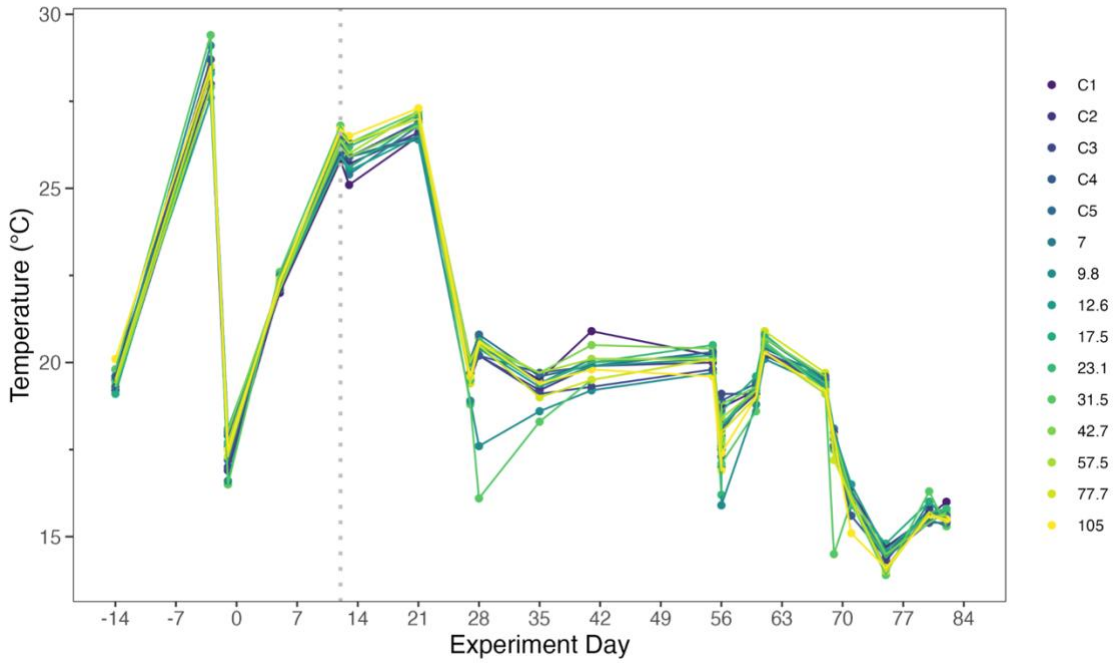
Appendix A

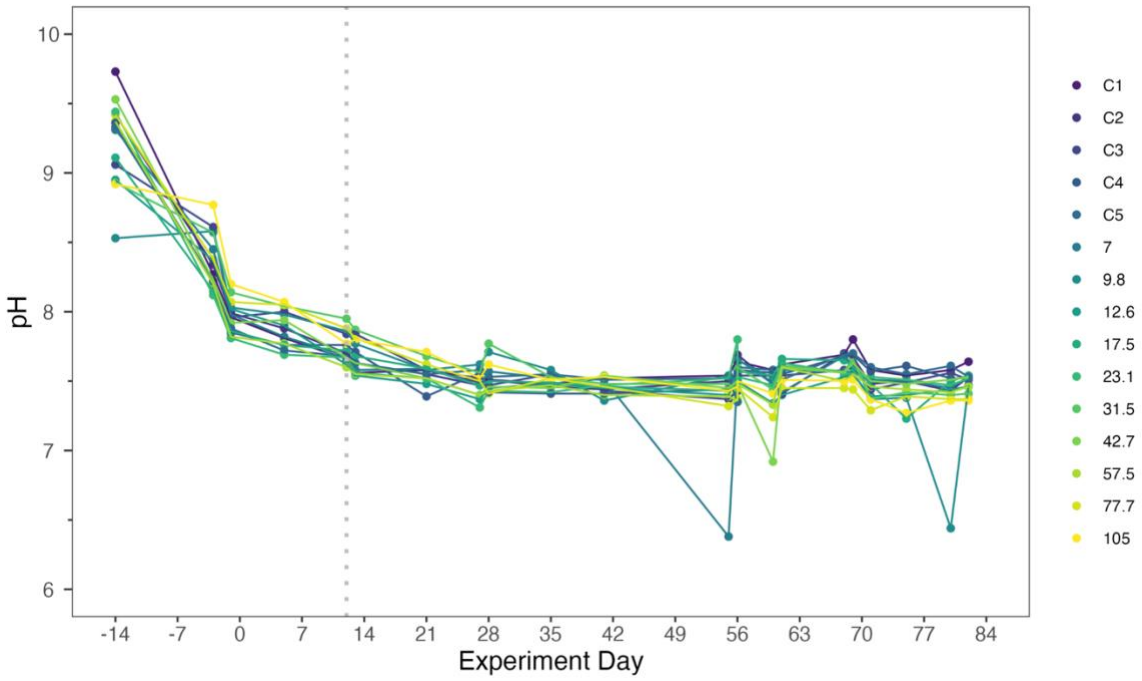
Supplemental Figures



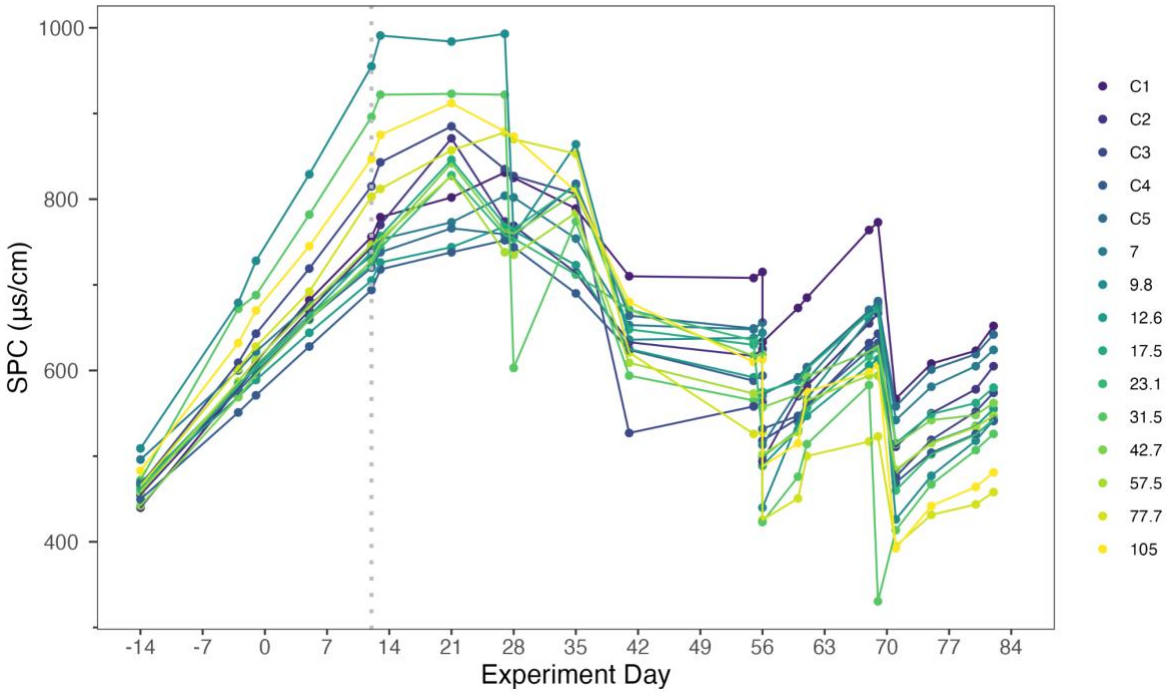
Supplemental Figure 1. Dissolved oxygen (mg/L) reading across five controls, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



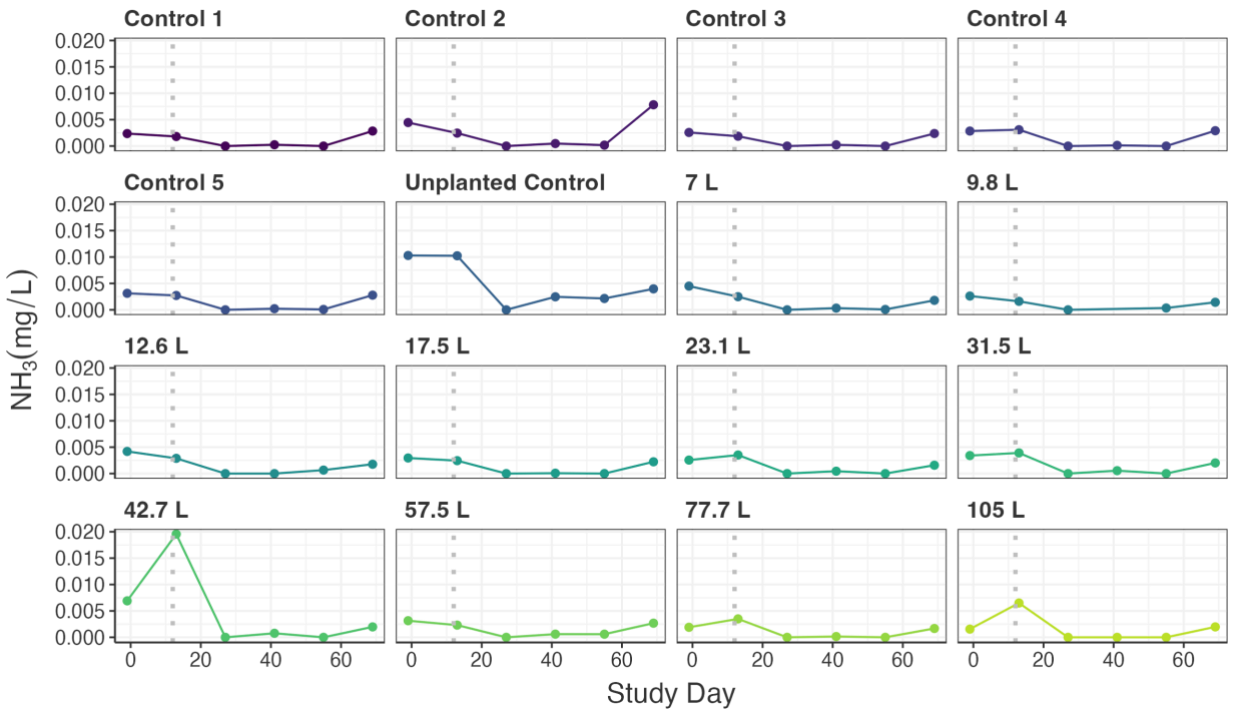
Supplemental Figure 2. Temperature (°C) readings across five controls, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



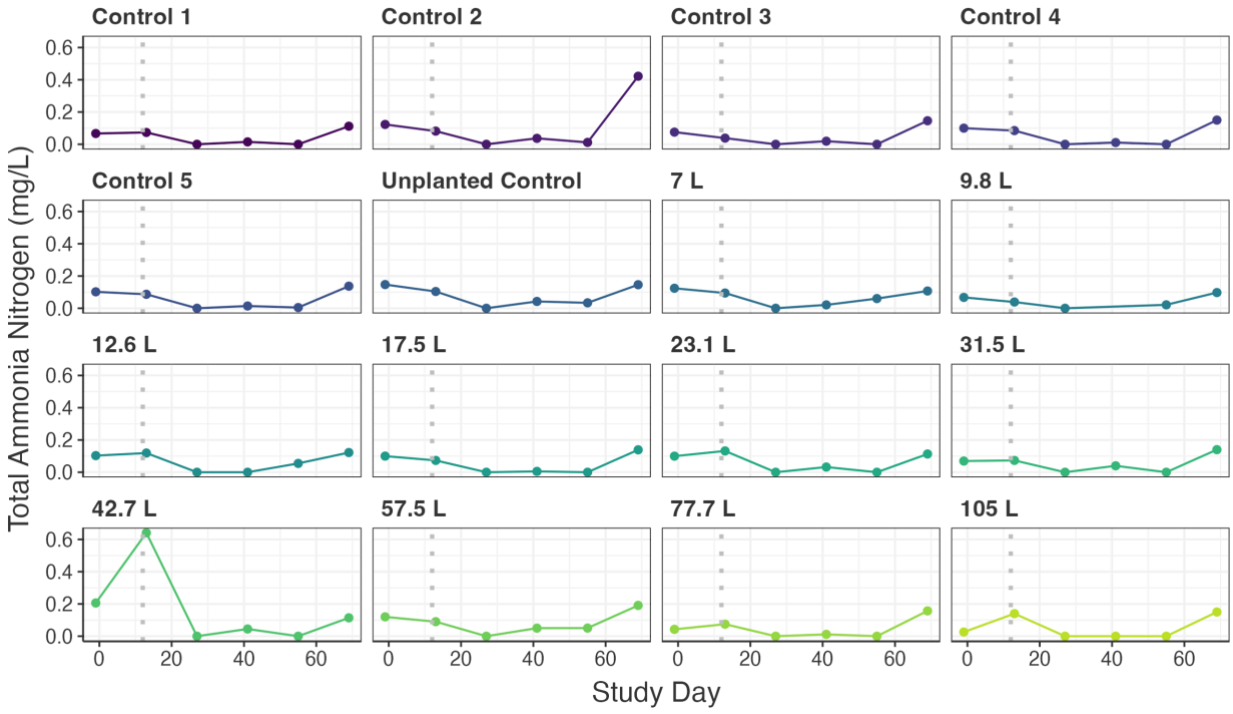
Supplemental Figure 3. pH readings across five controls, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



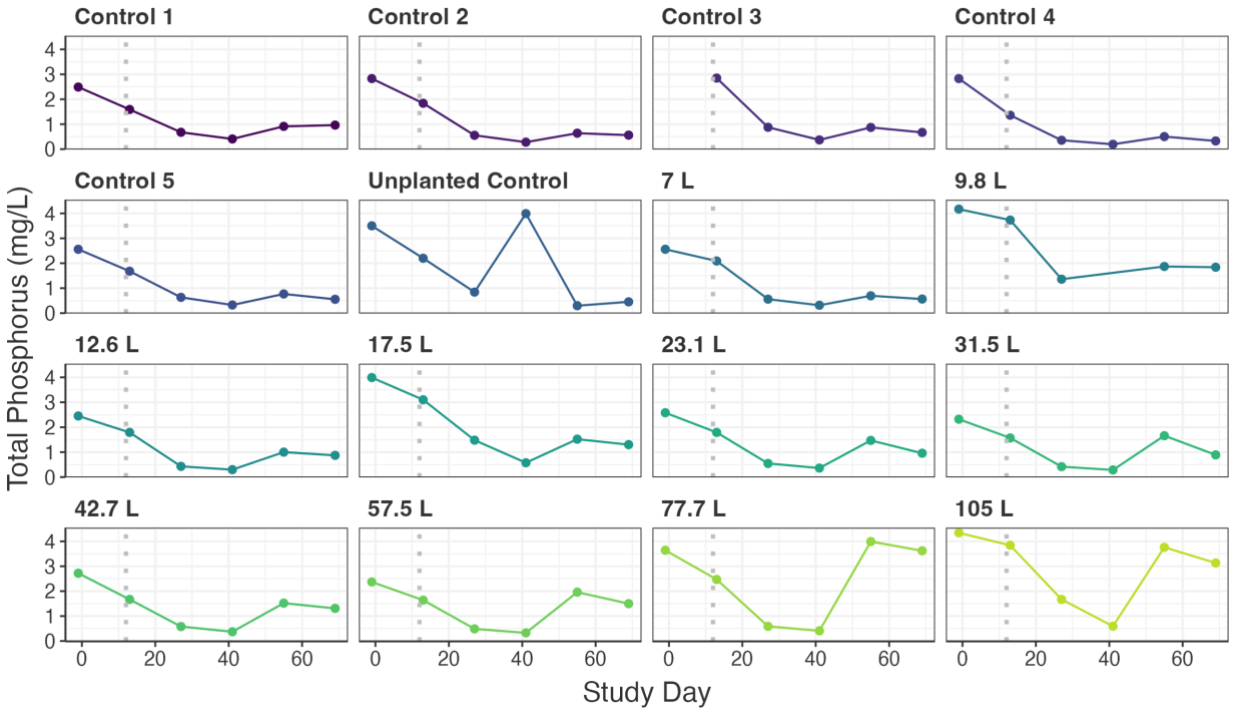
Supplemental Figure 4. Specific conductivity (µs cm^{-1}) readings across five controls, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



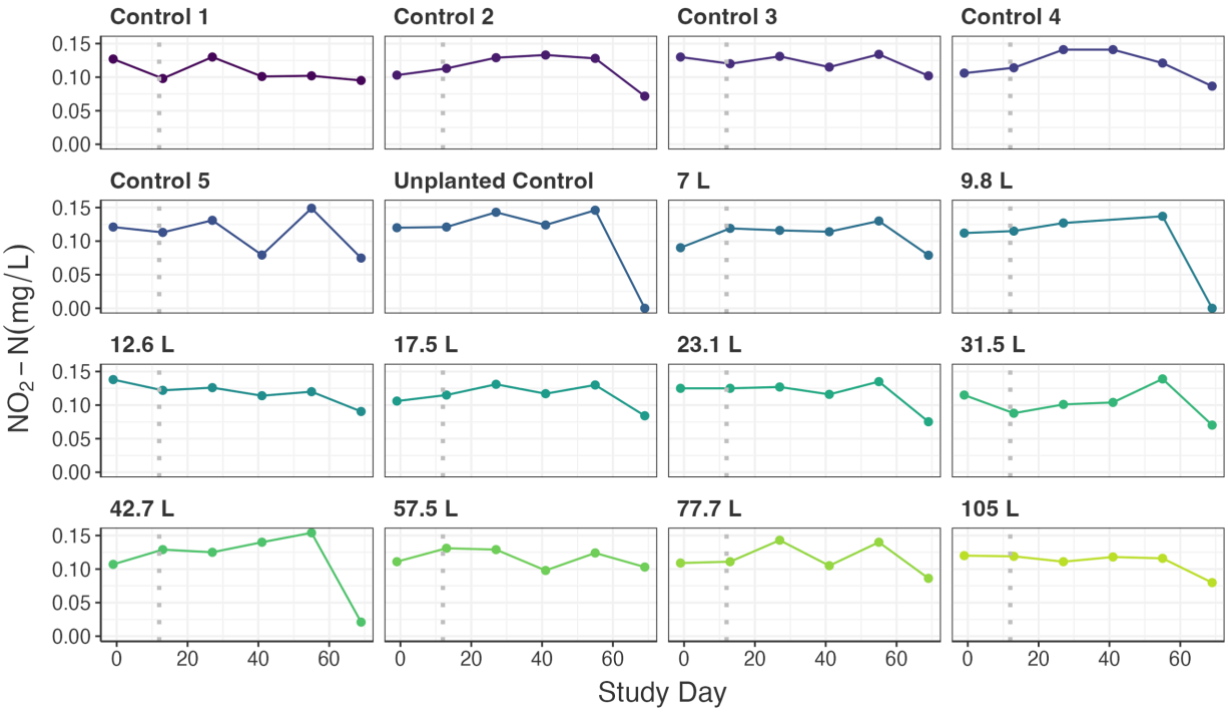
Supplemental Figure 5. Ammonia (NH_3) concentration in mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line). Ammonia was calculated using the following equations outlined in Delos and Erickson (1999): $Total\ ammonia = TAN * \left(\frac{17}{14}\right)$ and $NH_3 = \frac{Total\ ammonia}{1+10^{\left(0.09018+\left(\frac{2729.92}{Temp°C+273.15}\right)\right)-pH}}$.



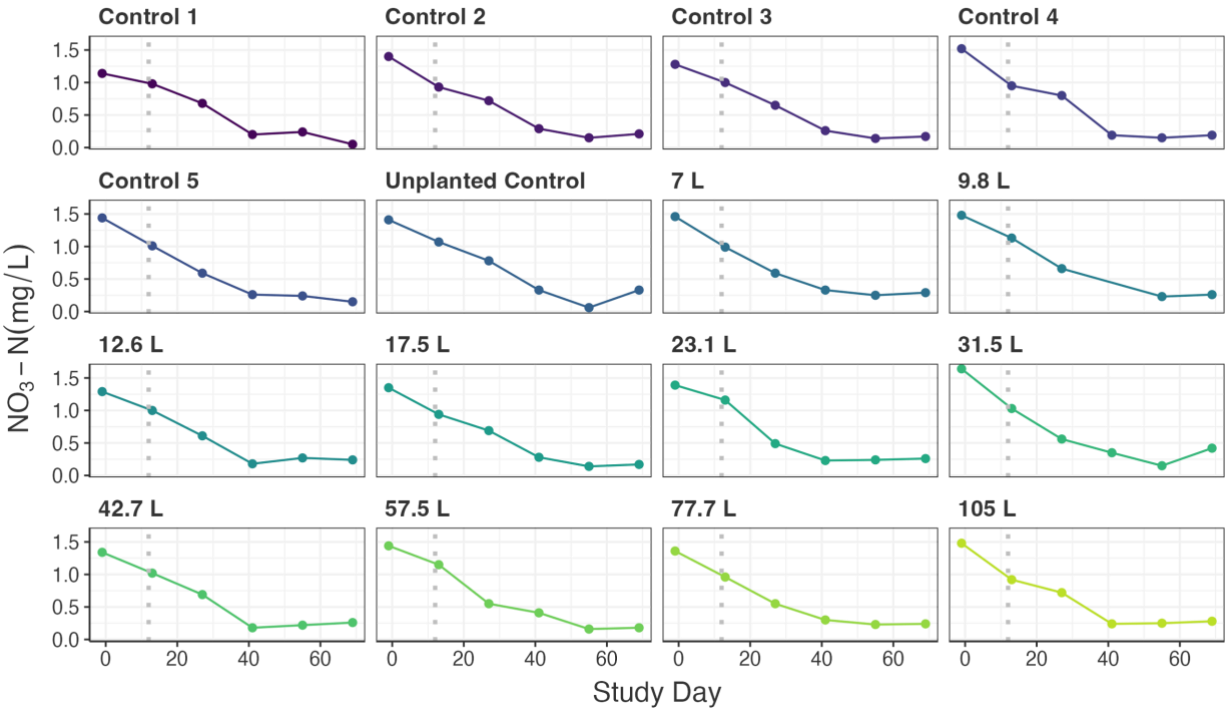
Supplemental Figure 6. Total ammonia nitrogen (TAN) mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



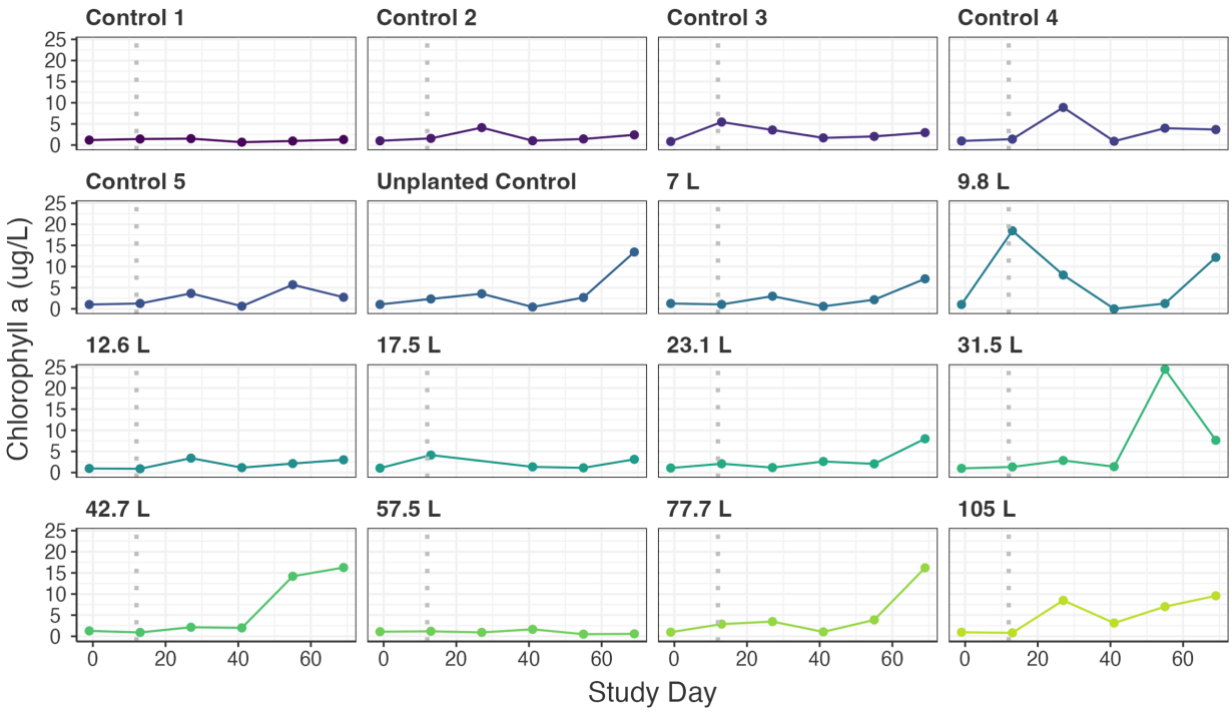
Supplemental Figure 7. Total phosphorus (TP) mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



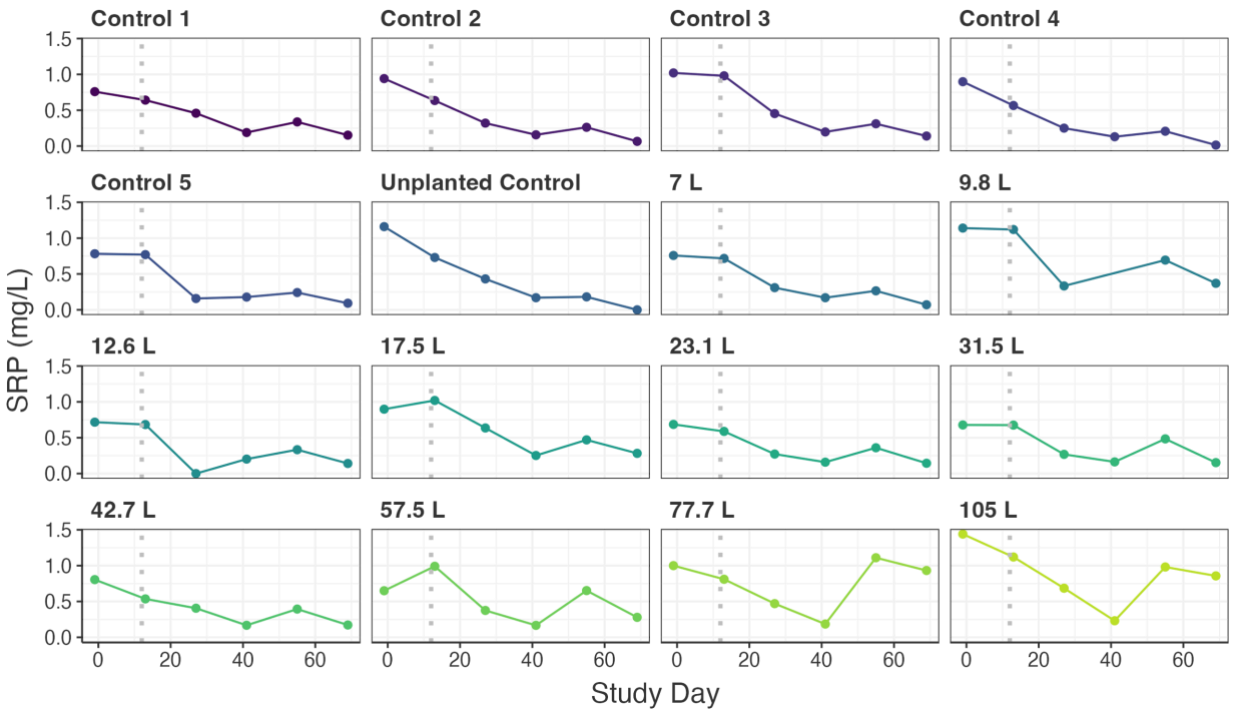
Supplemental Figure 8. Nitrite (NO2--N) mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



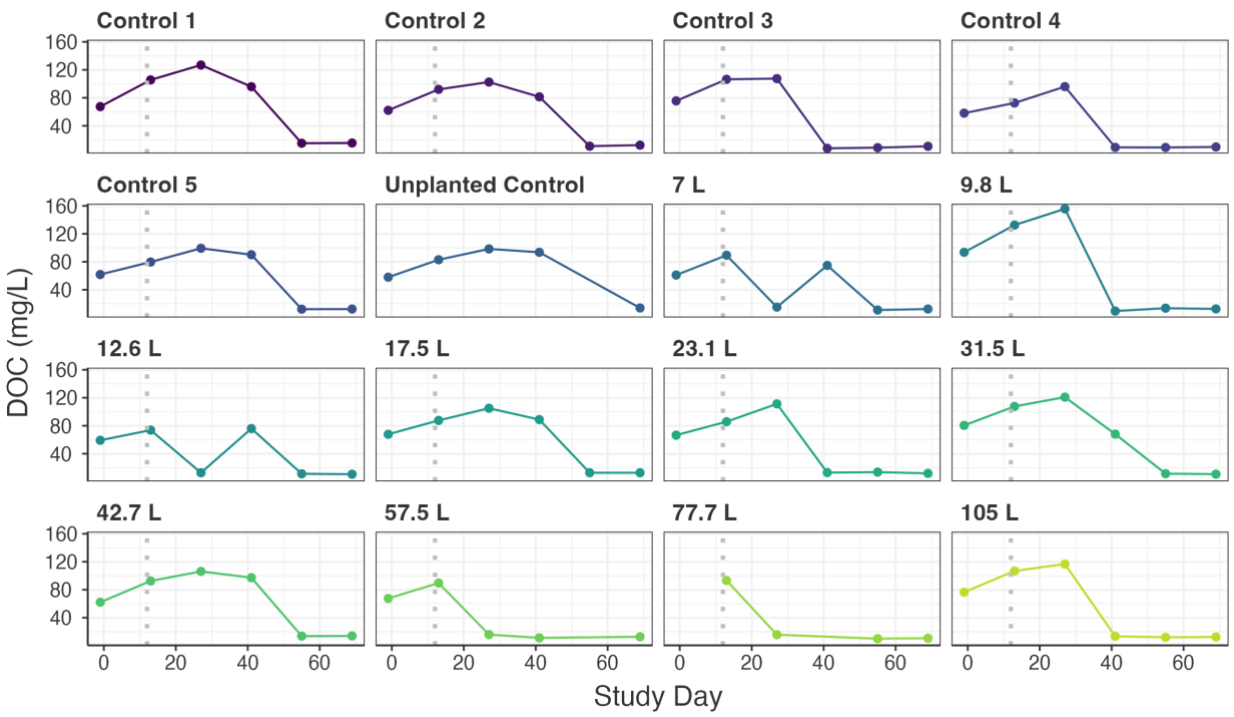
Supplemental Figure 9. Nitrate (NO_3^+- N) mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



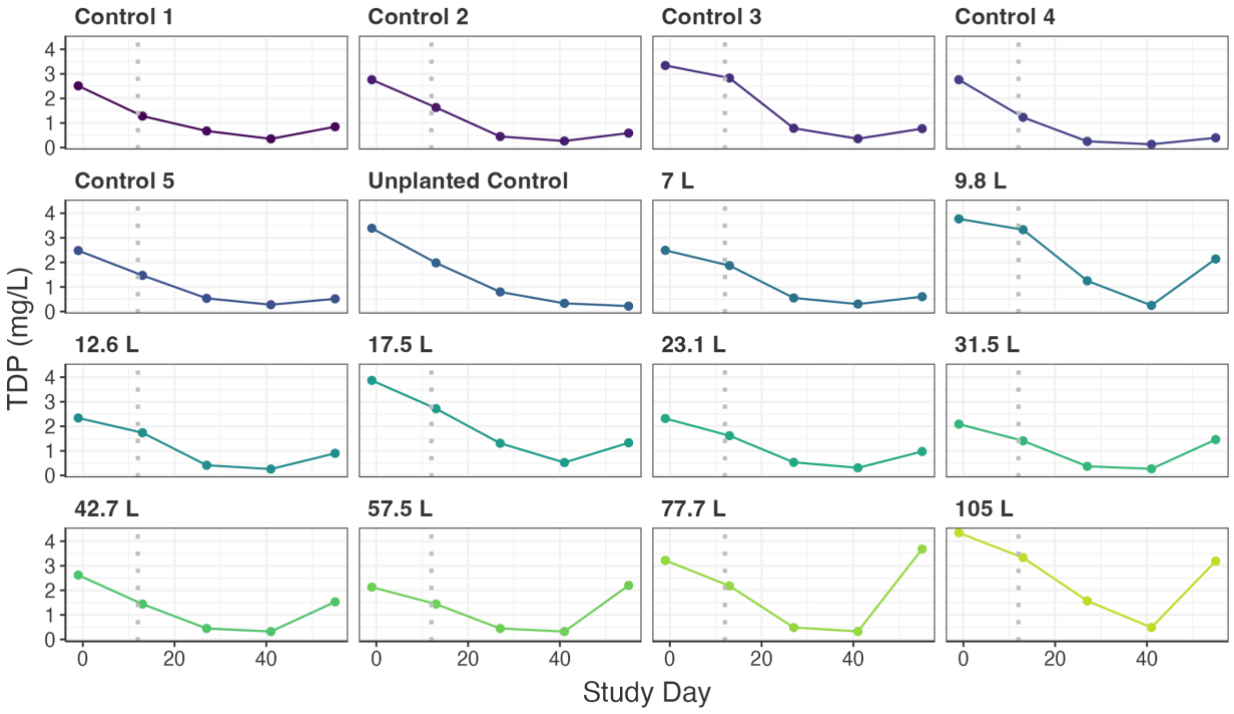
Supplemental Figure 10. Chlorophyll *a* ug L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



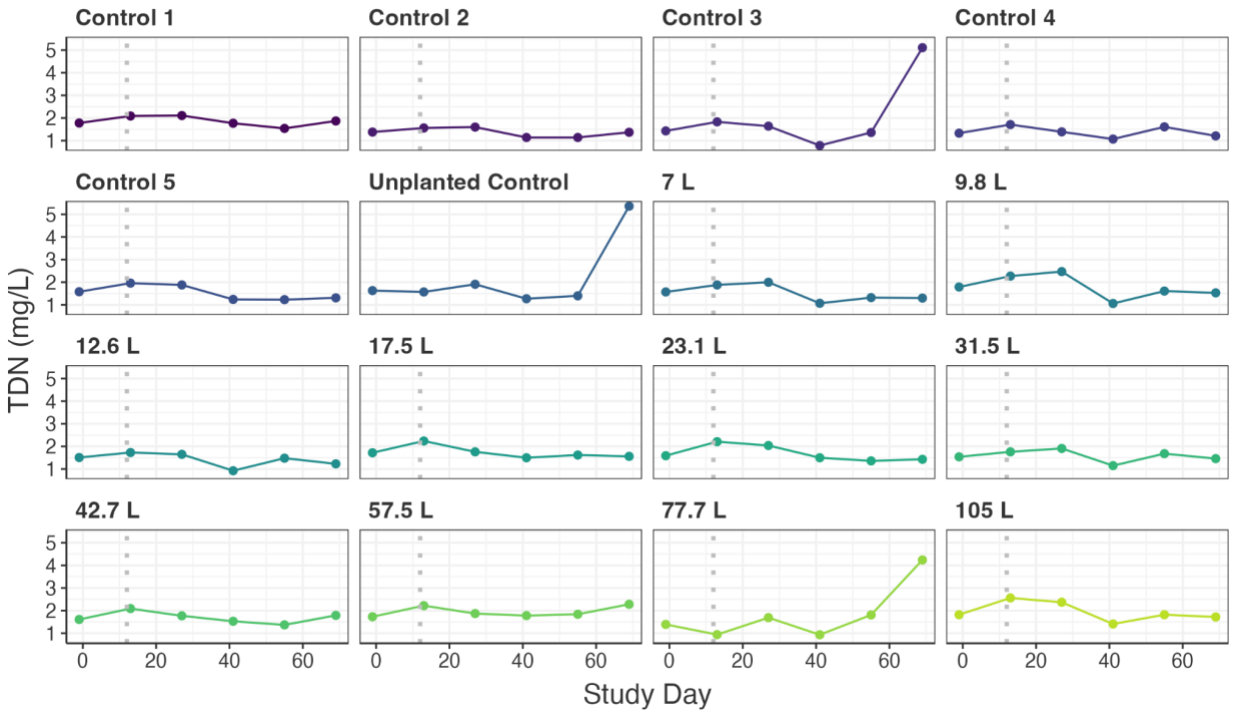
Supplemental Figure 11. Soluble reactive phosphorus (SRP) mg L-1 across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



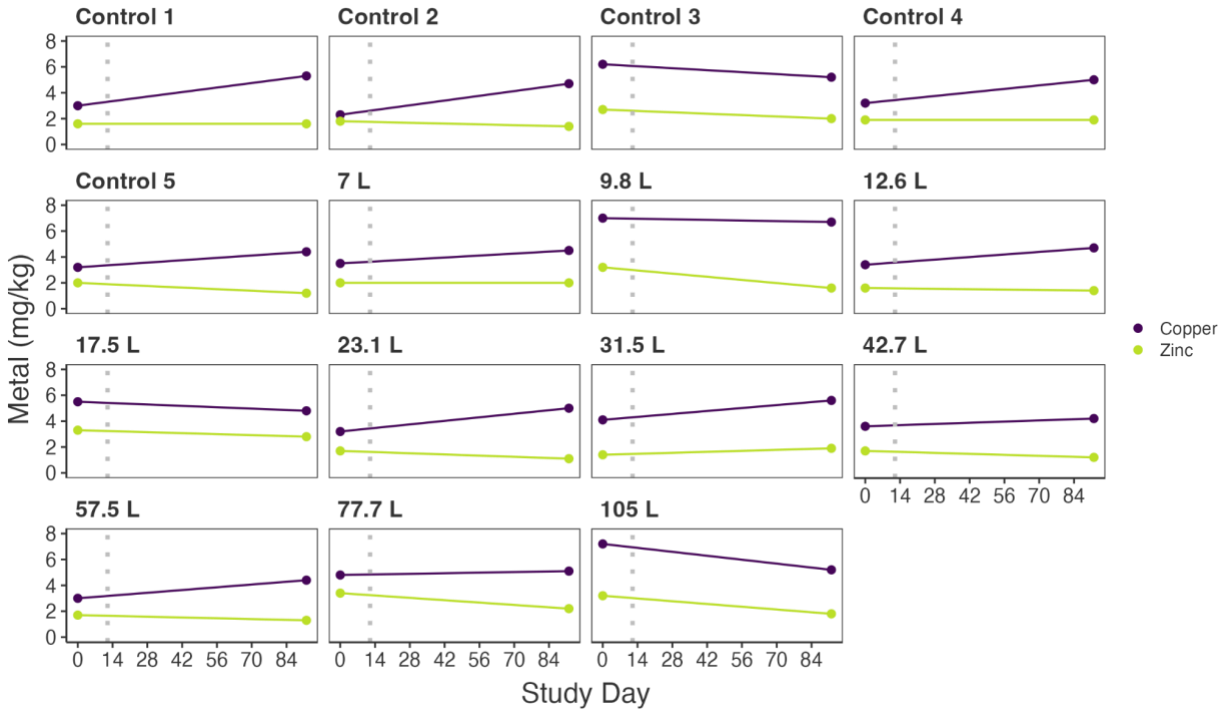
Supplemental Figure 12. Dissolved organic carbon (DOC) mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



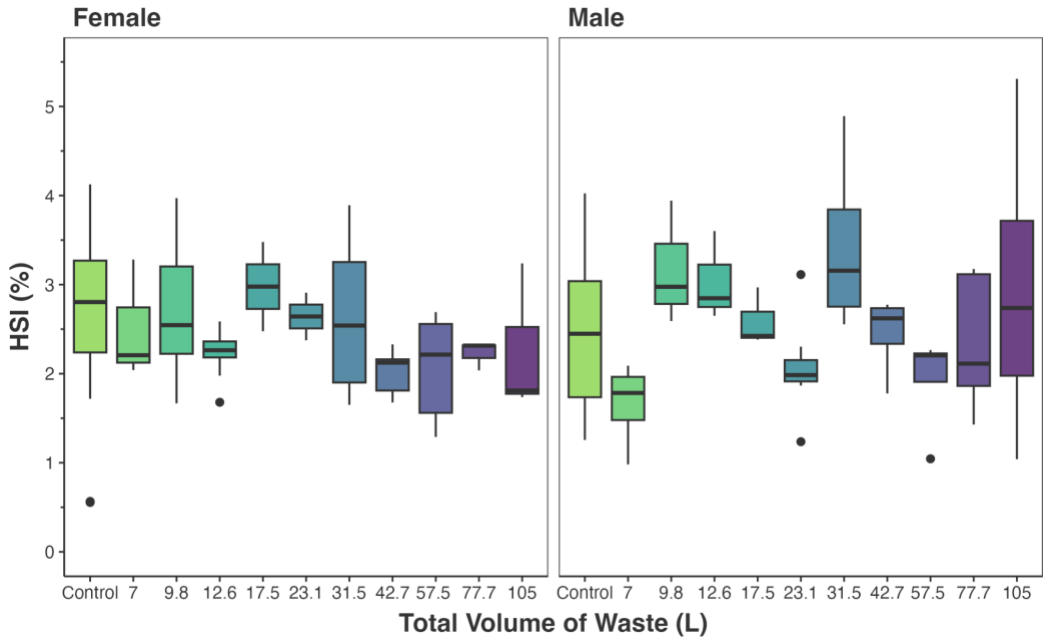
Supplemental Figure 13. Total dissolved phosphorus mg L-1 across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



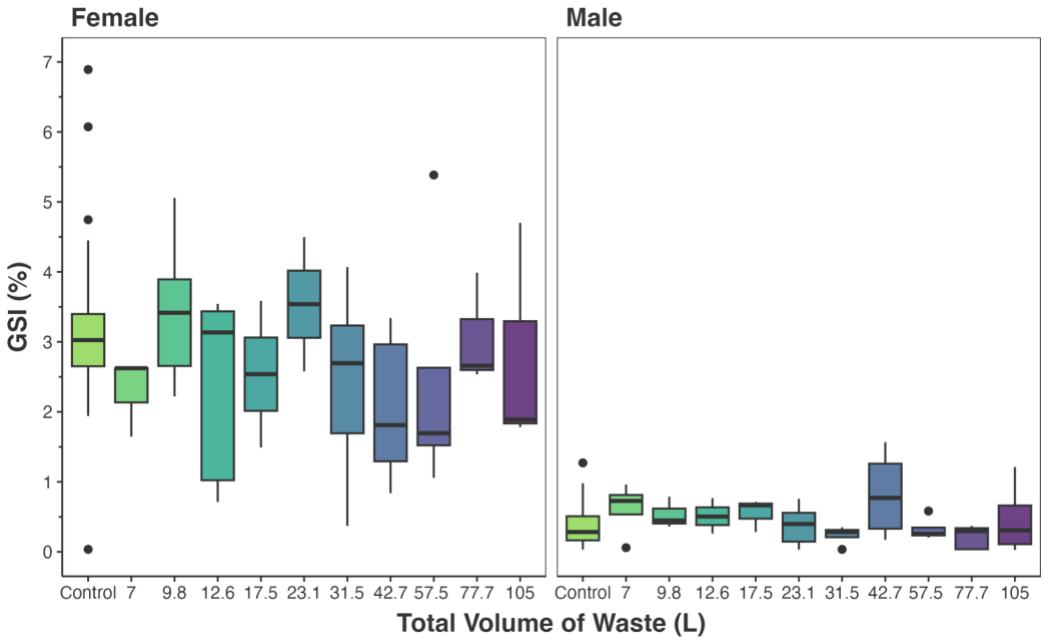
Supplemental Figure 14. Total dissolved nitrogen mg L^{-1} across five controls, one unplanted control, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line).



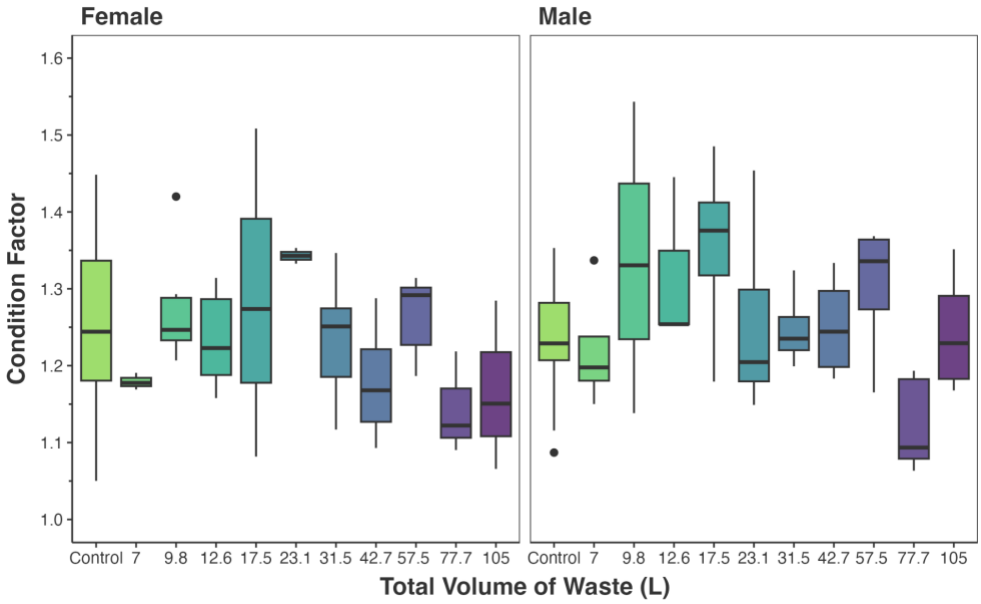
Supplemental Figure 15. Sediment metal content (mg kg^{-1}) across five controls, and 10 experimental mesocosms exposed to aquaculture wastewater (AWW) effluent. AWW was added on day zero and fathead minnows (*Pimephales promelas*) were added on day 12 (dotted line). Sediment was sampled on day zero prior to first wastewater addition, and on day 92.



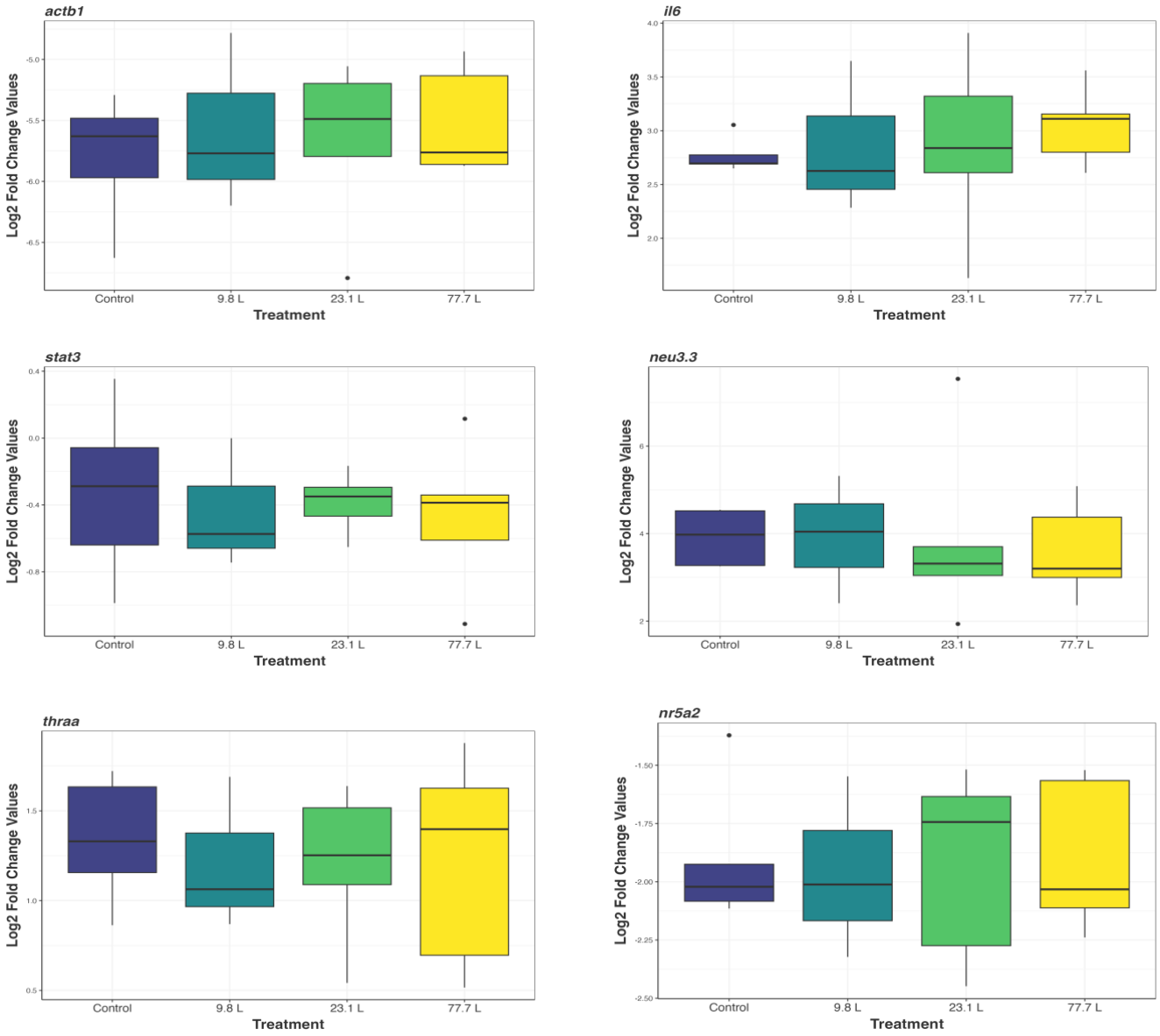
Supplemental Figure 16. Hepatosomatic Index (HSI; %) of adult male and female fathead minnows (*Pimephales promelas*) following a ~70 day exposure to increasing volumes of aquaculture wastewater effluent across five controls, and 10 experimental mesocosms. The dots outside of the boxes indicate outliers.



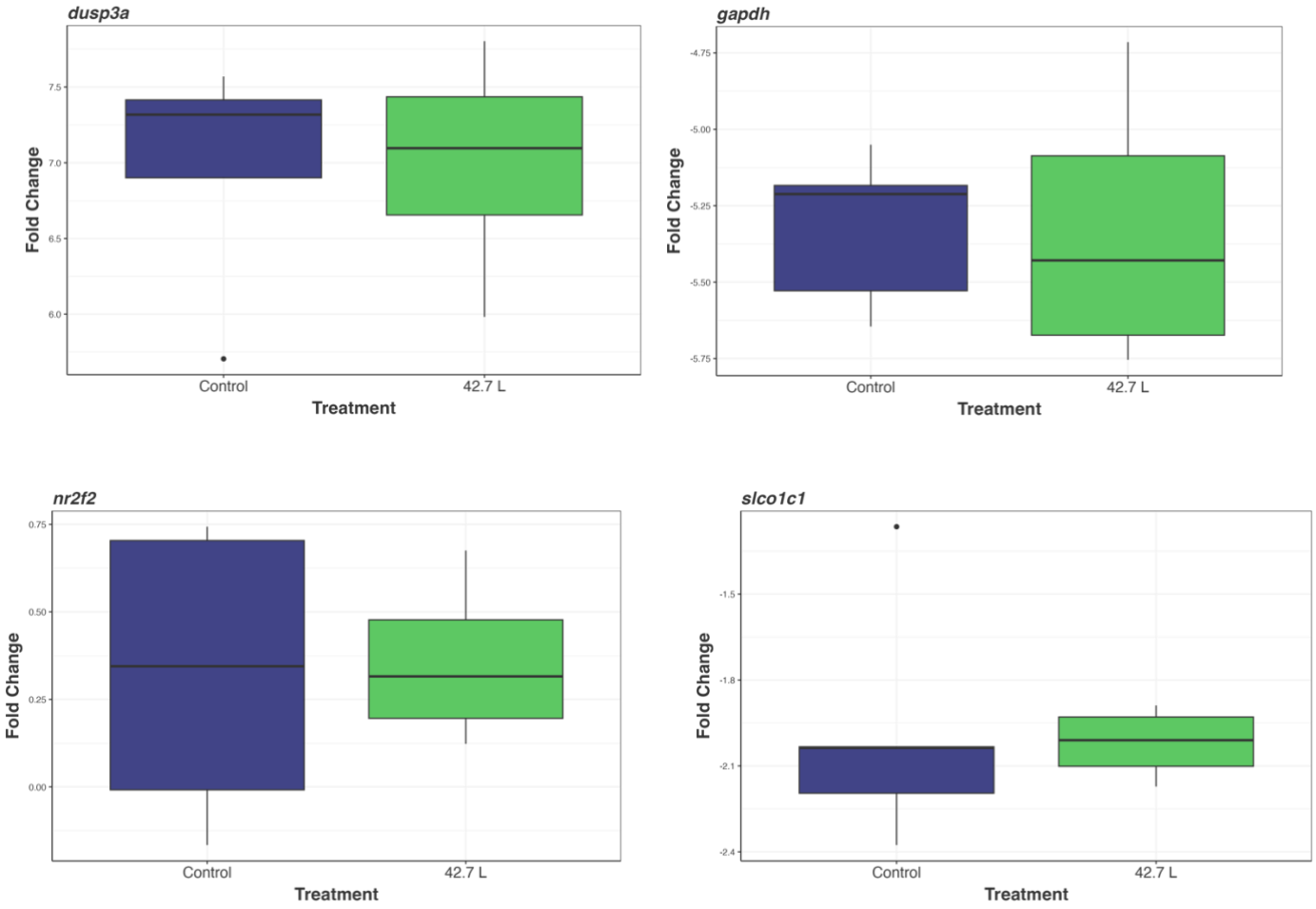
Supplemental Figure 17. Gonadosomatic index (GSI; %) of adult male and female fathead minnows (*Pimephales promelas*) following a ~70 day exposure to increasing volumes of aquaculture wastewater effluent across five controls, and 10 experimental mesocosms. The dots outside of the boxes indicate outliers.



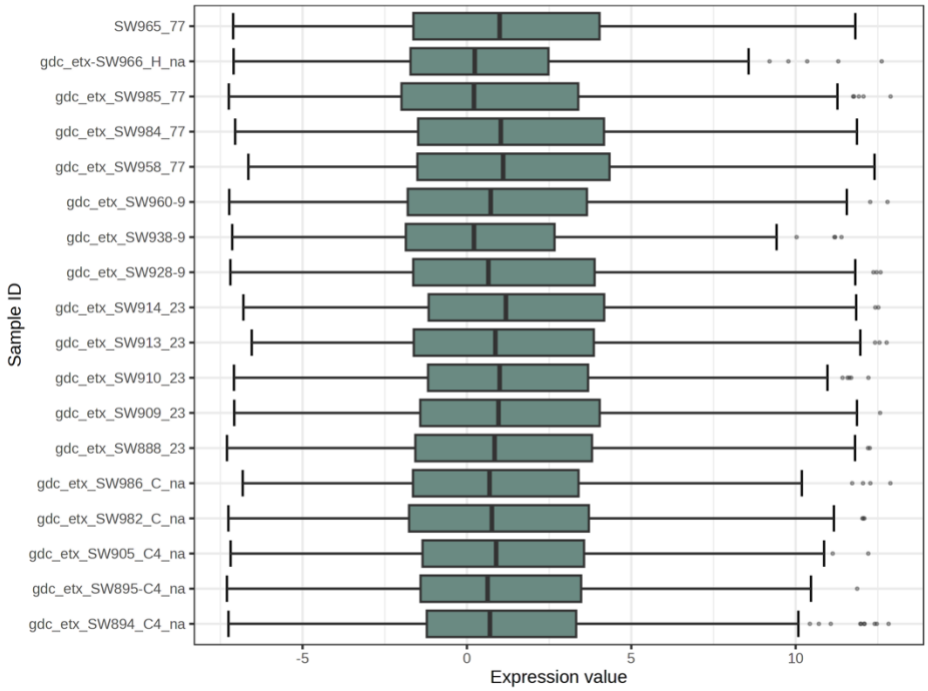
Supplemental Figure 18. Fulton's condition factor (K) of adult male and female fathead minnows (*Pimephales promelas*) following a ~70 day exposure to increasing volumes of aquaculture wastewater effluent across five controls, and 10 experimental mesocosms. The dots outside of the boxes indicate outliers.



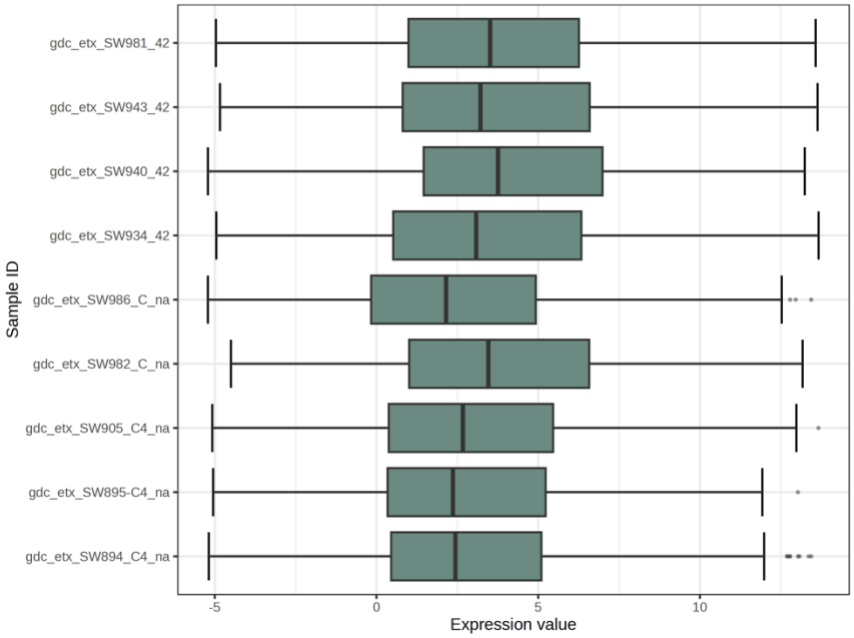
Supplemental Figure 19. Log2 fold-change values of six reference genes (*Actb1*, *il6*, *stat3*, *neu3.3*, *thraa*, *nr5a2*) used for deltaCt normalization in a transcriptional differential expression analysis of male fathead minnow (*Pimephales promelas*) liver tissues. The analysis compared four experimental groups: control, 9.8-L, 23.1-L, and 77.7-L, representing the total volume of aquaculture wastewater applied during a ~70-day mesocosm experiment.



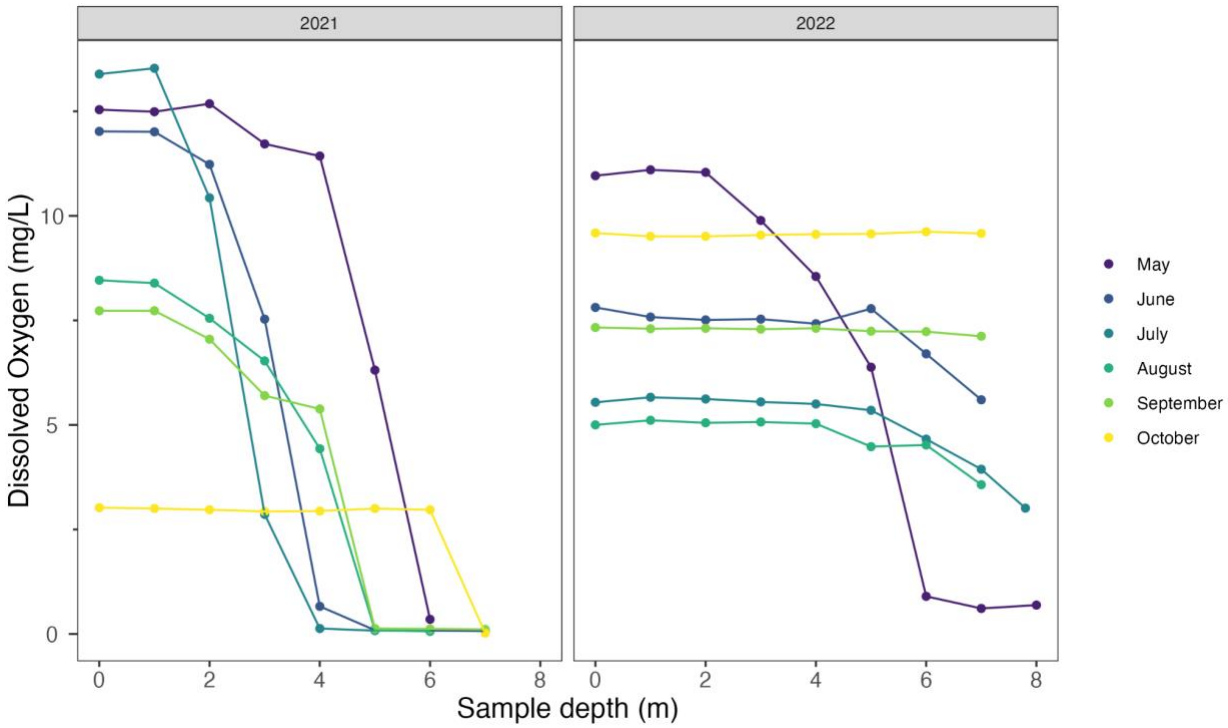
Supplemental Figure 20. Log2 fold-change values of four reference genes (*Dusp3a*, *gapdh*, *nr2f2*, *slco1c1*) used for deltaCt normalization in a transcriptional differential expression analysis of male fathead minnow (*Pimephales promelas*) liver tissues. The analysis compared four experimental groups: control, 9.8-L, 23.1-L, and 77.7-L, representing the total volume of aquaculture wastewater applied during a ~70-day mesocosm experiment.



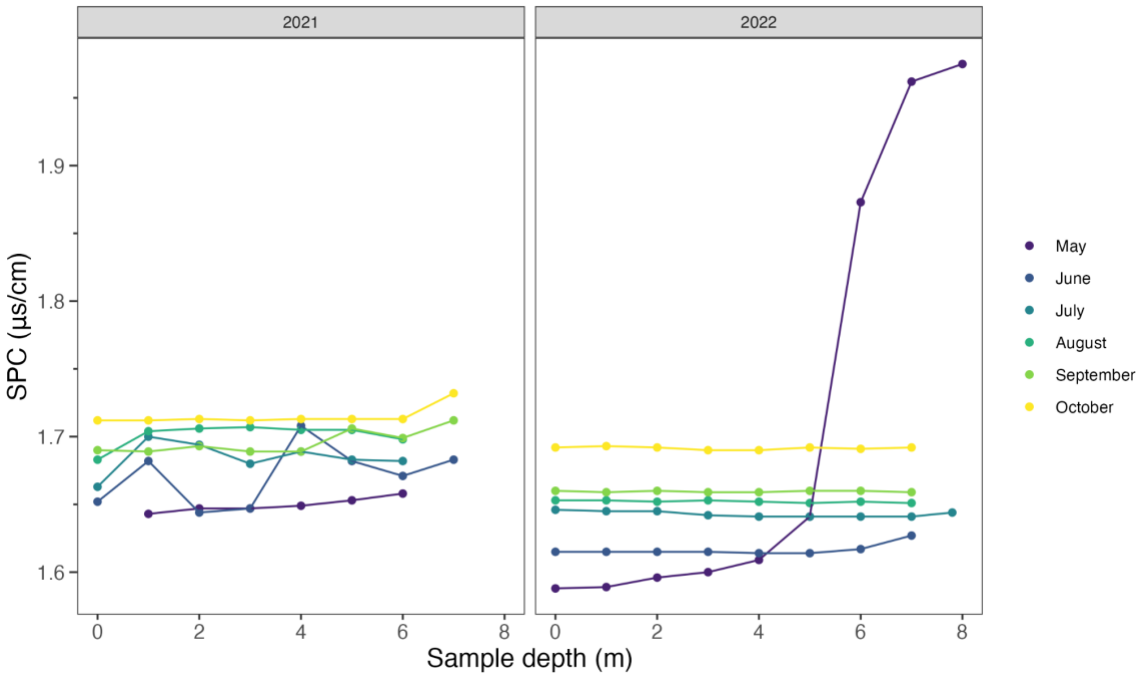
Supplemental Figure 21. Transcript expression values for control, 9.8-L, 23.1-L, and 77.7-L treatment samples following deltaCt normalization. Each sample is sourced from male fathead minnow (*Pimephales promelas*) liver tissues from either a control group, a 9.8 L, 23.1 L, or 77.7 L of total aquaculture wastewater effluent over a 70-day study period in wetland mesocosms.



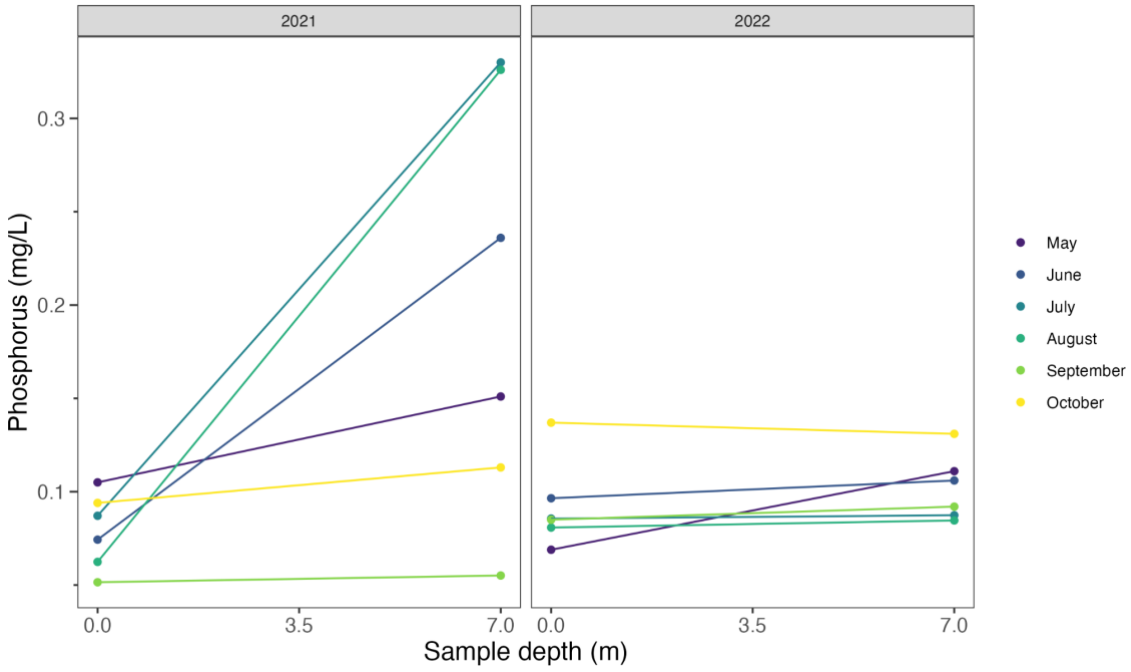
Supplemental Figure 22. Transcript expression values for control and 42.7-L treatment samples following deltaCt normalization. Each sample is sourced from male fathead minnow (*Pimephales promelas*) liver tissues from either a control group, or 42.7 L of total aquaculture wastewater effluent over a 70-day study period in wetland mesocosms.



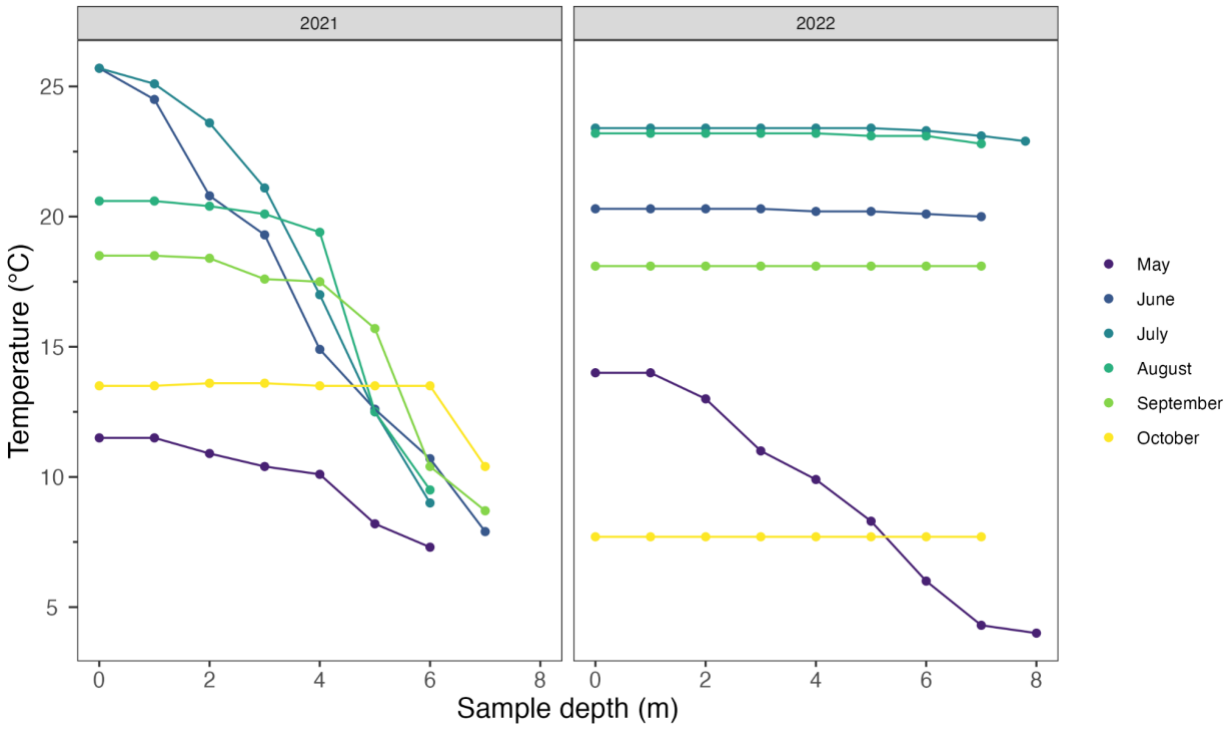
Supplemental Figure 23. Dissolved oxygen (mg/L) readings between 0- and 8-meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Readings were taken monthly during 2021 and 2022, with sample month denoted by colour.



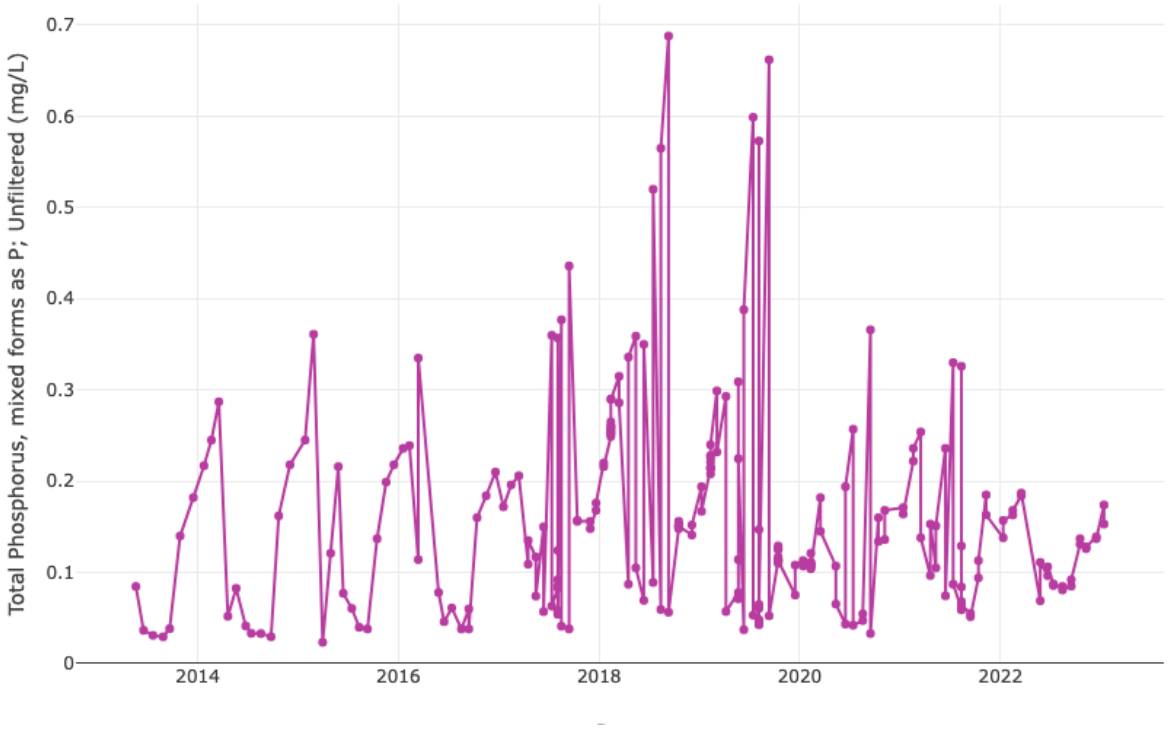
Supplemental Figure 24. Specific conductivity (µs/cm) readings between 0- and 8-meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Readings were taken monthly during 2021 and 2022, with sample month denoted by colour.



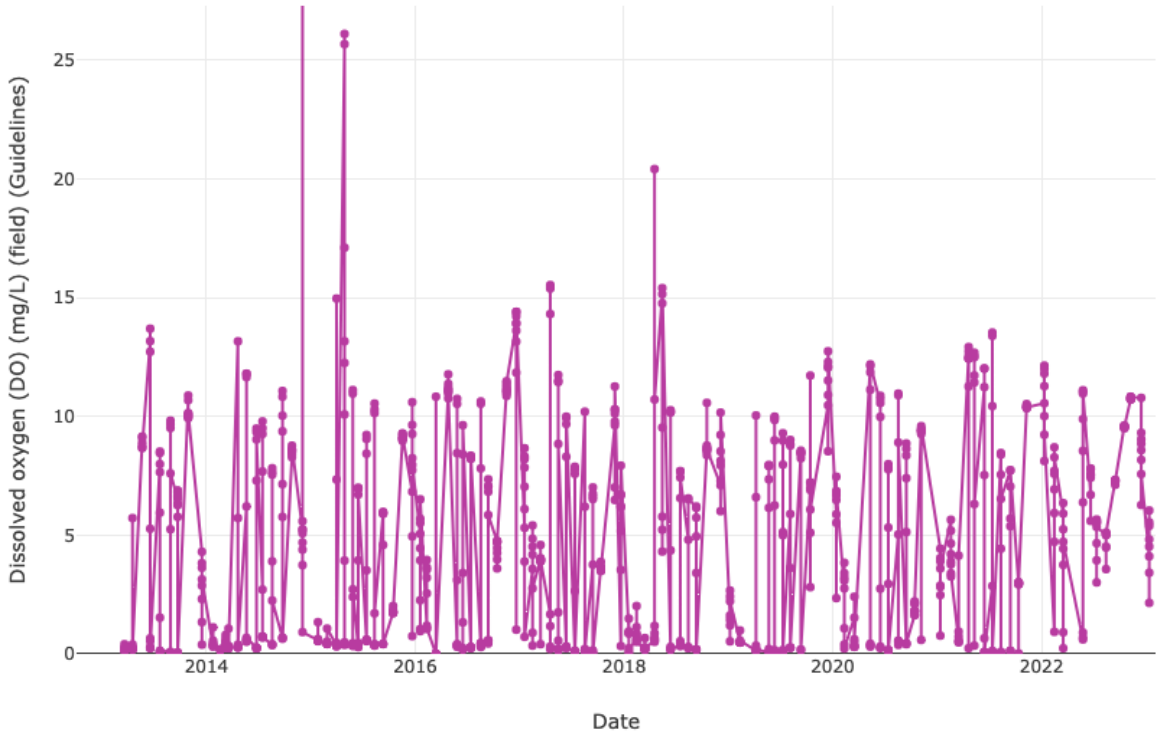
Supplemental Figure 25. Phosphorus (mg/L) concentrations at 0- and 7-meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Phosphorus samples were taken monthly during 2021 and 2022, with sample month denoted by colour.



Supplemental Figure 26. Temperature (°C) readings between 0- and 8-meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Temperature readings were taken monthly during 2021 and 2022, with sample month denoted by colour.



Supplemental Figure 27. Total Phosphorus as P between 0- and 8-meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Measurements were taken monthly between 2013 and 2023. Figure was taken from https://lakewinnipegdatastream.ca/.



Supplemental Figure 28. Dissolved oxygen (DO; mg L-1) between 0 and 8 meters depth in Lake Muir at Fort Whyte Alive, Winnipeg, Manitoba. Readings were taken monthly between 2013 and 2023. Figure was taken from https://lakewinnipegdatastream.ca/.

Supplementary Tables

Supplementary Table 1. Background nutrient content of mesocosm source water (adjacent pond) used for initial mesocosm set-up. Phosphorus is described as PO_4–P, and all nutrient parameters are reported in mg L^{-1}.

TP	TDP	SRP	TDN	TAN	NO_2–N	NO_3–N
2.319	1.950	0.770	2.550	0.657	0.182	1.48

Supplementary Table 2. Background chemical properties of mesocosm soil reported as a dry mass basis unless otherwise stated.

Parameter	Result	Units
N	7	mg kg^{-1}
Olsen P	46.0	mg kg^{-1} as PO_4–P
K	620	mg kg^{-1}
S	22	mg kg^{-1}
Ca	4700	mg kg^{-1}
Mg	820	mg kg^{-1}
Na	28	mg L^{-1}
pH	8.1	Unitless
Conductivity	0.79	dS m^{-1}
Organic matter	6.2	%
Cation exchange capacity	31.7	meq per 100 g
Wet density	1.60	g cm^{-3}
Dry density	0.63	g cm^{-3}

Supplementary Table 3. Water quality and nutrient parameters of the municipal water system in Carmen, MB, which was used to maintain mesocosm water volume following initial mesocosm set-up. Phosphorus parameters are described as PO_4–P, and nutrient units are reported in mg/L. The results are detailed as the mean (± SD when applicable) of two sampling events during the middle and end of the 2022 study.

TP	TDP	SRP	TDN	TAN	NO_2–N	NO_3–N	SRSi	DOC
0.058 ±0.012	0.048 ±0.019	0.076 ±0.032	0.295 ±0.005	<LOD	0.120 ±0.025	0.575 ±0.318	0.911	15.706 ±5.584

Supplementary Table 4. Nutrient Chemistry results for wastewater effluent samples. The first sample was analyzed using HACH kits due to unavailability at the commercial laboratory, and the other four additions were analyzed at Farmers Edge Laboratories.

Date sampled	**Date of addition**	**Experiment Day**	**TP (mg kg^{-1})**	**NH4-N (mg kg^{-1})**	**TKN (mg kg^{-1})**	**Density (g mL^{-1})**
2022-06-21	2022-06-30	0	48.75	285.00	296.17	0.998
2022-07-14	2022-07-14	14	48.00	185.00	245.64	0.998
2022-07-28	2022-07-28	28	39.80	223.12	267.21	0.998
2022-08-12	2022-08-11	42	417.00	236.00	955.02	0.985
2022-08-26	2022-08-29	60	275.00	291.18	291.18	0.996

Supplementary Table 5. The average (± SD) or range (min–max; when applicable) of total phosphorus (TP), total ammonia nitrogen (TAN), total Kjeldahl nitrogen (TKN), density, and pH of aquaculture wastewater (AWW) collection events added to treatment mesocosms. The first AWW collection was added to mesocosms on experiment day 0, 14, and 28, while the second AWW collection was added on experiment days 42 and 60. Samples were analyzed at room temperature (20–25°C).

AWW Collection event	Number of samples	TP (mg L^{-1})	TAN (mg L^{-1})	TKN (mg L^{-1})	Density (g mL^{-1})	pH
First	9	43.0 (5.8)	229.2 (44.6)	296.2 (132.6)	0.998 (0.001)	7.58 (0.09)
Second	2	275.0–417.0	236.0–291.2	291.2–955.0	0.985–0.996	7.65–7.68

Supplementary Table 6. Detailed study design for mesocosms. Each treatment is defined by the volume (L) of total wastewater applied to each mesocosm over the course of the study period. The volume capacity of each mesocosm is described in L. Five biweekly wastewater additions occurred over the 70-day study period. Nominal biweekly addition volumes are reported in L, with the volume of wastewater doubled during the last two additions.

Treatment	Mesocosm Capacity	Nominal biweekly addition volume (L)	
		Addition days 0, 14, and 28	Addition days 42 and 60
0	3000	0 (unplanted control)	0 (unplanted control)
0	1500	0 (control 1)	0 (control 1)
0	1500	0 (control 2)	0 (control 2)
0	1500	0 (control 3)	0 (control 3)
0	1500	0 (control 4)	0 (control 4)
0	1500	0 (control 5)	0 (control 5)
7	3000	1.0	2.0
9.8	1500	1.4	2.8
12.6	1500	1.8	3.6
17.5	3000	2.5	5.0
23.1	1500	3.3	6.6
31.5	1500	4.5	9.0
42.7	1500	6.1	12.2
57.4	1500	8.2	16.4
77.7	1500	11.1	22.2
105	1500	15.0	30.0

Supplementary Table 7. Total metal analysis results for aquaculture wastewater effluent, completed at ALS Laboratories reported in mg L^{-1}.

Analyte	June 22, 2022	July 28, 2022	August 29, 2022
Aluminum, total	0.2468	0.076	0.09750078
Antimony, total	0	0	0
Arsenic, total	0.00296	0.00308	0.00300002
Barium, total	0.1348	0.0884	0.12875103
Beryllium, total	0	0	0
Bismuth, total	0	0	0
Boron, total	0.548	0.648	0.5291709
Cadmium, total	0.0003068	0.0001	0.00013833
Calcium, total	87.2	57.6	71.25057
Cesium, total	0	0	0
Chromium, total	0	0	0
Cobalt, total	0.00808	0.0042	0.00608338
Copper, total	0.1	0.0464	0.05375043
Iron, total	2.7	0.8	1.7541807
Lead, total	0.002836	0.001332	0.00094584
Lithium, total	0.0356	0.0416	0.03166692
Magnesium, total	38.56	33.64	39.58365
Manganese, total	0.2736	0.0868	0.20375163
Molybdenum, total	0.003164	0.001248	0.00181668
Nickel, total	0.0086	0.00864	0.0079584
Phosphorus, total	39.28	24.92	45.8337
Potassium, total	10.84	13.56	9.833412
Rubidium, total	0.00748	0.00836	0.00587505
Selenium, total	0.002704	0.001728	0.00232502
Silicon, total	4.24	3.28	3.33336
Silver, total	0	0	0
Sodium, total	74.8	91.6	69.16722

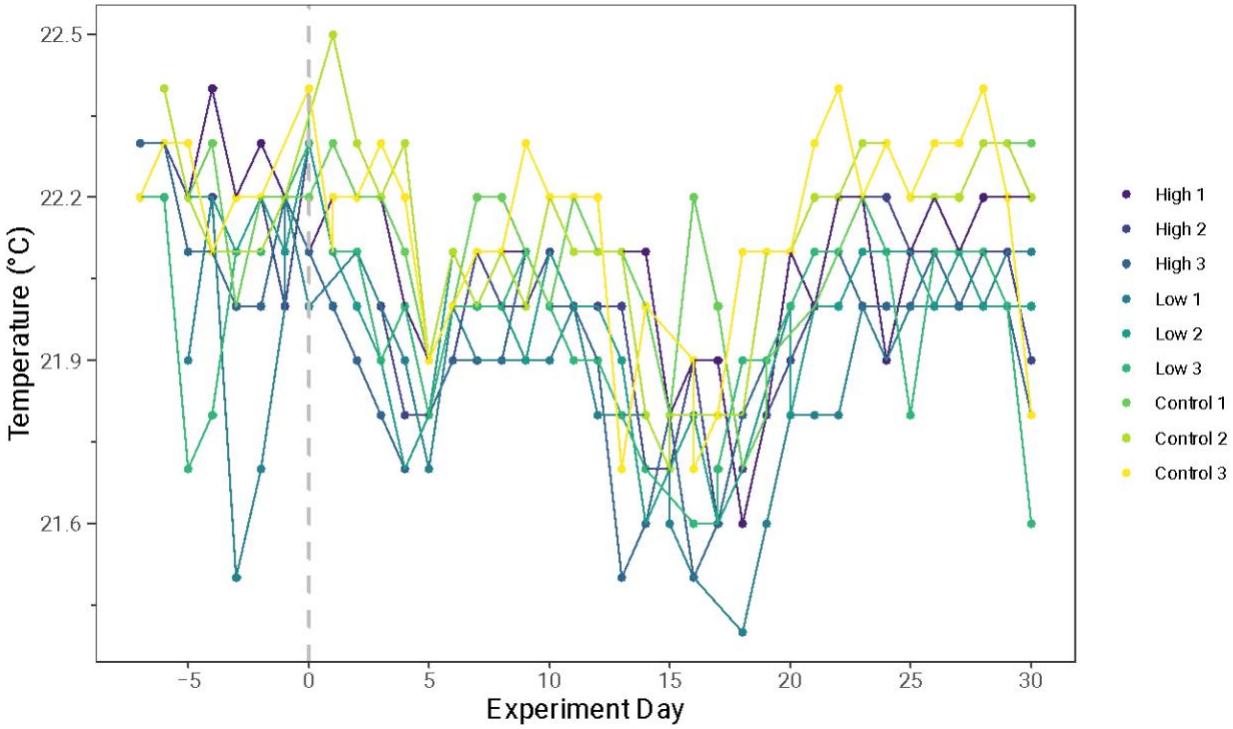
Strontium, total	0.3836	0.2656	0.30333576
Sulfur, total	13.96	12.88	11.958429
Tellurium, total	0	0	0
Thallium, total	0.00004	0	0
Thorium, total	0	0	0
Tin, total	0.00076	0	0
Titanium, total	0.0052	0.0024	0.00233335
Tungsten, total	0	0	0
Uranium, total	0.002208	0.00082	0.00105834
Vanadium, total	0	0	0
Zinc, total	0.664	0.2312	0.3791697
Zirconium, total	0	0	0

Supplementary Table 8. Results from linear mixed models (LMM) for fathead minnow (*Pimephales promelas*) growth indices using the equation y ~ treatment + sex + (treatment x sex) + (1 | mesocosm). The fixed effects are treatment and sex, the interaction effect is treatment x sex, and the random effect is (1 | mesocosm) to account for pseudoreplication from each mesocosm. For YSI water quality parameters, the model incorporated repeated measures and therefore the model is y ~ treatment + time + treatment x time + (1 | mesocosm). The fixed effects are treatment and time, while the interaction effect is treatment x time, and the random effect is (1 | mesocosm) to account for repeated measures over time.

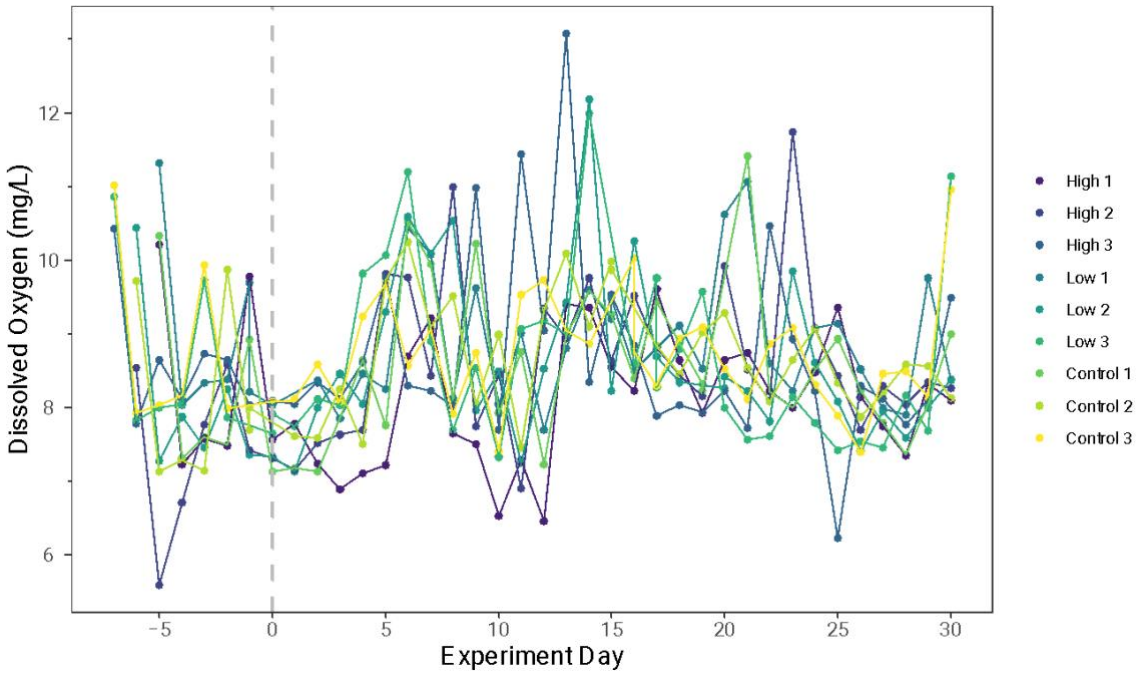
Response Variable	Treatment		Sex		Treatment x Sex	
	t-value	p-value	t-value	p-value	t-value	p-value
K	-1.662	0.106	0.520	0.604	0.609	0.544
LSI	-1.341	0.190	-0.847	0.399	1.579	0.117
GSI	-1.316	0.194	-11.352	<2e-16	0.991	0.324
Response Variable	Treatment		Time		Treatment x Time	
	t-value	p-value	t-value	p-value	t-value	p-value
Temp	0.370	0.712	-12.171	<2e-16	-0.454	0.650
DO	-0.646	0.518	-24.481	<2e-16	-0.938	0.349
pH	1.178	0.240	-11.393	<2e-16	-1.889	0.060
SPC	1.750	0.084	-3.697	2.6e-4	-3.129	0.002

Appendix B

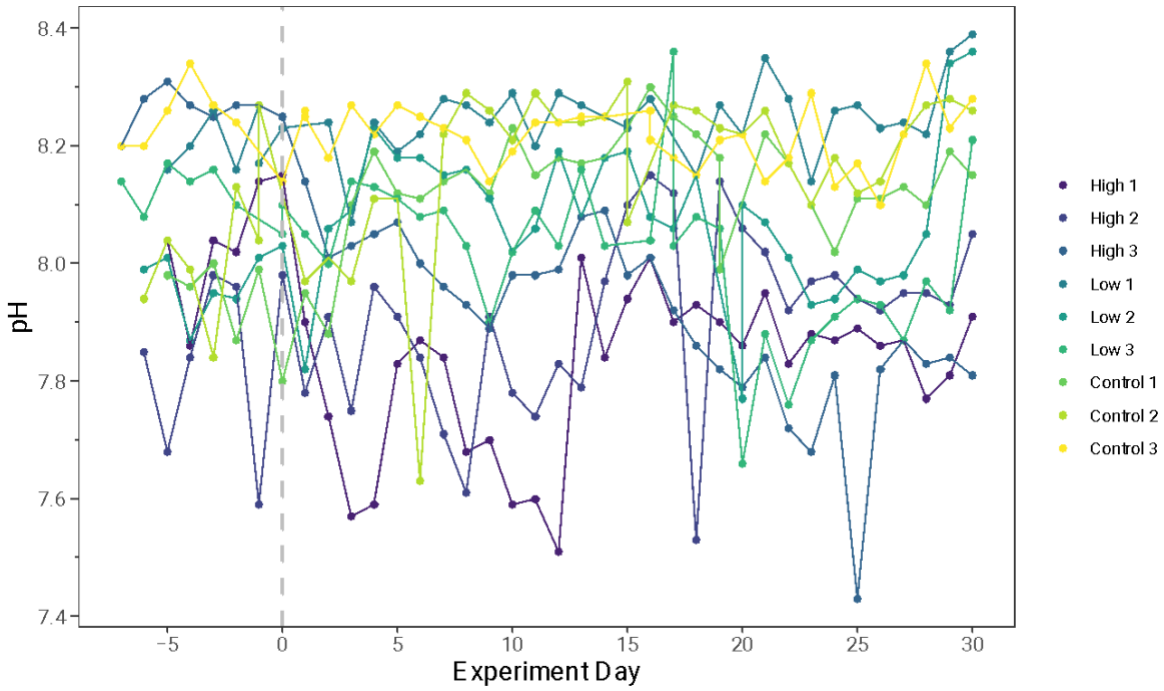
Supplemental Figures



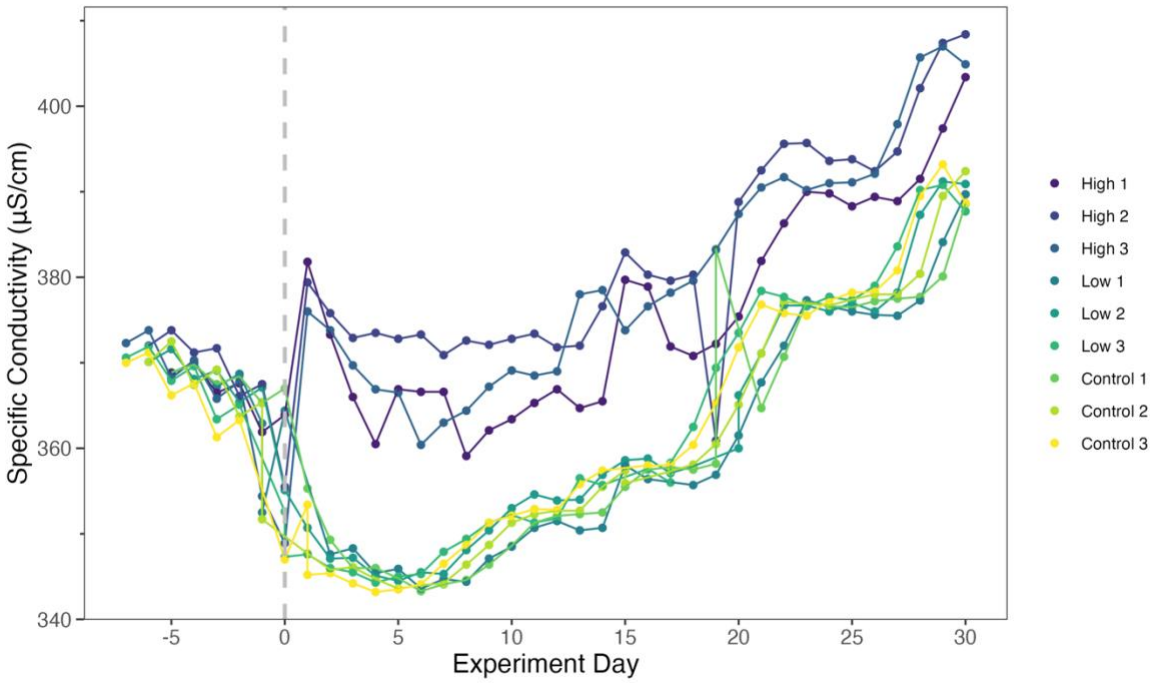
Supplemental Figure 29. Water temperature (°C) from day -7 to day 30 during nutrient exposures in 70-L tanks. Data are shown for 3 replicate tanks from three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



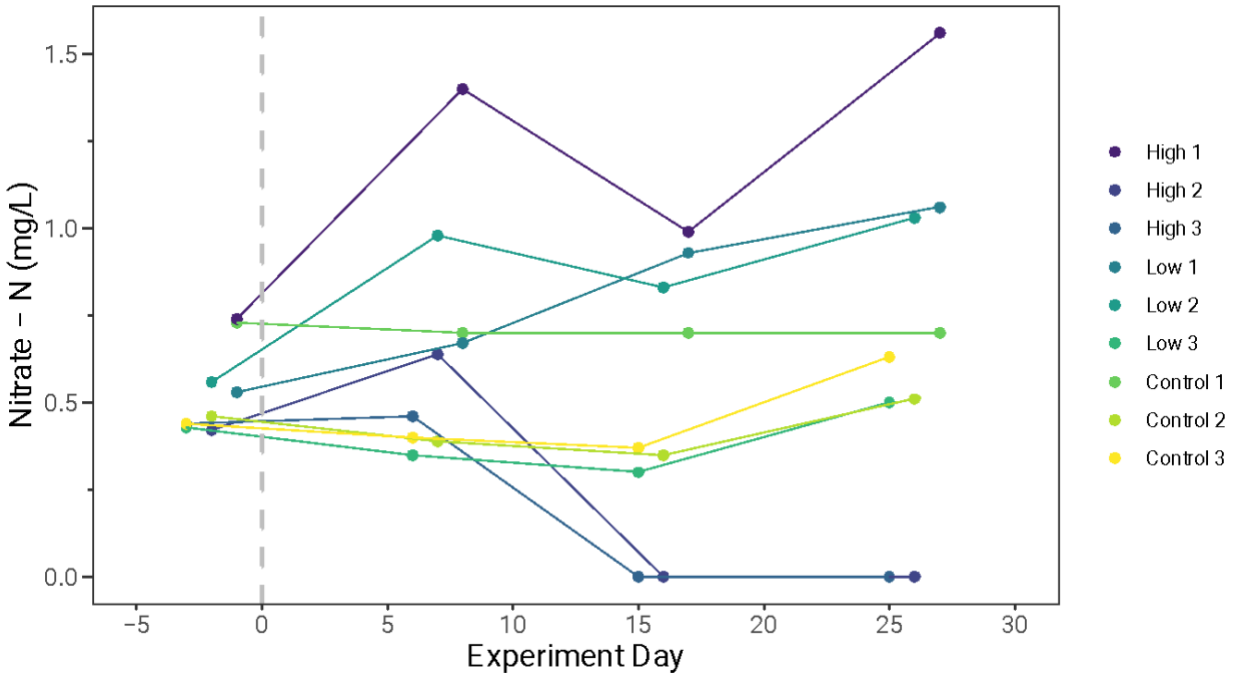
Supplemental Figure 30. The dissolved oxygen (mg L^{-1}) from day -7 to day 30 during nutrient exposures in 70-L tanks. Data are shown for 3 replicate tanks from three experimental groups, control, low nutrient exposure (0.39 mg $L^{-1}$of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO4– P) in triplicate. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



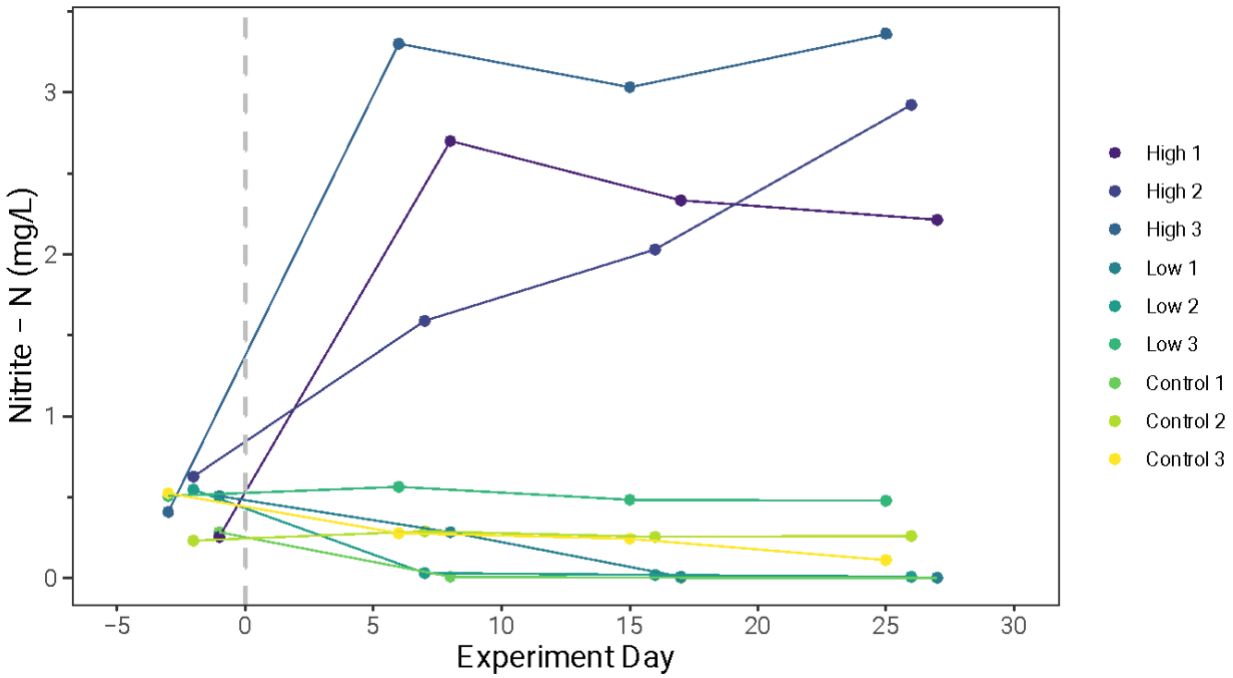
Supplemental Figure 31. The pH readings from day -7 to day 30 during nutrient exposures in 70-L tanks. Data are shown for 3 replicate tanks from three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



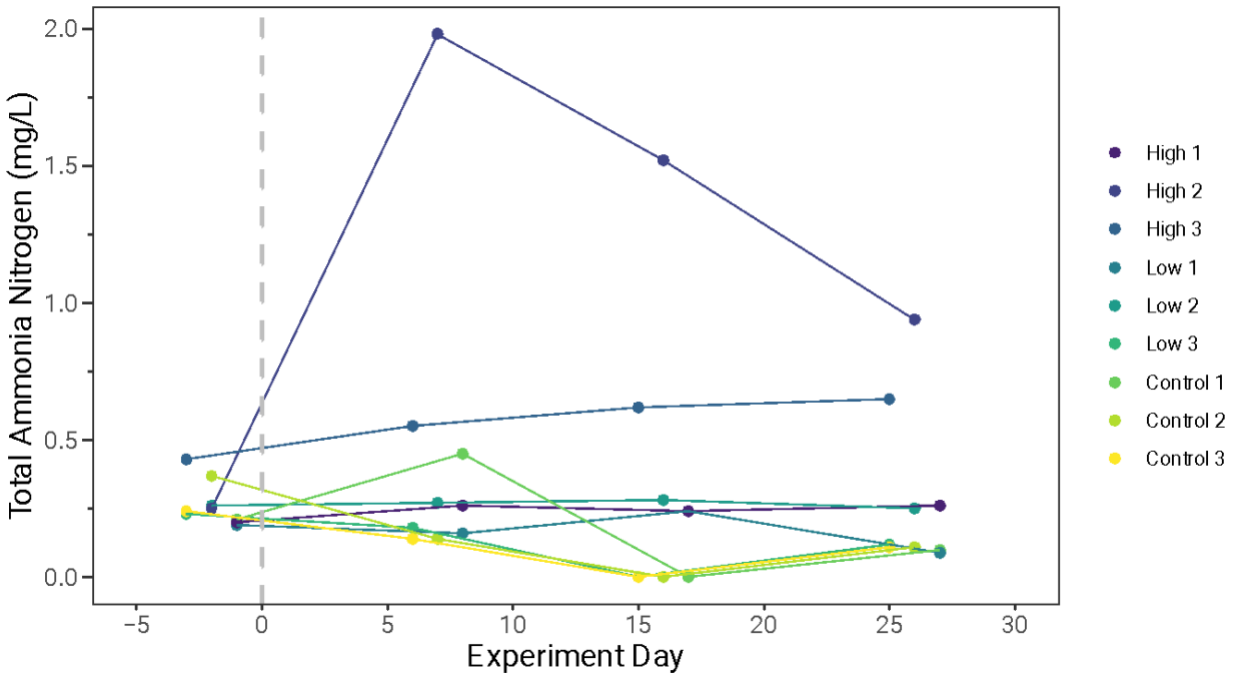
Supplemental Figure 32. The specific conductivity (µS cm^{-1}) from day -7 to day 30 during nutrient exposures in 70-L tanks. Data are shown for 3 replicate tanks from three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



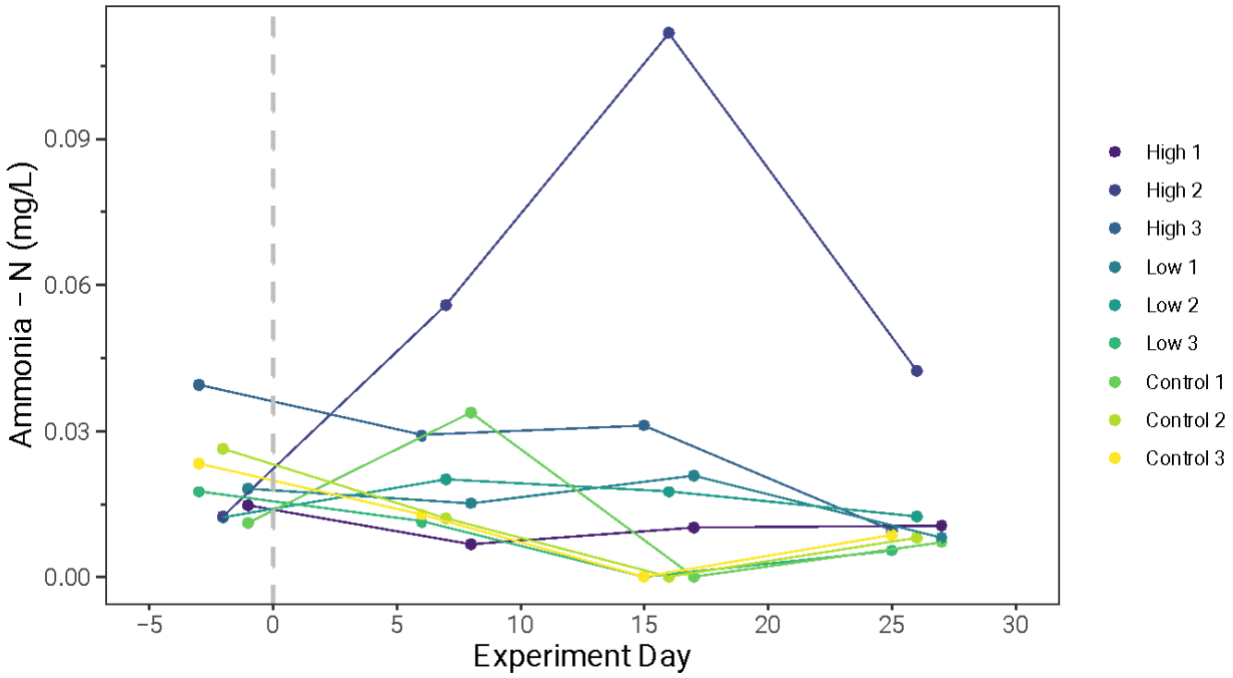
Supplemental Figure 33. Nitrate as N (mg L^{-1}) measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



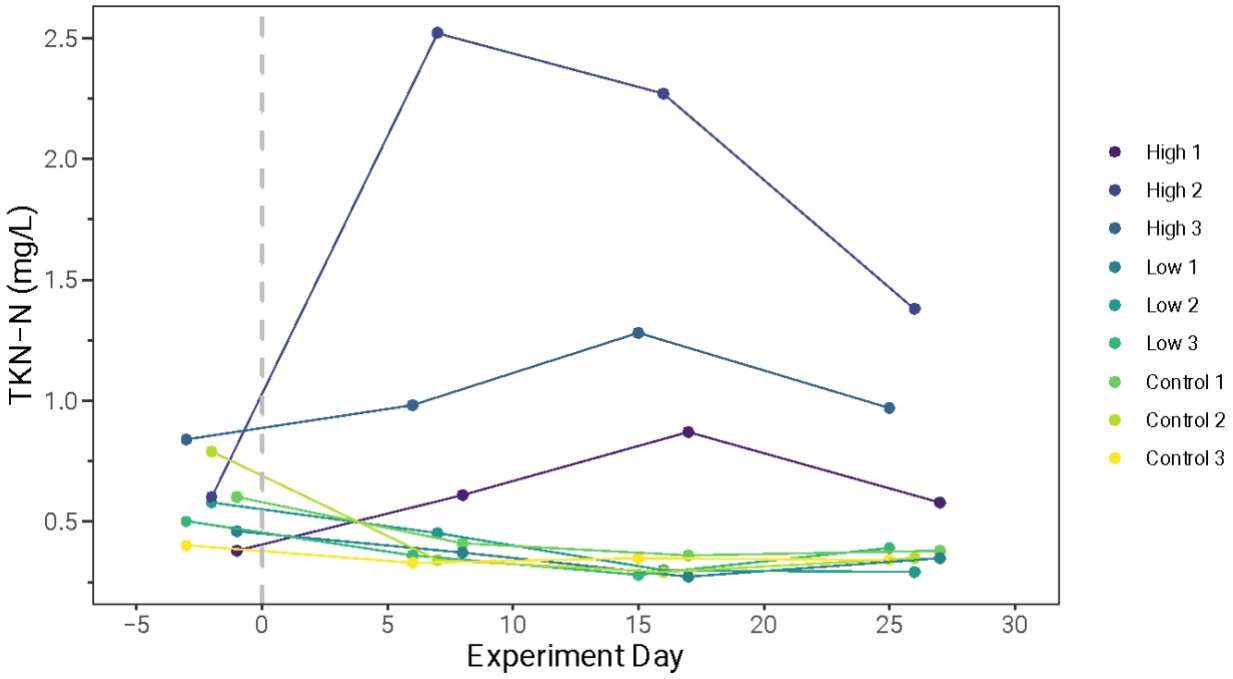
Supplemental Figure 34. Nitrite as N (mg L^{-1}) measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



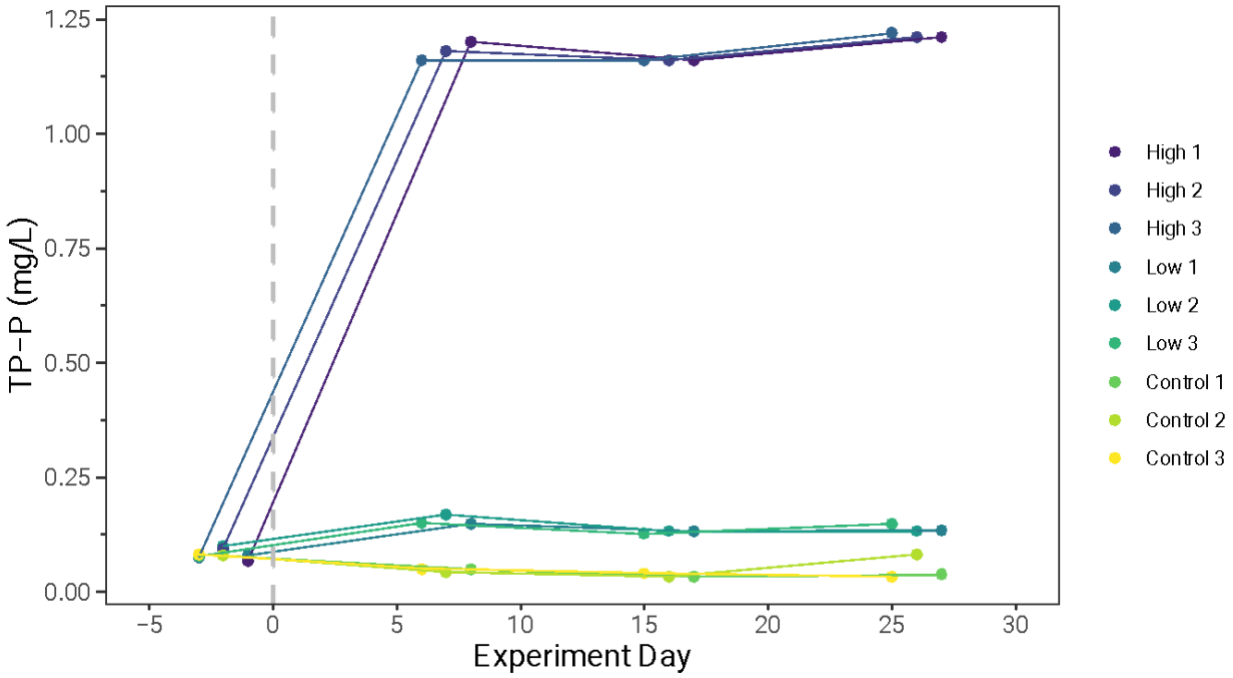
Supplemental Figure 35. Total ammonia nitrogen as N (mg L^{-1}) measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



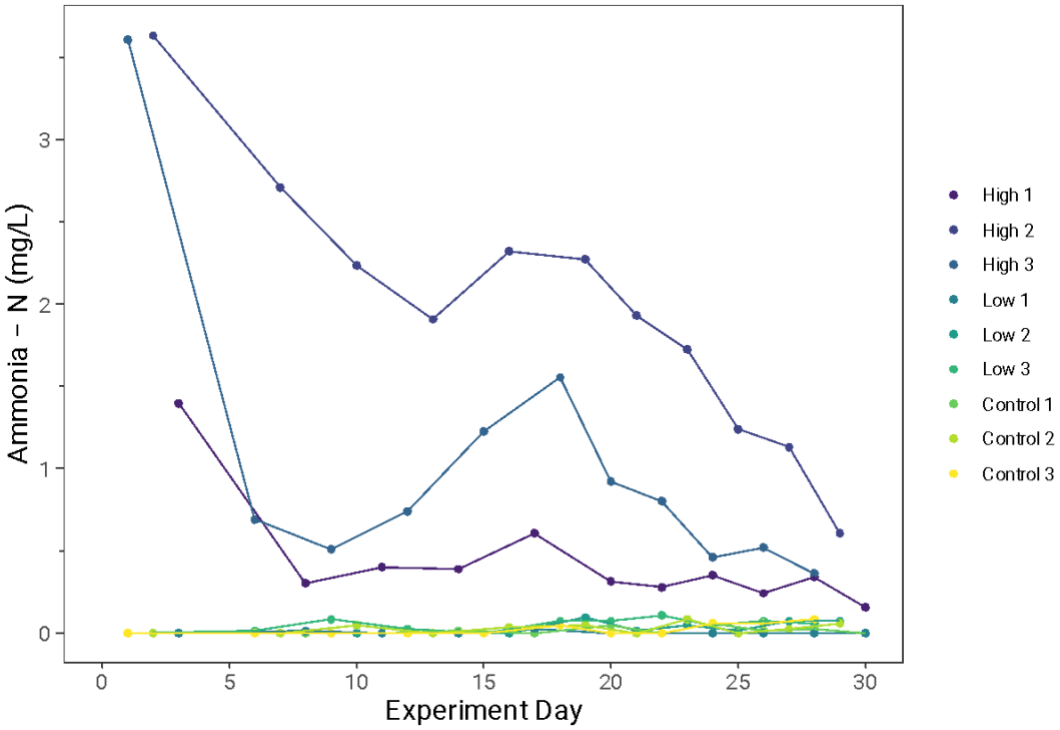
Supplemental Figure 36. Ammonia as N (NH_3-N; mg L^{-1}) calculated from TAN reading and pH reading, measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



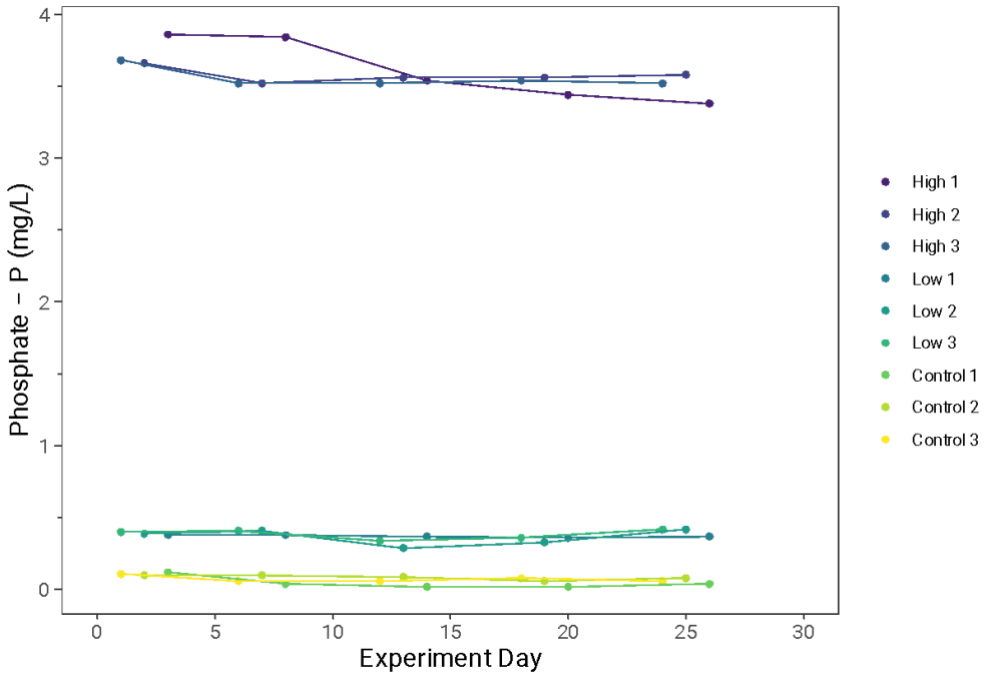
Supplemental Figure 37. Total Kjeldahl Nitrogen as N (TKN – N; mg L^{-1}) measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



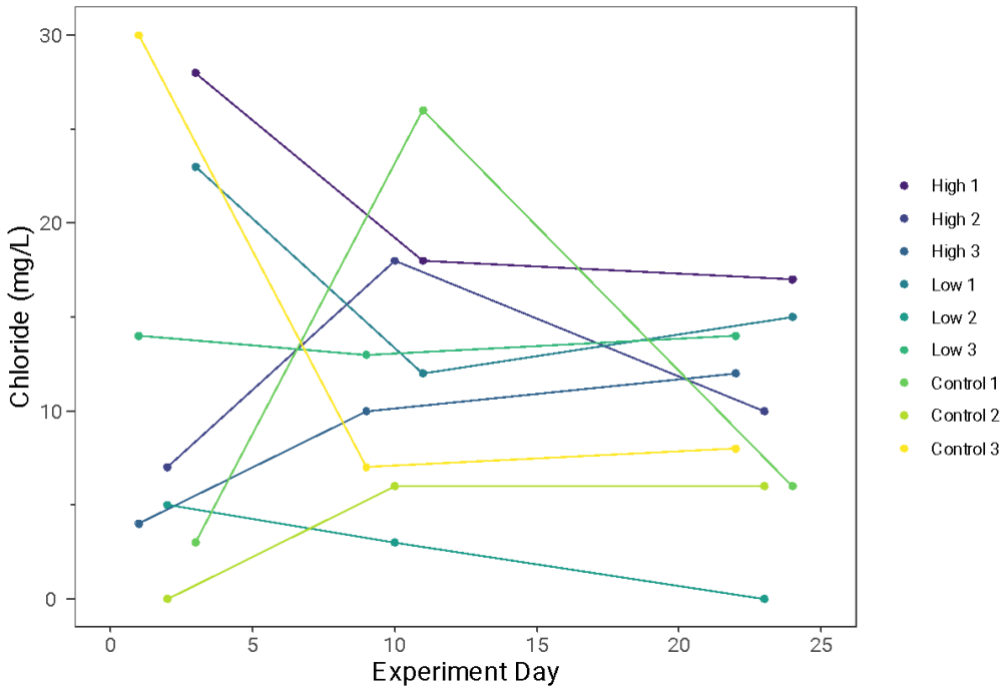
Supplemental Figure 38. Total phosphorus as P (TP – P; mg L^{-1}) measured in nine 70-L experimental tanks at four time points, each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The initial measurement was taken prior to experiment day 0, with the other three points being at the mid-points and final days of study. The grey dashed line on day 0 indicates when nutrients were first added to low and high treatment tanks.



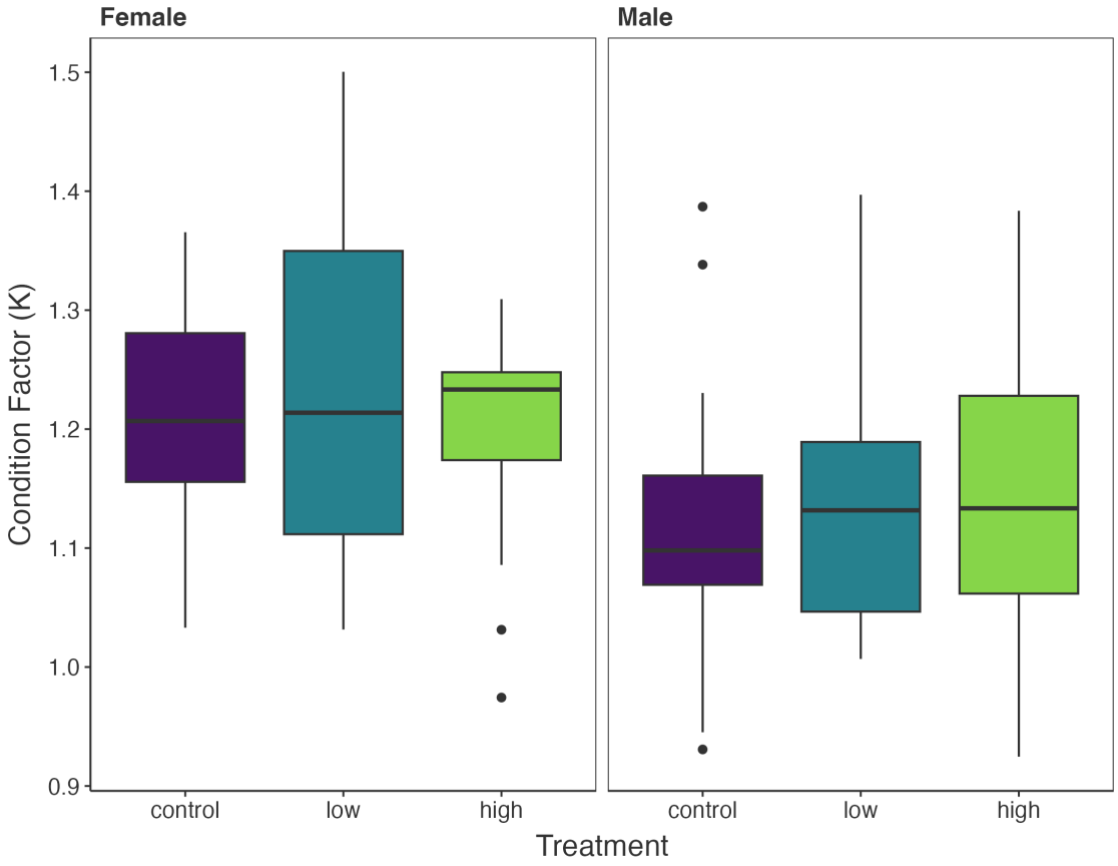
Supplemental Figure 39. Ammonia as N (mg L^{-1}) measurements obtained for each of the nine 70-L experimental tanks at several time points over a 30-day exposure period using a handheld colorimeter. Each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. Nutrients were added on day 0.



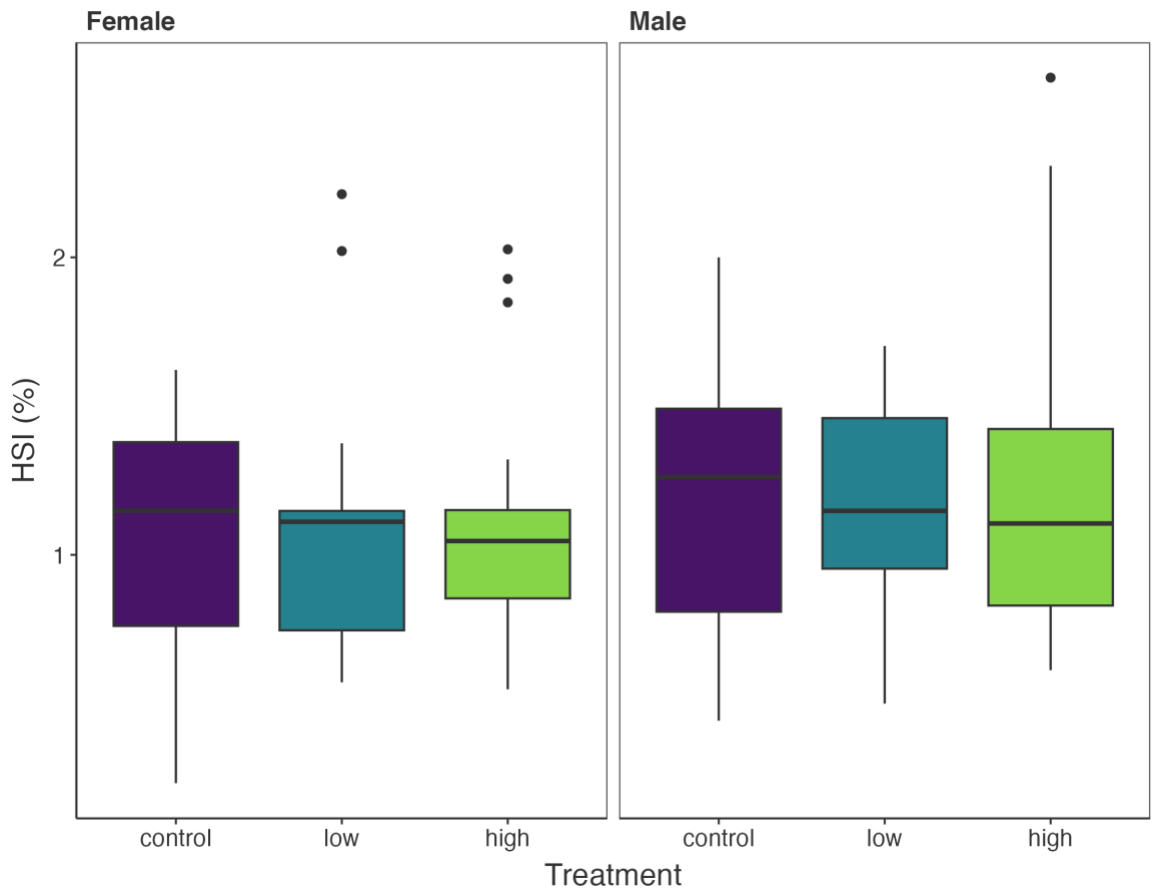
Supplemental Figure 40. Phosphate as P (mg L^{-1}) measurements ± 0.04 mg L^{-1} obtained for each of the nine 70-L experimental tanks at several time points over a 30-day exposure period using a handheld colorimeter. Each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. Nutrients were added on day 0.



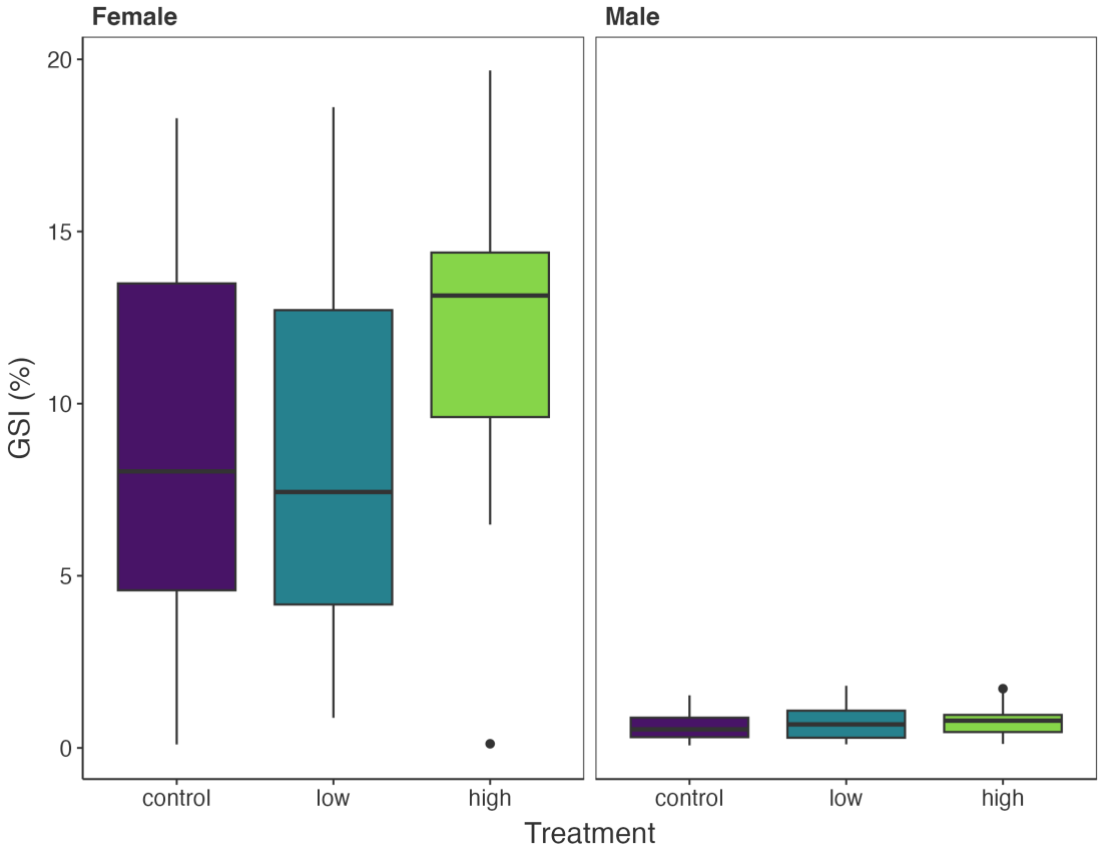
Supplemental Figure 41. Chloride (mg L^{-1}) measurements obtained for each of the nine 70-L experimental tanks at several time points over a 30-day exposure period using a handheld colorimeter. Each tank belonging to one of three experimental groups, control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. Nutrients were added on day 0. Chloride concentration remained below levels of concern following additions of ammonium chloride.



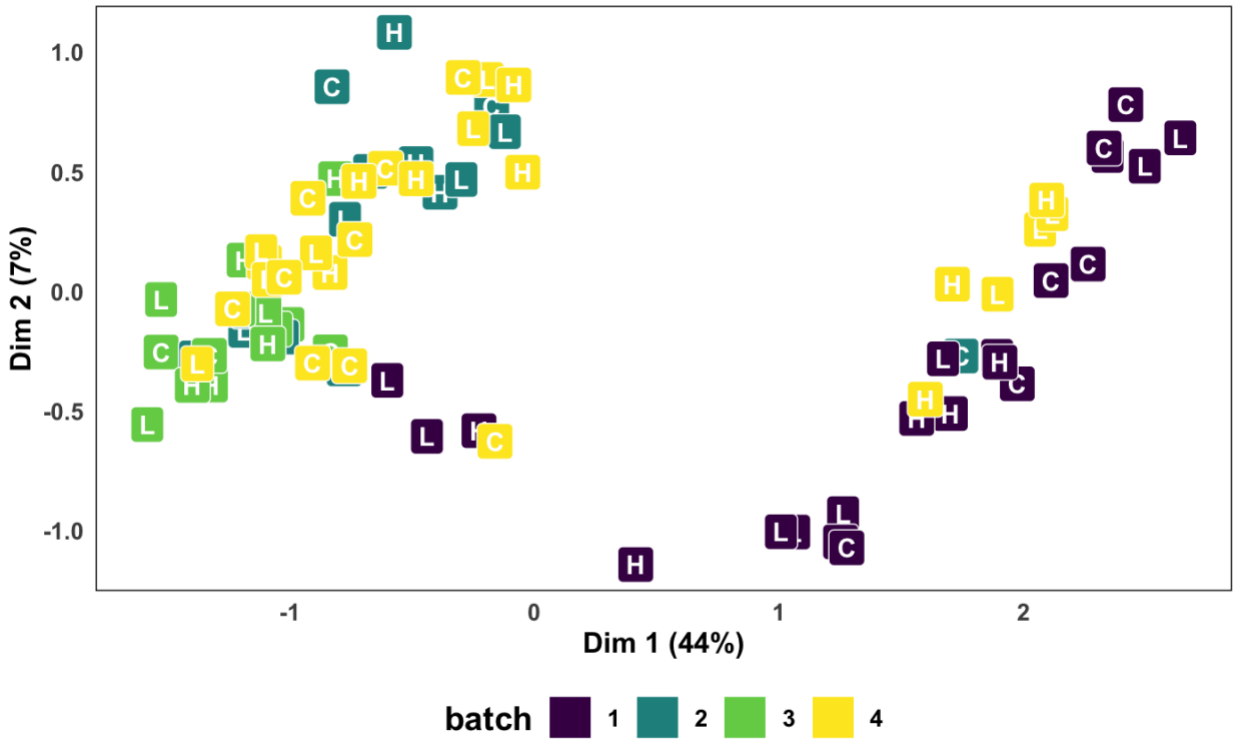
Supplemental Figure 42. Condition factor (K) of female and male fathead minnows chronically exposed to excess ammonium and phosphorus for 30-days. K is determined according to the equation $K = \frac{weight * 100}{length^3}$ using morphometrical data collected at the conclusion of the study. No significant differences were observed between treatments (n = 45). The experimental treatments consisted of control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The points lying outside of boxes indicate outliers.



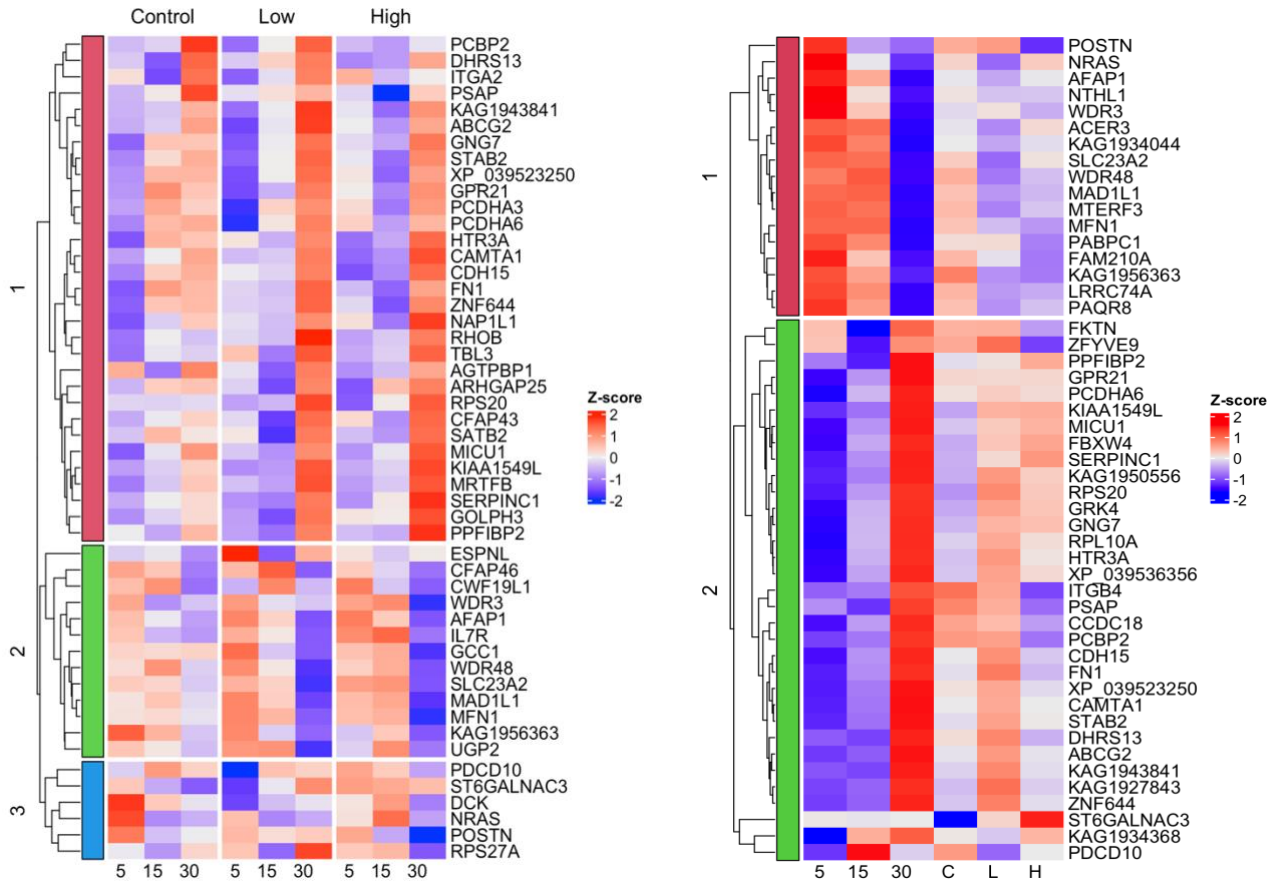
Supplemental Figure 43. Hepatosomatic index (HSI) of female and male fathead minnows chronically exposed to excess ammonium and phosphorus for 30-days. HSI is liver weight as a percentage of total body weight. No significant differences were observed between treatments (n = 45). The experimental treatments consisted of control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The points lying outside of boxes indicate outliers.



Supplemental Figure 44. Gonadosomatic index (GSI) of female and male fathead minnows chronically exposed to excess ammonium and phosphorus for 30-days. GSI is calculated as gonad weight as a percentage of total body weight. No significant differences were observed between treatments (n = 45). The experimental treatments consisted of control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) in triplicate. The points lying outside of boxes indicate outliers.

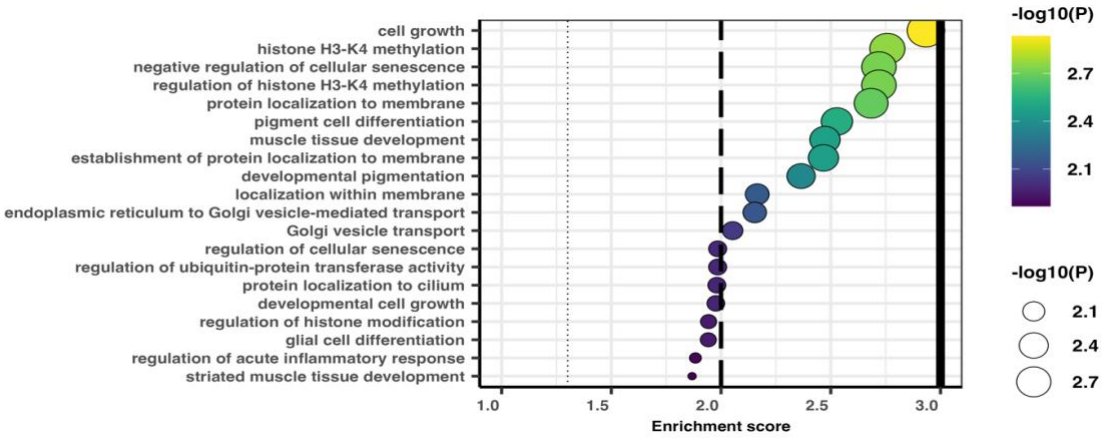


Supplemental Figure 45. Multidimensional scaling plot of fathead minnow mucus proteins, highlighting batch effects and experimental treatments, control (C), low exposure (L), and high exposure (H). Low nutrient exposure consisted of 0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P, and high nutrient exposure consisted of 4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P. Each data point is one sample containing epidermal mucus proteins pooled randomly from five fish residing in a control (n = 3), low (n = 3), or high (n = 3) nutrient exposure treatment. Each of the four batches indicate which samples were grouped to prepare proteins for LC-MS/MS protein quantification and identification. Batch one was prepared presumptively to analysis and likely degraded in storage.

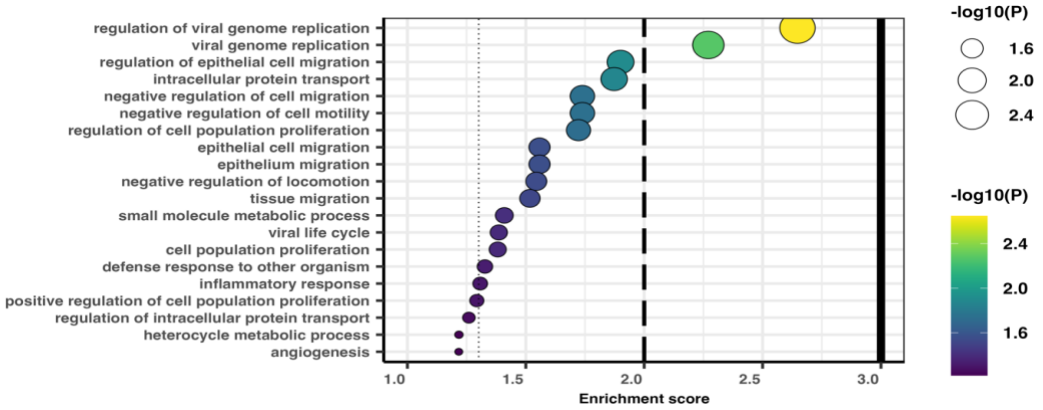


A. **B.**

Supplemental Figure 46. Heatmaps of the top 50 proteins in the fathead minnow mucus proteome which significantly changed in abundance due to treatment and exposure time (FDR < 0.2). The protein abundances are represented by z-scores, and the cluster blocks represent hierarchical clustering using Euclidean distances. The heatmaps are organized by the effect of individual exposure time in each treatment (**A**), and the overall effect of exposure time and treatment (**B**) on mucus proteins. Each column is labelled by either treatment: control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), and high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P; C, L, H), or sample day (Day 5, 15, or 30).

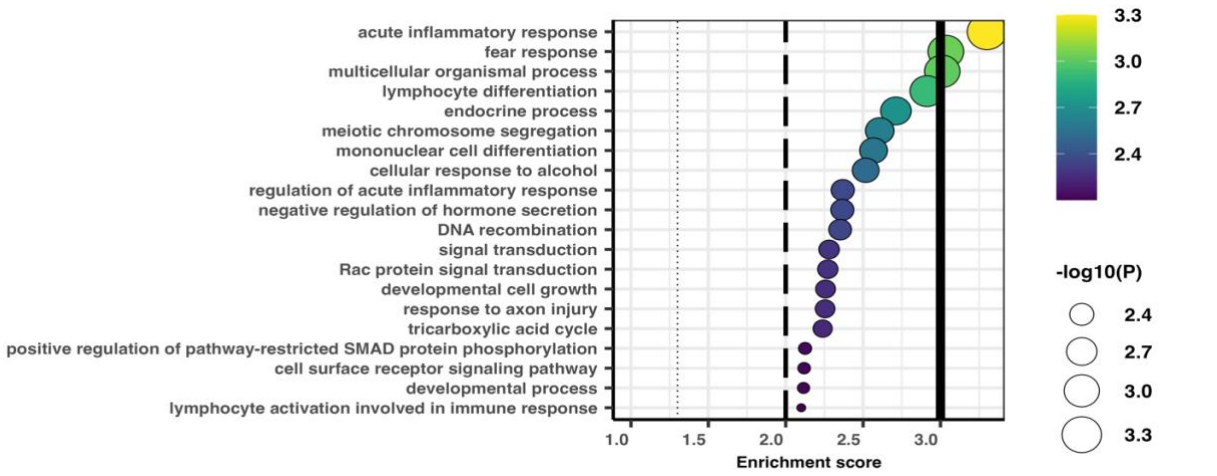


A.

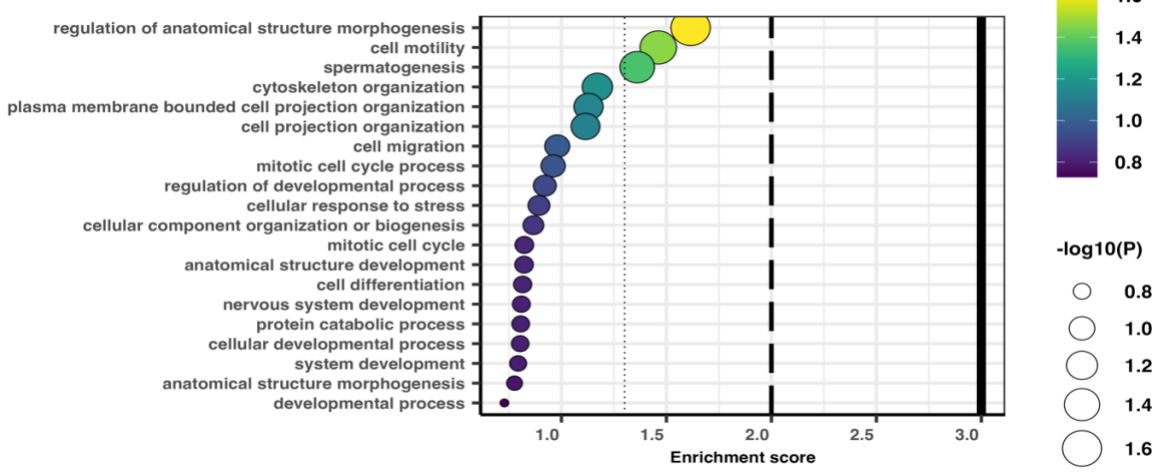


B.

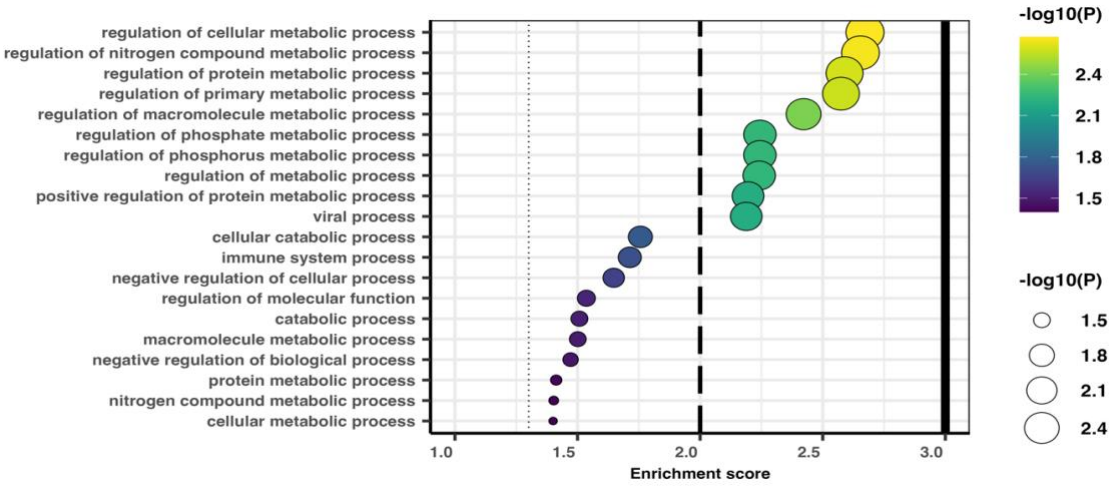
Supplemental Figure 47. The enrichment score (-log10(P-value)) of the top 20 biological process GO terms with at least three significant protein hits per term from mucus tissues of fathead minnows (*Pimephales promelas*) belonging to one of three experimental groups: low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), or high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P). Each plot contains GO terms derived from a differential expression analysis between experimental groups including high nutrient exposure **(A)** and the low nutrient exposure **(B)** on experiment day 30 compared to experiment day 5. Cut-off lines drawn at equivalents of p=0.05 (dotted), p=0.01 (dashed), p=0.001 (solid).


acute inflammatory response
fear response
multicellular organismal process
lymphocyte differentiation
endocrine process
meiotic chromosome segregation
mononuclear cell differentiation
cellular response to alcohol
regulation of acute inflammatory response
negative regulation of hormone secretion
DNA recombination
signal transduction
Rac protein signal transduction
developmental cell growth
response to axon injury
tricarboxylic acid cycle
positive regulation of pathway-restricted SMAD protein phosphorylation
cell surface receptor signaling pathway
developmental process
lymphocyte activation involved in immune response
1.0
1.5
2.0
2.5
3.0
Enrichment score
3.3
3.0
2.7
2.4
-log10(P)
2.4
2.7
3.0
3.3

A


regulation of anatomical structure morphogenesis
cell motility
spermatogenesis
cytoskeleton organization
plasma membrane bounded cell projection organization
cell projection organization
cell migration
mitotic cell cycle process
regulation of developmental process
cellular response to stress
cellular component organization or biogenesis
mitotic cell cycle
anatomical structure development
cell differentiation
nervous system development
protein catabolic process
cellular developmental process
system development
anatomical structure morphogenesis
developmental process
1.0
1.5
2.0
2.5
3.0
Enrichment score
1.4
1.2
1.0
0.8
-log10(P)
0.8
1.0
1.2
1.4
1.6

B.


regulation of cellular metabolic process
regulation of nitrogen compound metabolic process
regulation of protein metabolic process
regulation of primary metabolic process
regulation of macromolecule metabolic process
regulation of phosphate metabolic process
regulation of phosphorus metabolic process
regulation of metabolic process
positive regulation of protein metabolic process
viral process
cellular catabolic process
immune system process
negative regulation of cellular process
regulation of molecular function
catabolic process
macromolecule metabolic process
negative regulation of biological process
protein metabolic process
nitrogen compound metabolic process
cellular metabolic process
1.0
1.5
2.0
2.5
3.0
Enrichment score
-log10(P)
2.4
2.1
1.8
1.5
-log10(P)
1.5
1.8
2.1
2.4

C.

Supplemental Figure 48. The enrichment score (-log10(P-value)) of the top 20 biological process GO terms with at least three significant protein hits per term from mucus tissues of fathead minnows (*Pimephales promelas*) belonging to one of three experimental groups: control, low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P), or high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P). Each plot contains GO terms derived from a differential expression analysis between experimental groups including high nutrient exposure **(A)**, low nutrient exposure **(B)**, and the control **(C)** on experiment day 30 compared to experiment day 15. Cut-off lines drawn at equivalents of p=0.05 (dotted), p=0.01 (dashed), p=0.001 (solid).

Supplementary Tables

Supplemental Table 9. Background water chemistry following charcoal treatment used as a source for laboratory tanks at Ontario Tech University. Water samples were taken on March 11, 2022, and analyzed at York-Durham Regional Environmental Laboratory in Pickering, Ontario.

Parameter	Result	Unit
Calculated Conductivity	398	µS cm^{-1}
Calculated TDS	194	mg L^{-1}
Total Anions	3.55	meq L^{-1}
Total Cations	3.6	meq L^{-1}
Hardness (as CaCO3)	131	mg L^{-1}
pH	8.2	
Alkalinity as CaCO3	89.3	mg L^{-1}
Turbidity	<0.10	NTU
Ammonia and Ammonium as N	<0.25	mg L^{-1}
Bromide	<0.50	mg L^{-1}
Calcium	37.5	mg L^{-1}
Chloride	39.5	mg L^{-1}
Fluoride	0.49	mg L^{-1}
Magnesium	9.01	mg L^{-1}
Nitrate as N	0.51	mg L^{-1}
Nitrite as N	<0.05	mg L^{-1}
Phosphate as P	<0.05	mg L^{-1}

Potassium	1.74	mg L^{-1}
Sodium	21.6 (outside limit)	mg L^{-1}
Sulphate	28.2	mg L^{-1}
Aluminum	0.0406	mg L^{-1}
Antimony	<0.0005	mg L^{-1}
Arsenic	<0.0005	mg L^{-1}
Cadmium	<0.0005	mg L^{-1}
Chromium	<0.0005	mg L^{-1}
Cobalt	<0.0005	mg L^{-1}
Copper	0.0115	mg L^{-1}
Iron	0.0035	mg L^{-1}
Lead	<0.0005	mg L^{-1}
Manganese	<0.0005	mg L^{-1}
Molybdenum	<0.0025	mg L^{-1}
Nickel	0.0007	mg L^{-1}
Selenium	<0.0005	mg L^{-1}
Zinc	0.0015	mg L^{-1}

Supplemental Table 10. The top 20 biological processes in a gene ontology (GO) enrichment analysis of liver proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) for 30 days. The top GO terms are comparing the high nutrient treatment to the control. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	# of Proteins in GO Term	# of DE proteins (increased)	# of DE proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0045833	negative regulation of lipid metabolic process	20	0	3	1.000	0.004
GO:0062014	negative regulation of small molecule metabolic process	20	0	3	1.000	0.004
GO:0009895	negative regulation of catabolic process	66	0	5	1.000	0.004
GO:0042113	B cell activation	43	3	1	0.006	0.517
GO:0032760	positive regulation of tumor necrosis factor production	16	2	1	0.008	0.236
GO:1903557	positive regulation of tumor necrosis factor superfamily cytokine production	16	2	1	0.008	0.236
GO:0009894	regulation of catabolic process	187	0	8	1.000	0.011
GO:0090596	sensory organ morphogenesis	57	3	1	0.013	0.620
GO:1903076	regulation of protein localization to plasma membrane	21	2	1	0.014	0.298
GO:0042472	inner ear morphogenesis	23	2	1	0.017	0.321
GO:0009056	catabolic process	477	3	14	0.820	0.019
GO:0015031	protein transport	293	3	10	0.489	0.020
GO:0035303	regulation of dephosphorylation	25	2	1	0.020	0.344

GO:0032680	regulation of tumor necrosis factor production	25	2	1	0.020	0.344
GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	25	2	1	0.020	0.344
GO:0032640	tumor necrosis factor production	25	2	1	0.020	0.344
GO:0071706	tumor necrosis factor superfamily cytokine production	25	2	1	0.020	0.344
GO:0044248	cellular catabolic process	385	3	12	0.683	0.020
GO:0016241	regulation of macroautophagy	36	0	3	1.000	0.021
GO:0042471	ear morphogenesis	26	2	1	0.021	0.355

Supplemental Table 11. The top 20 biological processes in a gene ontology (GO) enrichment analysis of liver proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a low nutrient exposure (0.39 mg L^{-1} of total ammoniacal nitrogen (TAN), and 0.29 mg L^{-1} of PO_4– P)) for 30 days. The top GO terms are comparing the low nutrient treatment to the control. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0071495	cellular response to endogenous stimulus	268	34 (CASK, FUT8, PTPRA, PIK3CG, HTR3A, PDGFC, etc.)	11 (CAT, FGFRL1, ADAM17, etc.)	0.00028	0.66339
GO:0010453	regulation of cell fate commitment	12	5 (BMPR1A, IL12B, TNFSF18, CHD3, AR)	0	0.00083	1

GO:0009719	response to endogenous stimulus	309	36 (CASK, FUT8, PTPRA, PIK3CG, HTR3A, PDGFC, etc.)	13 (CAT, FGFRL1, ADAM17, etc.)	0.00098	0.63497
GO:0021535	cell migration in hindbrain	4	3 (ARL13B, EPHB2, EPHB1)	0	0.00127	1
GO:0071310	cellular response to organic substance	352	39 (PTPRA, PIK3CG, HTR3A, PDGFC, CARM1, etc.)	19 (IL1RAP, DAPK1, TMBIM6, ADAM17, ACTG1, etc.)	0.00158	0.21647
GO:0021602	cranial nerve morphogenesis	9	4 (HOXD3, EPHB2, EPHB1, PLXNA1)	0	0.00219	1
GO:1903078	positive regulation of protein localization to plasma membrane	9	4 (EPHB2, PRKCE, LRP1, RACK1)	0	0.00219	1
GO:0071417	cellular response to organonitrogen compound	137	19 (PTPRA, PIK3CG, HTR3A, PDGFC, BLM, EPHB2, etc.)	5 (FLNA, RPS6KB1, AKAP6, etc.)	0.00251	0.74265
GO:0009887	animal organ morphogenesis	213	26 (HOXD3, MYO7A, PDGFC, ARL13B, etc.)	7 (RPS6KB1, FGFRL1, PLEKHA1, ZNF335, etc.)	0.00278	0.85163
GO:2000316	regulation of T-helper 17 type immune response	5	3 (IL12B, EPHB2, TNFSF18)	0	0.00301	1
GO:0007568	aging	34	3 (CARM1, SLC1A2, GRB2)	6 (CAT, ENDOG, RPS6KB1, TP63, AMH, VCAM1)	0.4269	0.00336
GO:0071407	cellular response to organic cyclic compound	111	16 (PIK3CG, HTR3A, CARM1, BLM, HCN2, DIAPH2, CYP7A1, etc.)	5 (FLNA, RPS6KB1, AKAP6, etc.)	0.0037	0.55831
GO:0021953	central nervous system neuron differentiation	46	9 (GBA2, BMPR1A, EPHB2, CSF1R, etc.)	4 (ZNF335, NIN, CHD5, MDGA2)	0.00372	0.14685
GO:1901699	cellular response to nitrogen compound	144	19 (PTPRA, PIK3CG, HTR3A,	5 (FLNA, RPS6KB1, AKAP6, etc.)	0.00445	0.78138

			PDGFC, BLM, EPHB2, etc.)			
GO:0050962	detection of light stimulus involved in sensory perception	6	3	0	0.006	1.000
GO:0050908	detection of light stimulus involved in visual perception	6	3	0	0.006	1.000
GO:0072538	T-helper 17 type immune response	6	3	0	0.006	1.000
GO:0070661	leukocyte proliferation	49	9	3	0.006	0.375
GO:0090329	regulation of DNA-templated DNA replication	18	2	4	0.361	0.007
GO:1904377	positive regulation of protein localization to cell periphery	12	4	0	0.007	1.000

Supplemental Table 12. The top 20 biological processes in a gene ontology (GO) enrichment analysis of liver proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P), and a low nutrient exposure (0.39 mg L^{-1} TAN, and 0.29 mg L^{-1} of PO_4– P) for 30 days. The top GO terms are comparing the high nutrient treatment to the low. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0033143	regulation of intracellular steroid hormone receptor signaling pathway	15	6 (CRY2, TP63, DAB2, SRC, CNOT1, KMT2D)	2 (NCOR1, CRY1)	1.72E-05	0.100

GO:0007186	G protein-coupled receptor signaling pathway	136	8 (RIC8A, GPR55, etc.)	15 (PRKAR1A, JAK2, GNA12, BHLHA15, etc)	0.205	7.65E-05
GO:0033146	regulation of intracellular estrogen receptor signaling pathway	7	4 (TP63, SRC, CNOT1. KMT2D)	0	9.20E-05	1.000
GO:0030518	intracellular steroid hormone receptor signaling pathway	21	6 (CRY2, TP63, DAB2, SRC, CNOT1, KMT2D)	3 (JAK2, NCOR1, CRY1)	1.52E-04	0.038
GO:0033144	negative regulation of intracellular steroid hormone receptor signaling pathway	8	4 (CRY2, TP63, DAB2, CNOT1)	2 (NCOR1, CRY1)	1.78E-04	0.031
GO:0043401	steroid hormone mediated signaling pathway	25	6 (CRY2, TP63, DAB2, SRC, CNOT1, KMT2D)	3 (JAK2, NCOR1, CRY1)	4.32E-04	0.059
GO:0030520	intracellular estrogen receptor signaling pathway	10	4 (TP63, SRC, CNOT1, KMT2D)	0	5.01E-04	1.000
GO:0062014	negative regulation of small molecule metabolic process	20	3 (SIRT4, SOGA1, ACADVL)	5 (FMO4, AKT1, HDAC4, NCOR1, CRY1)	0.048	5.61E-04
GO:1903828	negative regulation of protein localization	41	7 (SIRT4, UBAC2, ASTN2, DERL2, MAP1A, DAB2, TMBIM1)	4 (LRRC15, ACTN2, ADRA2A, YOD1)	0.001	0.058
GO:0036035	osteoclast development	6	3 (SLC9B2, ATP6AP1, SRC)	0	0.001	1.000
GO:0010677	negative regulation of cellular carbohydrate metabolic process	7	1 (SOGA1)	3 (HDAC4, NCOR1, CRY1)	0.259	0.001

GO:0051668	localization within membrane	126	4 (SRP54, ATP6AP1, DAB2, TMBIM1)	12 (DENND4C, VPS26A, LRRC15, CD2, ACTN2, GRIN3B, etc.)	0.783	0.002
GO:0062012	regulation of small molecule metabolic process	68	9 (MLXIPL, SIRT4, SOGA1, APOA1, DAB2, ACADVL, etc.)	7 (FMO4, FABP1, AKT1, HDAC4, NCOR1, ADORA2B, etc.)	0.002	0.010
GO:0009755	hormone-mediated signaling pathway	33	6 (CRY2, TP63, DAB2, SRC, CNOT1, KMT2D)	3 (JAK2, NCOR1, CRY1)	0.002	0.114
GO:0010648	negative regulation of cell communication	227	19 (CRY2, GRIK2, SIRT4, TP63, BIRC6, UBAC2, etc.)	8 (ADRA2A, ARRDC3, AKT1, STAMBP, VASN, etc.)	0.002	0.584
GO:0023057	negative regulation of signaling	227	19 (CRY2, GRIK2, SIRT4, TP63, BIRC6, UBAC2, etc.)	8 ADRA2A, ARRDC3, AKT1, STAMBP, VASN, etc.)	0.002	0.584
GO:0045429	positive regulation of nitric oxide biosynthetic process	8	0	3 (JAK2, AKT1, ARPV1)	1.000	0.002
GO:1904407	positive regulation of nitric oxide metabolic process	8	0	3	1.000	0.002
GO:0071356	cellular response to tumor necrosis factor	27	2	5	0.312	0.002
GO:0035967	cellular response to topologically incorrect protein	17	1	4	0.517	0.003

Supplemental Table 13. The top 20 biological processes in a gene ontology (GO) enrichment analysis of mucus proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) for 30 days. The top GO terms are comparing the high nutrient treatment between experiment day 30 and day 5. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0016049	cell growth	110	12	3	0.001	0.636
GO:0051568	histone H3-K4 methylation	14	4	0	0.002	1.000
GO:2000773	negative regulation of cellular senescence	7	3	0	0.002	1.000
GO:0051569	regulation of histone H3-K4 methylation	7	3	0	0.002	1.000
GO:0072657	protein localization to membrane	138	4	11	0.808	0.002
GO:0050931	pigment cell differentiation	8	3	0	0.003	1.000
GO:0060537	muscle tissue development	93	10	2	0.003	0.767
GO:0090150	establishment of protein localization to membrane	51	2	6	0.609	0.003
GO:0048066	developmental pigmentation	9	3	0	0.004	1.000
GO:0051668	localization within membrane	161	4	11	0.892	0.007
GO:0006888	endoplasmic reticulum to Golgi vesicle-mediated transport	20	4	0	0.007	1.000
GO:0048193	Golgi vesicle transport	60	7	1	0.009	0.836
GO:2000772	regulation of cellular senescence	12	3	1	0.010	0.302

GO:0051438	regulation of ubiquitin-protein transferase activity	12	3	0	0.010	1.000
GO:0061512	protein localization to cilium	16	1	3	0.478	0.010
GO:0048588	developmental cell growth	62	7	2	0.011	0.550
GO:0031056	regulation of histone modification	35	5	1	0.011	0.650
GO:0010001	glial cell differentiation	47	2	5	0.563	0.011
GO:0002673	regulation of acute inflammatory response	13	3	0	0.013	1.000
GO:0014706	striated muscle tissue development	65	7	2	0.014	0.576

Supplemental Table 14. The top 20 biological processes in a gene ontology (GO) enrichment analysis of mucus proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a low nutrient exposure (0.39 mg L^{-1} TAN, and 0.29 mg L^{-1} of PO_4– P) for 30 days. The top GO terms are comparing the low nutrient treatment between experiment day 30 and day 5. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0045069	regulation of viral genome replication	BP	13	0	2	1
GO:0019079	viral genome replication	BP	20	1	2	0.15113779
GO:0010632	regulation of epithelial cell migration	BP	61	3	0	0.01258739
GO:0006886	intracellular protein transport	BP	180	5	1	0.01339853

GO:0030336	negative regulation of cell migration	BP	70	3	0	0.01824165
GO:2000146	negative regulation of cell motility	BP	70	3	0	0.01824165
GO:0042127	regulation of cell population proliferation	BP	290	1	5	0.9165401
GO:0010631	epithelial cell migration	BP	82	3	0	0.02767284
GO:0090132	epithelium migration	BP	82	3	0	0.02767284
GO:0040013	negative regulation of locomotion	BP	83	3	0	0.02855721
GO:0090130	tissue migration	BP	85	3	0	0.03037138
GO:0044281	small molecule metabolic process	BP	349	3	5	0.55204794
GO:0019058	viral life cycle	BP	58	1	2	0.38001711
GO:0008283	cell population proliferation	BP	355	1	5	0.95376976
GO:0098542	defense response to other organism	BP	171	4	1	0.04705189
GO:0006954	inflammatory response	BP	150	1	3	0.71488859
GO:0008284	positive regulation of cell population proliferation	BP	152	1	3	0.71973591
GO:0033157	regulation of intracellular protein transport	BP	47	2	1	0.05500583
GO:0046483	heterocycle metabolic process	BP	963	5	9	0.92943709
GO:0001525	angiogenesis	BP	112	3	0	0.06067484

Supplemental Table 15. The top 20 biological processes in a gene ontology (GO) enrichment analysis of mucus proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a high nutrient exposure (4.62 mg L^{-1} TAN, and 3.45 mg L^{-1} PO_4– P) for 30 days. The top GO terms are comparing the high nutrient treatment between experiment day 30 and day 15. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0002526	acute inflammatory response	26	5	0	5.01E-04	1.000
GO:0042596	fear response	8	3	0	9.23E-04	1.000
GO:0032501	multicellular organismal process	1446	53	45	9.74E-04	0.899
GO:0030098	lymphocyte differentiation	76	3	9	0.329	0.001
GO:0050886	endocrine process	16	0	4	1.000	0.002
GO:0045132	meiotic chromosome segregation	17	0	4	1.000	0.002
GO:1903131	mononuclear cell differentiation	85	3	9	0.395	0.003
GO:0097306	cellular response to alcohol	29	1	5	0.544	0.003
GO:0002673	regulation of acute inflammatory response	13	3	0	0.004	1.000
GO:0046888	negative regulation of hormone secretion	10	1	3	0.237	0.004
GO:0006310	DNA recombination	75	0	8	1.000	0.004
GO:0007165	signal transduction	1064	40	38	0.005	0.505
GO:0016601	Rac protein signal transduction	14	3	0	0.005	1.000
GO:0048588	developmental cell growth	62	6	4	0.006	0.175
GO:0048678	response to axon injury	21	0	4	1.000	0.006

GO:0006099	tricarboxylic acid cycle	11	1	3	0.257	0.006
GO:0010862	positive regulation of pathway-restricted SMAD protein phosphorylation	12	0	3	1.000	0.007
GO:0007166	cell surface receptor signaling pathway	522	23	18	0.008	0.588
GO:0032502	developmental process	1300	46	41	0.008	0.857
GO:0002285	lymphocyte activation involved in immune response	36	1	5	0.623	0.008

Supplemental Table 16. The top 20 biological processes in a gene ontology (GO) enrichment analysis of mucus proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) exposed to a low nutrient exposure (0.39 mg L^{-1} TAN, and 0.29 mg L^{-1} of PO_4– P) for 30 days. The top GO terms are comparing the low nutrient treatment between experiment day 30 and day 15. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0022603	regulation of anatomical structure morphogenesis	213	2	2	0.164	0.024
GO:0048870	cell motility	386	4	1	0.035	0.403
GO:0007283	spermatogenesis	99	2	0	0.044	1.000
GO:0007010	cytoskeleton organization	367	1	2	0.739	0.067
GO:0120036	plasma membrane bounded cell projection organization	387	2	2	0.392	0.074
GO:0030030	cell projection organization	394	2	2	0.401	0.077
GO:0016477	cell migration	342	3	0	0.105	1.000

GO:1903047	mitotic cell cycle process	167	2	1	0.110	0.193
GO:0050793	regulation of developmental process	505	2	2	0.538	0.120
GO:0033554	cellular response to stress	373	3	0	0.128	1.000
GO:0071840	cellular component organization or biogenesis	1365	7	3	0.136	0.212
GO:0000278	mitotic cell cycle	202	2	1	0.150	0.230
GO:0048856	anatomical structure development	1196	6	3	0.192	0.151
GO:0030154	cell differentiation	870	5	1	0.153	0.720
GO:0007399	nervous system development	585	3	2	0.326	0.155
GO:0030163	protein catabolic process	207	2	1	0.156	0.235
GO:0048869	cellular developmental process	877	5	1	0.157	0.723
GO:0048731	system development	883	5	2	0.161	0.307
GO:0009653	anatomical structure morphogenesis	611	3	2	0.353	0.167
GO:0032502	developmental process	1300	6	3	0.261	0.187

Supplemental Table 17. The top 20 biological processes in a gene ontology (GO) enrichment analysis of mucus proteins (FDR < 0.2) from fathead minnows (*Pimephales promelas*) in an experimental control group for 30 days. The top GO terms are comparing the control group between experiment day 30 and day 15. A minimum of three significant protein hits was required in each GO term.

Accession ID	GO Term	Number of Proteins in GO Term	Number of differentially expressed proteins (increased)	Number of differentially expressed proteins (decreased)	P-value (increased)	P-value (decreased)
GO:0031323	regulation of cellular metabolic process	935	5	1	0.002	0.937
GO:0051171	regulation of nitrogen compound metabolic process	943	5	1	0.002	0.939
GO:0051246	regulation of protein metabolic process	499	4	1	0.003	0.743
GO:0080090	regulation of primary metabolic process	978	5	1	0.003	0.946
GO:0060255	regulation of macromolecule metabolic process	1049	5	2	0.004	0.797
GO:0019220	regulation of phosphate metabolic process	278	3	0	0.006	1.000
GO:0051174	regulation of phosphorus metabolic process	278	3	0	0.006	1.000
GO:0019222	regulation of metabolic process	1140	5	2	0.006	0.841
GO:0051247	positive regulation of protein metabolic process	289	3	0	0.006	1.000
GO:0016032	viral process	84	2	1	0.006	0.192
GO:0044248	cellular catabolic process	413	3	0	0.018	1.000
GO:0002376	immune system process	428	3	1	0.019	0.684
GO:0048523	negative regulation of cellular process	882	4	1	0.023	0.925

GO:0065009	regulation of molecular function	496	3	1	0.029	0.741
GO:0009056	catabolic process	508	3	0	0.031	1.000
GO:0043170	macromolecule metabolic process	1602	5	5	0.032	0.366
GO:0048519	negative regulation of biological process	984	4	2	0.034	0.760
GO:0019538	protein metabolic process	1021	4	3	0.039	0.497
GO:0006807	nitrogen compound metabolic process	1676	5	5	0.040	0.418
GO:0044237	cellular metabolic process	1678	5	4	0.040	0.690

Calculations

Experimental tanks required a mixture of ammonium phosphate dibasic and ammonium chloride to achieve desired exposure concentrations. The high treatment initial dosing was achieved using the following calculation:

$$\left(3.45\frac{mg}{L}_{PO_4}\right)(64.96L) = 0.2241\ g_{PO_4}$$

$$\frac{0.2241\ g_{PO_4}}{94.97\frac{g}{mol}_{PO_4}} = 0.0023598\ mol_{PO_4}$$

$$(0.0023598\ mol_{PO4})\left(132.06\ \frac{g}{mol}_{(NH_4)_2HPO_4}\right) = 0.3116\ g\ (NH_4)_2HPO_4$$

$$\left(0.0023598\ mol_{PO_4}\right)(2) = 0.0047196\ mol_{NH_4}$$

$$\left(4.615\frac{mg}{L}_{NH_4}\right)(64.96\ L) = 0.29979\ g_{NH_4}$$

$$\frac{0.29979\ g_{NH_4}}{18.04\frac{g}{mol}_{NH_4}} = 0.016618\ mol_{NH_4}$$

$$0.016618\ mol_{NH_4}\ total - 0.0047196\ mol_{NH_4} from\ \ (NH_4)_2HPO_4 = 0.0118984\ mol_{NH_4} needed$$

$$\left(0.118984\ mol_{NH_4}\right)\left(53.49\frac{g}{mol}_{NH_4Cl}\right) = 0.6364\ g\ NH_4Cl$$