Application of Environmental Metabolomics for Assessment of Aquatic Invertebrate Sensitivity to Naphthenic Acids

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2023

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Naphthenic acids (NAs) are a class of chemicals found in oil sands process waters (OSPW) from the extraction of bitumen from surface mined oil sand. In Alberta, Canada, OSPW is currently stored indefinitely in tailing ponds and there are environmental concerns about seepage or spillage. NAs are known to be toxic to aquatic organisms. However, NA toxicity data are limited to primarily acute effects in a few species. Due to difficulties in comparing NA toxicity results, an extracted solution referred to as naphthenic acid fraction components (NAFCs) can be used as it is representative of OSPW toxicity and is well characterized. The goal of this research is to inform NA standards by performing chronic toxicity tests to derive metabolomic responses and estimates of survival. I exposed the ramshorn snail (*Planorbarius corneus*), and nymphs of two dragonflies, the common whitetail (*Plathemis lydia*) and the eastern pondhawk *(Erythemis* spp.), to 0, 6, 12, and 25 mg/L NAFCs for 21-days (snails) or 14-days (dragonflies) in laboratory microcosm experiments. Survival was altered only in the common whitetail with NAFC exposure. Snails laid approximately double the number of egg masses at higher concentrations of NAFCs. The metabolome responded differently among taxa. NAFC exposure altered the metabolome of only the common whitetail. Metabolite analysis for the common whitetail found that most altered metabolites were amino acids. The identified metabolites were involved primarily in pathways related to energy metabolism and protection from oxidative stress. My findings indicate that sensitivity to NAFCs is taxon-specific and demonstrate that an organism's metabolome can provide insight into toxic effects of NAFCs.

Plain Language Summary

Naphthenic acids (NAs) are contaminants thought to be the primary toxic components of industrial wastewater produced during oil sands extraction and is referred to as oil sands process waters (OSPW). In Alberta, Canada, large quantities of OSPWs are stored indefinitely in tailing ponds and there are environmental concerns surrounding potential seepage or spillage. NAs are known to be toxic. However, sublethal effects have rarely been studied and thus are not well understood. To assess NA toxicity, an extract from OSPWs, referred to as naphthenic acid fraction components (NAFCs) can be used to compare results more easily as it is chemically well understood. My goal was to assess the sublethal toxicity of NAFCs to aquatic organisms using a technique referred to as metabolomics. Metabolomics assesses changes in an organism's metabolism by investigating the amount of metabolites, such as sugars, and amino acids, involved in pathways of metabolism. This is useful in revealing sublethal effects of contaminants and to reveal information about metabolic pathways affected by exposure to contaminants. To achieve my goal, the ramshorn snail, and nymphs of two dragonflies, the common whitetail, and the eastern pondhawk, were exposed to 0, 6, 12, and 25 mg/L NAFCs in 21-day (snails) and 14-day (dragonflies) laboratory experiments. A slight decrease in survival was observed in the common whitetail, as well as alterations in the metabolome compared to the control at all exposure levels. The alterations in the metabolome indicated that the abundance of amino acids was affected and metabolites were associated primarily with energy metabolism and protection from stress. Snails laid approximately double the number of eggs at 12 and 25 mg/L NAFCs. However, there was no clear impact to snail survival or the metabolome. For the eastern pondhawk, there was no clear impact to survival or the metabolome. Knowledge gained from this study can be used to develop chronic toxicity standards of NAs for protection of aquatic ecosystems. My findings indicate that sensitivity to NAFCs depends on the investigated organism. In the common whitetail, a more sensitive organism to NAFC concentrations, metabolomics revealed changes in metabolism with exposure to sublethal concentrations of NAFCs and may be an excellent indicator taxon of NAs in the environment.

Acknowledgements

I would like to first thank my supervisor, Dr. Adam Yates, and my co-supervisor, Dr. Bob Brua, for your mentorship. I wholeheartedly thank you both for the help you have given me over the past few years, and your willingness to support me during my time completing this degree. You were both patient, and more than willing to help with any and all questions I had. I also want to thank Dr. Paul Craig, of my committee. Thank you for your guidance and feedback throughout this experience.

Thank you to Daryl Halliwell and Samantha Matheson of Environment and Climate Change Canada. Your help, feedback, troubleshooting and suggestions throughout my project were extremely valuable.

I would like to thank Dr. Richard Frank and Dr. Mark Hewitt of Environment and Climate Change Canada, for your advice throughout, and for the use of your NAFC solution. I would also like to Dr. John Headley and Ian Vander Meulen of Environment and Climate Change Canada, for your help with the characterization and quantification of the NAFC solution, as well for being willing to answer questions I had.

To my friends in the StrEAMS lab, thank you all for being there to listen to me, providing advice, helping me with statistics, and for giving me reassurance.

Finally, I could not have done this without my friends, family, and partner. The support and encouragement I received from you helped to make this possible.

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1.0 Introduction

Naphthenic acids (NAs) are a group of compounds naturally present in crude oil and bitumen (Brient et al., 2000). A common source of these acids, among other contaminants, is highly concentrated industrial wastewater from oil extraction, hereafter referred to as oil sands process waters (OSPWs). These OSPWs are produced during the extraction of bitumen from the oil sands and subsequently stored in tailing ponds (Clemente & Fedorak, 2005; Frank et al., 2006). Presently, OSPWs are indefinitely stored in these tailing ponds and the potential for seepage or spillage are a cause for environmental concern (Hewitt et al., 2020). OSPWs are known to be toxic to aquatic organisms and toxicity is primarily attributed to NAs (Hughes et al., 2017). However, knowledge of NA toxicity, especially chronic and sublethal toxicity, remains limited.

Safe concentrations for NAs in the environment are not known and the Canadian Council of Ministers of the Environment (CCME) are currently developing guidelines for NAs and are in need of chronic toxicity data for the protection of aquatic organisms (Government of Canda, Federal Environmental Quality Guidelines, 2023; Reynolds et al., 2022). The majority of prior NA toxicity research thus far has focused on acute impacts in a small number of taxa (Reynolds et al., 2022). Moreover, comparing results of existing studies is often difficult due to different sources of NAs having differences in toxicity (Bartlett et al., 2017; Frank et al., 2006; Holowenko et al., 2002). To address the challenge of comparing toxicity results, an extracted solution referred to as naphthenic acid fraction components (NAFCs) can be used. NAFCs are samples extracted from OSPWs and contain the NAs found in OSPWs to standardize the toxicity testing of NAs found in OSPWs. These samples are thought to be more representative of OSPW toxicity than commercially purchased NA samples, are well characterized and have been utilized by others allowing for the comparison of toxicity results (Bartlett et al., 2017; Morandi et al., 2015; Robinson et al., 2023). However, no previous studies exist investigating NAFCs in chronic, toxicity tests and understanding the potential sublethal effects is a major challenge.

Environmental metabolomics is a technique increasingly used in toxicity testing (Bedia, 2022). Environmental metabolomics utilizes changes in an organism's metabolome to detect changes that may point towards stress, such as in response to a contaminant (Bundy et al., 2008; Lankadurai et al., 2013). The metabolome consists of all metabolites (biochemical products involved in metabolism) within an organism (Bundy et al., 2008; Lankadurai et al., 2013). Changes in these metabolite levels may indicate impacted metabolism pathways and is thought to be a highly sensitive indicator (Taylor et al., 2018). Combining environmental metabolomics with traditional toxicity testing could reveal biochemical pathways altered with contaminant exposure or fill the need for a sensitive indicator for sublethal stress with exposure to contaminants. Due to the lack of acceptable NA threshold concentrations and a poor understanding of the overall ecological effects of sublethal, chronic exposure to NAs, utilizing metabolomics may help to reveal the potential impacts of these contaminants. Herein, macroinvertebrates will be chronically exposed to NAFCs and use metabolomics to assess sublethal stress. This will aid in the development of sublethal NA toxicity guidelines by providing toxicity endpoints, assessing sublethal stress, and assessing potential pathways impacted by NAFC exposure for different taxa.

1.1 Literature Review

1.1.1 Naphthenic Acids in the Environment

Naphthenic acids (NAs) are a complex group of surfactant carboxylic acids containing one or more aromatic ring structures and an alkyl group of the general chemical formula $C_nH_{2n-z}O_2$ (Brient et al., 2000). NAs are naturally present in crude oil and bitumen and are commercially used in tire production, paint production, asphalt-paving, as a wood preservative, an additive in some lubricants, and as a catalyst for the drying of some coatings (Brient et al., 2000). While NAs are ubiquitous in ecosystems with bitumen deposits there are environmental concerns as the extraction of bitumen results in the production of large quantities of wastewater containing concentrated NAs.

One of the largest potential sources of NAs to the environment are oil sand process waters (OSPWs). In the oil sands regions of Alberta, Canada, the hot water extraction technique required to separate the useful bitumen from soil and clay results in the production of two to four barrels of OSPWs (which contain NAs) for every barrel of oil (Barrow et al., 2010; Giesy et al., 2010; Kannel & Gan, 2012). To reduce water consumption, 80-88% of consumed water is recycled, however, this recycling of wastewater increases the concentration of contaminants, including NAs, and is thought to increase toxicity (Kannel & Gan, 2012; Yergeau et al., 2012). Furthermore, there is a zero-discharge policy for OSPWs in Alberta, Canada, thus resulting in a growing quantity of OSPWs (Wu et al., 2019). As of 2018, approximately 170,000 m^3 of OSPW is stored indefinitely in tailing ponds (Redman et al., 2018).

The OSPW in tailing ponds contains NAs, as well as heavy metals, polycyclic aromatic hydrocarbons, and other organic compounds (Gagné et al., 2011). Within tailing ponds, measured NA concentrations range from 20-120 mg/L (Bartlett et al., 2017; Holowenko et al., 2002). The composition, concentration, and toxicity of NA compounds found in these ponds varies dramatically based on location, size, and age of tailing ponds (Frank et al., 2016; Scott et al., 2020). There are environmental concerns regarding the storage of OSPWs as accidental release could occur following weather events or through seepage to waterways. Furthermore, groundwater and river sediments near tailing ponds are chemically similar to ponds, suggesting seepage could be occurring (Holden et al., 2011; Yergeau et al., 2012). In the Athabasca River, 100 km upstream of Fort McMurray, measured NA concentrations ranged from 0.1-0.9 mg/L (Kannel & Gan, 2012; Schramm et al., 2000; Scott et al., 2008). NAs have also been found in groundwater when sampling near tailing ponds in Alberta, Canada, finding concentrations between 0.4-51 mg/L (Clemente & Fedorak, 2005; W.P. Marsh, M.D. MacKinnon, and P.M. Fedorak, reported as unpublished data by Clemente & Fedorak, 2005). The ecological impacts of NAs are presently not well understood. Thus, investigating the ecological impacts of NAs is vital to understanding the potential impacts of the storage or accidental release of this contaminated water.

1.1.2 Toxicity of Naphthenic Acids

NAs are thought to be the primary toxic component of all contaminants in OSPWs. They are known to be acutely toxic to aquatic organisms and could potentially cause multigenerational changes, such as declining populations of some taxa (Hughes et al., 2017; Philibert et al., 2019; Scott et al., 2020). A present challenge with assessing NA toxicity is that the majority of prior research on NA and naphthenic acid fraction component (NAFC) toxicity primarily focuses on finding lethal endpoints in a limited number of species. Fish and aquatic invertebrates appear to be more sensitive to NA exposure than plants, with LC_{50}s as low as 1.9 mg/L for the fish *Pimephales promelas*, compared to 56.2 mg/L in the plant species *Typha latifolia* (Kinley et al., 2016). In another study, toxicity to fish was determined to be between 4-78 mg/L NAs (LC_{50} among fish species) depending on factors such as water hardness, temperature, dissolved oxygen concentration, or the length of organism exposure to the contaminant (Kannel & Gan, 2012). While there are a few studies in which bean plants sprayed with NAs saw stimulated growth (Wort et al., 1973; Wort & Patel, 1970), all other studies (including taxa of plants, fish, mammals, and bacteria) reviewed by Clemente & Fedorak, (2005) observed deleterious effects, such as increased mortality.

There is presently a limited understanding of chronic OSPW, NA, or NAFC toxicity. In a 75-day exposure study, *Lithobates pipiens* tadpoles were observed to have reduced growth and development to sublethal levels of 1 and 2 mg/L NAs (Melvin et al., 2013). In the white sucker (*Catostomus commersonii*), smaller testes and ovaries, as well as reduced body growth, were observed after four months of exposure to OSPWs in 5 experimental ponds with NA concentrations measured as <1, 2, 3, 6, and 14 mg/L (Arens et al., 2015). In a 21-day exposure (NA concentration measured at 28.6 µg/L) study, recently hatched *Daphnia magna* had reduced reproduction (reduced neonates per female) and intergeneration effects were observed with a decrease in population over the following generation (Cardoso et al., 2020). Therefore, it appears that NAs may have chronic toxicity implications, such as impacting growth, reproduction, or survival. However, to confirm these effects, further

investigation into the chronic effects of NAs on a wider range of taxa would be useful, and utilizing a common extract would allow for a better comparison of toxicity results. It is often difficult to compare NA toxicity results as toxicity is source-dependent (i.e., commercial vs extracted, or NA differences between tailing ponds). Furthermore, antagonistic or synergistic effects of chemical interactions in OSPWs may also contribute to, or be responsible for, this toxicity (Hughes et al., 2017; Li et al., 2017). However, while it is important to recognize these toxicity differences, it remains useful to consider previous studies investigating commercial NAs as they may indicate more sensitive taxa or provide context for toxicity (i.e., potential impacted metabolic pathways).

Many previous studies utilize commercially purchased NA samples, which are useful as they are well characterized, allowing for results to be compared. However, they are thought to be more toxic than NA samples extracted from OSPWs and contain a less diverse mixture of NAs (Bartlett et al., 2017; Marentette et al., 2015a; Robinson et al., 2023; Scott et al., 2020). For example, Johnston, (2015) investigated the impacts of commercially purchased NAs on the snail *Lymnaea stagnalis*, which experienced an LC_{50} between 25 – 50 mg/L with hatching rate and embryo growth (embryo-to-egg ratio) affected above 20 and 30 mg/L, respectively. In a 21-day study involving *Hexagenia* spp. mayflies chronically exposed to low concentrations of commercial NAs (between 0.0001 – 100 µg/L), no observable impacts to growth and only 6% mortality across a concentration gradient was found (Pomfret et al., 2021). The response of different aquatic invertebrates and fish was investigated by Kinley et al., (2016), observing 7-day LC_{50} responses of 1.9 mg/L for the fathead minnow (*P. promelas*), 2.8 mg/L for water fleas (*Ceriodaphnia dubia*), 4.1 mg/L for an amphipod (*Hyalella azteca*), and 6.5 mg/L for midges (*Chironomus dilutus*). Thus, aquatic organisms appear to be sensitive to commercially purchased NAs, however, at low concentrations ($\leq$ 0.1 mg/L), survival remains high. Furthermore, Marentette et al. (2017) observed that commercial NAs were more toxic than OSPW-derived NAs, suggesting that commercial NAs are not directly representative of the toxicity of OSPWs.

Due to the variation in toxicity of NA to organisms, utilizing an extracted solution common to other studies is useful in allowing for improved comparisons of results and to better understand the effects of the composition of NAs (Bartlett et al., 2017). Although comparing to toxicity results using commercially purchased NA samples is beneficial, they are more toxic and comprised of smaller and simpler acids than NA samples extracted from OSPWs (Bartlett et al., 2017; Marentette et al., 2015a; Scott et al., 2020). Thus, with differences in NA composition between tailing ponds, and commercial NAs being less representative of OSPW toxicity, there is a need for comparable, environmentally relevant, NA toxicity results. In an effort to standardize the investigation of OSPWs, Frank et al. (2006) developed a technique to extract NAs of different oxygenation states (Frank et al., 2006; Headley et al., 2013; Marentette et al., 2015a). This extracted solution was referred to as naphthenic acid fraction components (NAFCs) and is thought to be more representative of OSPWs than commercially purchased NAs because NAFCs contain the major toxic components of OSPWs (Hughes et al., 2017; Morandi et al., 2015; Robinson et al., 2023). Furthermore, as OSPW composition dramatically varies, and characterization is difficult, utilizing a standardized mixture is useful for the comparison of toxicity testing results and to better understand the chemical composition. Thus, using NAFCs in toxicity testing is beneficial as NAFCs are more reflective of OSPWs than commercially purchased samples and are well characterized compared to raw OSPWs. There is currently a pressing need for NA standard development to understand the potential impacts of stored OSPWs, and the use of NAFC samples offers an opportunity to develop comparable, and environmentally relevant toxicity results.

There has recently been interest in utilizing NAFC samples for toxicity testing as these samples are relatively well characterized and more representative of the toxicity of OSPWs than commercial NAs (Frank et al., 2006; Robinson et al., 2023). These studies include that of Marentette et al., (2015a) who compared NAFC toxicity with commercial NAs to *Pimephales promelas*, finding that NAFCs were less toxic than commercial NAs. In a different, early life stage study, walleye (*Sander vitreus*) and the fathead minnow (*P.*

promelas) were observed to have EC_{50}s (hatch success and deformities) of 10-11 mg/L and 22-25 mg/L, respectively (Marentette et al., 2015b). Adverse effects were observed in *P. promelas* exposed to NAFCs (2.5-54 mg/L) for the initial days post-hatching before being raised in an uncontaminated lake (Reynolds et al., 2022). It was observed that exposed fish experienced a decrease in swim activity and an increase in swim burst events, suggesting that embryonic exposure to NAFCs may have persistent sublethal effects (Reynolds et al., 2022). Aquatic invertebrates appeared to be slightly less sensitive than fish, where Bartlett et al. (2017) investigated aquatic invertebrates exposed to NAFCs finding that the crustacean *Hyalella azteca* was relatively sensitive compared to other investigated taxa with an LC_{50} of 16.7 – 27.4 mg/L, while the freshwater muscle *Lampsilis cardium* had an EC_{50} (viability) of 7.05 – 123.0 mg/L. Offspring of the wood frog (*Rana sylvatica*) exposed to 10 mg/L NAFCs had impacted survival, slower growth, and additional abnormalities during development (Robinson et al., 2023). In humans, immobilized human trophoblast cells exposed to 125 mg/L NAFCs saw upregulation of transcription factors related to GDF15 production compared to the control (however, not NAFCs $\leq$ 25 mg/L), indicating that high NAFC concentration exposure may alter embryonic development (Jamshed et al., 2022). There are remaining knowledge gaps for NAFC toxicity research, in particular, these extracts have been investigated in a limited number of species and most previous studies have assessed acute toxicity. Thus, there is a pressing need for NA and NAFC toxicity data to aid in toxicity guideline development.

1.1.3 Naphthenic Acid Toxicity Guideline Development

Water quality guidelines for the protection of aquatic life are recommended concentrations of contaminants and nutrients at which there is low toxicity risk to an aquatic ecosystem (Nugegoda & Kibria, 2013). The development of toxicity guidelines is important for the protection of aquatic ecosystems and human health. In Canada, the Canadian Water Quality Guidelines (CWQG) for the Protection of Aquatic Life include benchmark concentrations of anthropogenic stressors to protect freshwater, marine, and estuarine aquatic

life for long term exposure to substances (*Canadian Council of Ministers of the Environment | Le Conseil Canadien Des Ministres de l'environment*, 2023). The CWQG guidelines generally includes information, such as a summary of the contaminant in the environment (i.e., contaminant sources, relevant concentrations), safe environmental concentrations, and a summary of prior acute and chronic toxicity studies (Canadian Council of Ministers of the Environment, 1999).

When developing environmental guidelines for contaminants in ecosystems, it is important that numerous taxa are considered to ensure the protection of the most species possible (Nugegoda & Kibria, 2013). Different organisms have different responses to stress from contaminants and there is significant variation in sensitivity to contaminants. Thus, developing guidelines based on a wide range of taxa is beneficial (Li et al., 2017). One widely used approach for contaminant risk assessment is the use of species sensitivity distributions (Fox et al., 2021; Nugegoda & Kibria, 2013). Species sensitivity distributions (SSDs) are statistical estimates of a concentration hazardous to less than a specified proportion of the population, and they are developed using prior toxicity data (Fox et al., 2021). Alternatively, SSDs can statistically estimate the number of species in an ecosystem affected by a specified contaminant concentration (Fox et al., 2021). The use of SSDs is particularly beneficial when there is a thorough knowledge base for the toxicity of the contaminant in question, as by having a more thorough dataset, the statistical power is stronger, lending more confidence when developing guidelines (Fox et al., 2021). One major challenge is small sample bias, where SSDs built utilizing small sample sizes (less than 8 species) creates uncertainty and overfitted data (Fox et al., 2021). Therefore, it is important to consider a wide range of different taxa when developing SSDs and when utilizing toxicity data for risk assessment as a whole.

Another consideration is functional feeding groups (FFGs), where organisms are grouped by food acquisition methods, such as grazers ingesting algae growing on surfaces (Cummins & Klug, 1979). Previous studies have demonstrated that toxicity may depend on FFG in some cases, such as that of Liess et al. (2017), where it was observed that predators

were less sensitive to heavy metals than other investigated taxa. Therefore, considering the FFGs of investigated organisms is useful in understanding potential ecological implications. Furthermore, when developing guidelines for contaminants, such as NAFCs, it is important to investigate taxa of different FFGs as there may be toxicity differences or differences in response to contaminants. For example, some taxa may have reduced reproduction with sublethal exposure to certain contaminants, while others may not. There is also presently a need for additional toxicity data to use in SSDs for NA and NAFC toxicity guideline development and thus, it is beneficial to investigate a variety of taxa.

1.1.4 Modes of Action

The mode of action is the critical steps in which the toxin affects an organism, such as the biochemical pathways affected by contaminant exposure (Borgert et al., 2004). The primary pathways and modes of action related to NA toxicity are not well understood at present. However, NA toxicity is thought to be primarily attributed to oxidative stress, endocrine disruption, and energy metabolism, but other impacts such as narcosis may contribute to toxicity (Bartlett et al., 2017). Oxidative stress occurs when there are imbalances in the amount of reactive oxygen species present since the organism cannot detoxify these reactive oxygen species. This causes organismal damage to cellular processes, such as apoptosis and immune response (Deavall et al., 2012; Pizzino et al., 2017). Previous studies have observed that the expression of genes related to apoptosis and oxidative stress were increased with NA exposure (Marentette et al., 2017; Raez-Villanueva et al., 2019; Wang et al., 2015b). Oxidative stress also leads to decreases in energy metabolism, improper cell signaling, and alterations in other cellular functions, such as immune response, inflammation, or DNA damage (Mahjoub & Masrour-Roudsari, 2012).

Endocrine disruption results in alterations of an organism's use and production of hormones, such as alterations in sex steroids or hormone receptors (Wang et al., 2015a). Endocrine disruption has been observed in early life-stage zebrafish (*Danio rerio*) following NA exposure where the transcription of genes related to estrogen receptors and estrogen

synthesis were induced, and an increased occurrence of egg yolk sac edema deformities (Wang et al., 2015a). Leclair et al. (2015) also observed that NAs may act as antiandrogenics and antiestrogenics (inhibiting androgens and estrogens), which may have reproductive impacts, however, further investigation is needed.

While related to both endocrine disruption and oxidative stress, energy metabolism and energy storage may also be impacted by NA toxicity, where exposure to NAs may impede an organism's ability to metabolize lipids, resulting in a decrease in energy stored by the organism (Deavall et al., 2012; Melvin et al., 2013). Previous studies of fish suggest that as a stress response, a reallocation of energy resources may occur due to an observed decrease in sex steroid concentrations (Kavanagh et al., 2012). Another potential toxic effect of NAs involves the hydrophobicity of NAs, which may cause alterations of cellular membrane properties (the compounds are able to disrupt membrane function) resulting in alterations in surface tension, the permeability of cell membranes, and ultimately, narcosis of the cell (Frank et al., 2009). Overall, further research must be conducted to better understand impacted pathways and modes of action related to NA toxicity, and there are opportunities with novel toxicology techniques, such as metabolomics, to reveal these impacts.

1.1.5 Metabolomics for Toxicity Testing

Environmental metabolomics monitors changes in the metabolome in response to stressors in the environment. The metabolome contains all metabolites within an organism. Metabolites are biochemical products involved in metabolism, such as amino acids, sugars, and nucleotides (Bundy et al., 2008; Lankadurai et al., 2013). Changes in the abundance of specific metabolites may point to changes in metabolism pathways, such as the Krebs cycle, nucleotide biosynthesis, or the urea cycle (Hines et al., 2010; Lankadurai et al., 2013). Since metabolites are intermediates or products in these metabolic pathways, altered metabolite abundance suggests that changes are occurring within the organism, such as in the case of a stress response (Taylor et al., 2018). Thus, if metabolite abundance changes in response to exposure to stressors, it is possible to detect stress at sublethal, or chronic levels of exposure.

By analyzing differences in the abundance of specific metabolites it is possible to infer metabolic changes, such as alterations in specific pathways based on the difference in metabolite abundance. It is also possible to reveal information about which pathways are altered with exposure (Robertson, 2005). These metabolic pathway alterations may indicate the cause for greater impacts to the organism, such as impacted growth or reproduction, which could lead to population, community or ecosystem level changes over time (Hines et al., 2010; Lankadurai et al., 2013; Taylor et al., 2018). There are different methods to investigate changes in the metabolome, such as assessing the whole metabolome at once, or instead assessing specific metabolites depending on the goal of the study.

In environmental metabolomics, untargeted approaches are utilized to investigate the metabolome as a whole or a large number of metabolites in a biological sample at once, while targeted approaches instead investigate a specific group of metabolites (Haleem et al., 2008; Lankadurai et al., 2013). Untargeted approaches are useful in exploring potential modes of action and to identify a broad array of metabolites to potentially capture unexpected effects (Patti et al., 2012). Furthermore, untargeted metabolomics may be particularly useful when investigating toxicology of a variety of organisms as different organisms have variations in metabolite abundance and different responses to contaminant exposure (Longnecker et al., 2015). In contrast, targeted approaches are particularly useful in revealing biomarkers or for monitoring changes in key metabolites of interest (Lankadurai et al., 2013). As the toxicity of NAs is not well understood, untargeted metabolomics offers an opportunity to explore modes of action and investigate a broad range of metabolites for any observable changes. Furthermore, assessing the metabolome of different taxa helps to build the metabolomic knowledge base as a whole.

In toxicology, metabolomics is a relatively novel tool for assessing the impact of toxins and to potentially reveal toxicity pathways (Olesti et al., 2021). However, a major challenge is linking changes in the metabolome with changes to organism fitness (Olesti et al., 2021; Pomfret et al., 2021). Recent studies have demonstrated a linkage between fitness and metabolomic impacts. For example, Lv et al. (2022) observed lead exposure to alter the

metabolome of *Rana omeimontis* tadpoles, as well as impair growth and development. In another study, copper and pentachlorophenol exposure resulted in impacted growth and alterations in the metabolome of the marine mussel *Mytilus edulis* (Hines et al., 2010). Only one prior study has investigated the impact of NA exposure using metabolomics. Pomfret et al. (2021) exposed *Hexagenia* spp. mayflies to a range of sublethal concentrations of sodium naphthenate (0.0001 – 100 μg/L), and found only a slight decrease in survival at the highest concentration, but a shift in the metabolome was correlated with increasing NA concentrations (Pomfret et al., 2021). Affected metabolites appeared to be involved in energy metabolism, oxidative stress, and regulation of apoptosis (Pomfret et al., 2021). Overall, the metabolome shows promise as an endpoint in toxicity tests and may be useful in revealing implicated pathways (Lankadurai et al., 2013). However, further research must be completed to develop the usefulness of environmental metabolomics in toxicity testing.

1.2 Goals and Objectives

The goal of my thesis was to inform development of NA toxicity standards by using metabolomics to evaluate the chronic toxicity of NAFCs to aquatic benthic invertebrates.

This goal was achieved through a series of laboratory exposure experiments to address two research objectives:

1) Assess the survival, egg laying (snails only), and changes in the metabolome of three different aquatic invertebrate taxa (*Planorbarius corneus*, *Plathemis lydia,* and *Erythemis* spp.) chronically exposed to environmentally relevant concentrations of NAFCs.
2) Compare the metabolome among three different aquatic invertebrate taxa (*Planorbarius corneus*, *Plathemis lydia,* and *Erythemis* spp.).

My predictions based on these objectives were:

1) High survival at 0, 6, and 12 mg/L NAFCs, with most deaths occurring among individuals exposed to 25 mg/L NAFCs.

This prediction assumes that NAFCs will cause deaths to be observed among organisms, with the highest deaths being in the 25 mg/L NAFC group. The NAFC concentrations for my study were selected based on previously outlined studies using the same NAFC samples in aquatic invertebrates, such as that of Marentette et al., (2015b), where it was found that LC_{50}s were 16.7 mg/L – 27.4 mg/L for the amphipod *Hyalella azteca*. While there are no studies involving dragonflies exposed to NAs, for the pond snail *Lymnaea stagnalis*, an LC_{50} between 25 – 50 mg/L was observed for commercial NAs (Johnston, 2015). Thus, with a maximum concentration of 25 mg/L, I expect to observe impacted survival among organisms only at the highest concentration (25 mg/L), likely below 50% mortality (as LC50s laid

around the highest selected concentrations), and ideally will be able to observe potential sublethal impacts of NAFCs at other concentrations.

2) Snails will lay less eggs masses with exposure to NAFCs at the 25 mg/L exposure level.

I expect that at the highest selected NAFC concentration (25 mg/L) stress will occur in snails leading to a decrease in laying of egg masses. Previous acute toxicity studies utilizing other contaminants have observed similar results, finding that at higher levels of exposure, a decrease in snail egg laying occurs (Das & Khangarot, 2011; Seeland et al., 2013; Tripathi & Singh, 2004). Thus, I would expect stress from exposure to NAFCs will cause snails to re-allocate resources away from reproduction. Interestingly, it appears that in some cases, exposure to certain contaminants in sublethal levels will result in an increase in egg laying (Das & Khangarot, 2011; Tripathi & Singh, 2004). While other studies have found that at sublethal levels of contaminant exposure, no changes in the amount of egg laying occur (Johnson & Crowley, 1980; Seeland et al., 2013). Thus, I expect that at the investigated sublethal concentrations of NAFCs there will either be no change in the amount of egg masses laid or a slight increase will be observed.

3) Metabolites driving differences between exposure levels will be associated with energy metabolism.

When analyzing which metabolites are responsible for differences in the metabolomes of individuals exposed to NAFCs, I expect that metabolites will be mostly related to energy metabolism. Numerous previous studies have observed that energy metabolism is implicated with NA toxicity (Melvin et al., 2013; Pomfret et al., 2021; Zhang et al., 2023). While there are other potential modes of toxicity, such as narcosis, endocrine disruption, or oxidative stress, energy metabolism is often also implicated (Marentette et al., 2017; Raez-Villanueva et al., 2019; Wang et al., 2015b). Thus, I expect that broadly, energy metabolism will be

altered with exposure to NAFCs, but other metabolites may be associated with the repair of oxidative stress, reproductive implications from endocrine disruption, or preventing narcosis.

4) The metabolome of individuals exposed to 25 mg/L will be more different from the control than other concentrations (6, or 12 mg/L).

I expect that exposure to naphthenic acids will have a measurable change in the metabolome. It is known that NAFCs are toxic to aquatic organisms and cause stress to the organism (Hughes et al., 2017). Environmental metabolomics detects differences in the level of metabolites within an organism, which are thought to be precursors to other responses such as impacted survival or reproduction (Taylor et al., 2018). It is also well documented that different taxa have different sensitivity to stressors (Li et al., 2017). Therefore, differences in the sensitivity among the metabolomes of investigated taxa are expected. Differences in sensitivity to NAs and NAFCs among taxa is also well documented, such as fish and aquatic invertebrates being more sensitive to NA exposure than plants, and different effects of toxicity, such as altered reproduction in *Daphnia magna* exposed to NAs (Cardoso et al., 2020; Kinley et al., 2016). Thus, I would expect that NAFC exposure will cause differences in the metabolome, but these responses will differ by taxa.

5) The metabolomes of all taxa will be different from each other, but dragonflies will be more similar to each other than to snails.

Among taxa, I expect that the metabolomes of non-exposed individuals will differ. As different organisms have different morphological traits, diet, and energy requirements, it is possible to distinguish between taxa on the basis of the metabolome as these traits are closely related to metabolites and metabolic pathways (Zelentsova et al., 2022). Furthermore, in a study of several species of the genus *Centaurium*, it was possible to distinguish between these highly related taxa using metabolomics (Banjanac et al., 2017). Thus, differences in the base metabolism of macroinvertebrates would be expected. However, if taxa are closely

related it may be more difficult to distinguish metabolomes. Furthermore, despite expecting it to be possible to distinguish between dragonflies, they will likely be more similar to each other, than to snails. Banjanac et al. (2017) observed that the metabolomes of related taxa were more similar than less related taxa. Thus, I expect a similar trend in this study, as dragonflies are of the same family, I expect that their metabolomes will be more similar to each other than to snails.

2.0 Methods

2.1 Study Organisms

My study assessed three aquatic invertebrate taxa, the ramshorn snail (*Planorbarius corneus*), and two Libellulid dragonflies; the common whitetail (*Plathemis lydia*), and the eastern pondhawk (*Erythemis* spp.; **Figure 1**). Taxa were selected to enable comparisons between closely (i.e., between dragonflies) and distally (i.e., between snails and dragonflies) related taxa and to examine differences between functional feeding groups (FFGs; i.e., between grazers (snails) and predators (dragonflies)).

Ramshorn snails were selected as they live in freshwaters and are herbivorous grazers consuming primarily plant material and detritus (Pavlica et al., 2000; Wang et al., 2022). Snails may uptake contaminants that have settled in sediments or through the ingestion of periphyton (Pastorino et al., 2020). Alternatively, snails may absorb contaminants directly through skin as they lack an exoskeleton (Pastorino et al., 2020).

The two dragonflies of the Libellulidae family were selected as they live in freshwater and are predators primarily consuming other aquatic invertebrates (Suhling et al., 2015). Predators are thought to uptake contaminants by filtering water through their gills or via the ingestion of prey (Pastorino et al., 2020). While there are presently no studies investigating the sensitivity of dragonflies to NAFCs, dragonflies of the Libellulidae family are generally considered to be sensitive to contaminants (Caixeta et al., 2022; von der Ohe & Liess, 2004).

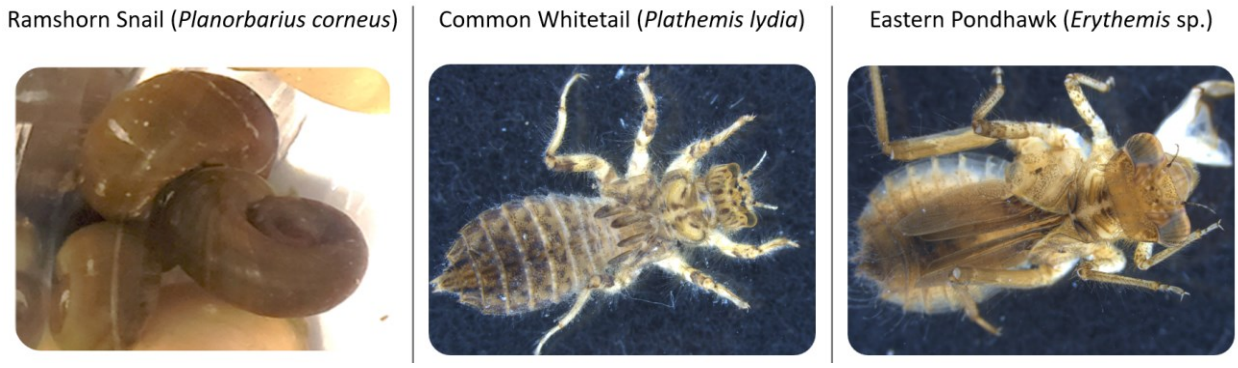

Figure 1. Photos of organisms investigated in this study. Snail image source: Robert Brua, 2022. Dragonfly images source: Adam Martens, 2022.

2.2 Naphthenic Acid Fraction Component Stock Solution

The NAFC stock solution used in this study was extracted from water samples collected from a tailings pond in Fort McMurray, Alberta, Canada in 2011 (Industry B Fresh, as described by Marentette et al., (2015)). A bulk extraction technique was developed by Frank et al. (2006), where diethylaminoethyl-cellulose, is used to remove soil organic matter, then dichloromethane is used to separate polycyclic aromatic hydrocarbons and other neutral organic compounds from the sodium naphthenate fraction, which was recovered and concentrated for use in future NAFC studies. This resulting NAFC solution contained naphthenic acids with a composition similar to the naphthenic acids of fresh OSPWs, however, there was a reduction in lower molecular weight NAFCs compared to fresh OSPWs (Frank et al., 2006). The stock solution used in my study used the extraction technique developed by Frank et al. (2006), but was collected in 2011, with a previously measured NAFC concentration of 1243 mg/L (Marentette et al., 2015a). This NAFC concentration was determined through liquid chromatography/mass spectrometry-quadrupole time of flight mass spectrometry direct injection (Brunswick et al., 2015; Marentette et al., 2015a). Characterization of this NAFC solution found that the relative abundance of O_2 species was 73.6%, with 2 and 3 ringed compounds being the most abundant (Marentette et al., 2015a). It is important to note that this characterization method is considered semi-quantitative due to the lack of naphthenic acid reference standards.

2.3 Experimental Design

The responses of snails, the common whitetail and the eastern pondhawk dragonflies to NAFC exposure were investigated through chronic toxicity tests lasting 21 days for snails and 14 days for dragonflies. By reviewing the lethal concentrations of previous NA and NAFC studies in aquatic invertebrates, the NAFC concentrations of 0, 6, 12, and 25 mg/L were selected. Snails and common whitetails were exposed to four concentrations of NAFCs (0, 6, 12, 25 mg/L), whereas eastern pondhawks were exposed to three concentrations (0, 12, 25 mg/L; **Figure 2)**. Concentrations were selected to be environmentally relevant, based on NA concentrations in tailing ponds (20-120 mg/L; (Bartlett et al., 2017; Holowenko et al., 2002)), rivers (0.1 – 0.9 mg/L; (Kannel & Gan, 2012)), and aquifers (0.4 - 51 mg/L; (Kannel & Gan, 2012)) in the Athabasca Oil Sands region.

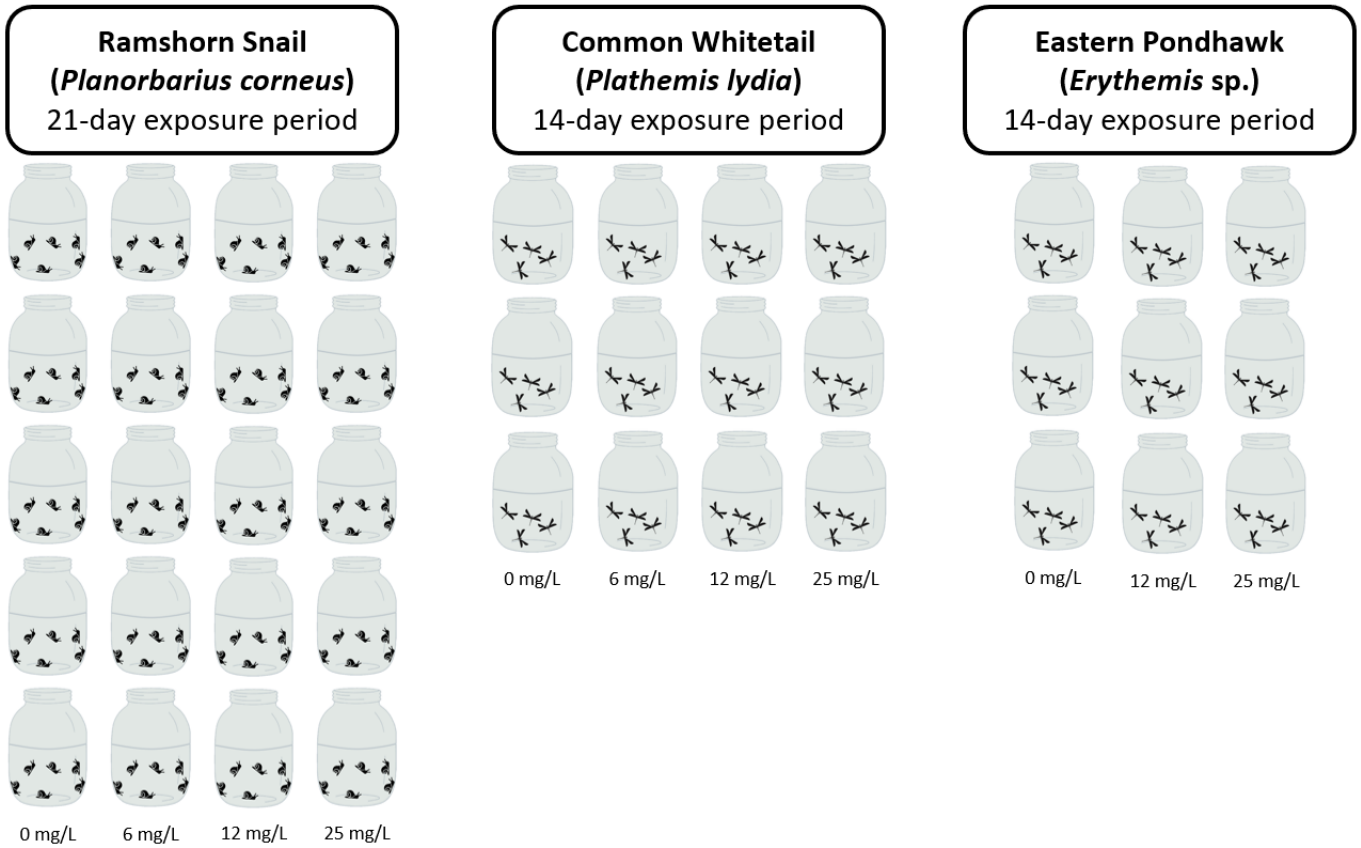

Figure 2. The experimental design illustrating the assignment of organisms to aquaria with respective NAFC concentration. Each aquaria contained either 6 snails or 4 dragonflies, with 5 replicate aquaria for snails and 3 replicate aquaria for dragonflies. Concentrations were 0, 6, 12, and 25 mg/L NAFCs for snails and the common whitetail, and 0, 12, and 25 mg/L for the eastern pondhawk.

For snails, there were 6 individuals per aquarium and 5 aquaria per concentration (20 aquaria; n = 120). common whitetails and eastern pondhawks each had 4 individuals per aquarium and 3 aquaria per concentration (common whitetails: 12 aquaria, n = 48; eastern pondhawk: 9 aquaria, n = 36). Four individual dragonfly nymphs were assigned randomly to each aquarium to limit intraspecific competition and antagonistic behaviours. Similarly, six individual snails were assigned randomly to each aquarium to limit cleaning frequency.

2.4 Preparation of Equipment and Sourcing of Organisms

Prior to the toxicity tests, aquaria, air tubing, and terracotta pots were cleaned using a 3% HCl acid bath for at least 2 hours. Equipment was then rinsed and scrubbed using phosphate free Extran MA 05, except for terracotta pots, which were instead soaked in Millipore Milli-Q water for at least 4 hours. All equipment was rinsed with Millipore Milli-Q water and dried overnight on drying paper.

Aquaria used in the toxicity testing experiment were 2 L, glass jars, and contained an air bubbler. Holes were drilled into aquaria lids to accommodate air tubing for bubblers. Dragonfly aquaria also contained shelters fabricated from the terracotta pots split into approximately 4 cm^2 pieces.

Organisms were purchased from Boreal Science® (ramshorn snails) and Merlan Scientific® (common whitetail nymphs, and eastern pondhawk nymphs) and shipped to the BioMES Lab at the National Hydrology Research Centre, Environment and Climate Change Canada in Saskatoon, SK, Canada. Upon arrival, organisms were transferred to 19 L aquaria, half filled with tap water filtered using a Culligan® Aqua-Cleer® 4 filter system (without reverse osmosis) to remove sediment and chlorine and to slightly boost alkalinity. Organisms were allowed to acclimate for 3-5 days at 21°C with a 16h:8h light/dark cycle in a climate-controlled room and were fed *ad-lib* during acclimation. Snails were fed rinsed spinach dropped on the surface of water. Common whitetails and eastern pondhawks were fed live Blackworms (*Lumbriculus variegatus*) purchased from Merlan Scientific®.

2.5 Experimental Procedure

To each aquarium, 1 L of solution containing the respective NAFC concentrations of 0, 6, 12, and 25 mg/L (snails and common whitetail), or 0, 12, and 25 mg/L (eastern pondhawk) were added (**Figure 2**). Individual organisms were removed from acclimation aquaria and randomly grouped to 6 (snails) or 4 (dragonflies) individuals. Wet mass of each group was then measured by gently blotting individuals on blotting paper, transferring all grouped individuals to a tared weighing dish, and using a Mettler Toledo PM400 balance (0.001 g precision) to determine the wet mass for each group. Wet mass was recorded by group as individuals within aquaria were indistinguishable. Grouped individuals were transferred to their assigned aquarium and placed in the climate-controlled room. Aquaria were organized using a stratified random system, where each concentration was represented in each row, but randomly organized per row inside of the climate-controlled room.

During the exposure period, organism feeding continued ad lib and the climate-controlled room remained at 21°C with a 16h:8h light/dark. Temperature, percentage of dissolved oxygen saturation, dissolved oxygen concentration, specific conductivity, and pH were monitored every 7 days using a YSI DSS Pro probe. The probe was thoroughly rinsed with deionized water between readings.

Snails were exposed to NAFCs in a static-renewal test, where in lieu of cleaning, snails were transferred to new aquaria on the 14^{th} day of exposure. This was performed by preparing new, labelled aquaria with the same air stone and NAFC concentration as the beginning of the exposure period. Organisms were gently and quickly transferred to these new aquaria. These newly prepared aquaria containing the study organisms were then returned to the same location in the climate-controlled room. For snails, the number of egg masses found within the aquaria were counted when transferring snails to new aquaria (day 14), and 7 days after (at the end of the 21-day exposure period). Dragonflies were statically exposed to NAFCs, as aquaria did not require cleaning during the 14-day exposure period.

2.5.1 Naphthenic Acid Fraction Component Quantification and Characterization

Water samples were taken from two snail aquaria of each concentration at 0, 7, and 14 days to investigate NAFC composition and approximate concentration. These samples were prepared for analysis using a solid-phase extraction method previously reported by Headley et al. (2002). The analysis on extracted samples was performed using a Thermo Fisher LTQ Orbitrap Velos Elite™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA), with a resolution of 240,000 measured at 400 m/z in full-scan negative-ion electrospray mode, using the same conditions and spectra processing as Vander Meulen et al. (2021). As stated by prior studies, this method is considered semi-quantitative due to the lack of naphthenic acid reference standards (Bartlett et al., 2017; Marentette et al., 2015a; Robinson et al., 2023). Extraction, quantification, and characterization of NAFCs was carried out by personnel from Environment and Climate Change Canada at the National Hydrology Research Centre (Saskatoon, SK, Canada).

2.5.2 End of Exposure Periods

At the end of the exposure periods, surviving organisms were gently dried on blotting paper then transferred to a tared weighing dish to be re-weighed. Final mass and the number of surviving individuals was measured to estimate the change in mass per individual by dividing the final mass by the number of surviving individuals. All surviving organisms were individually transferred into labelled cryovials and immediately flash frozen in liquid nitrogen. For each snail, tissue was separated from shell with only tissue being frozen. All vials were stored at -80 °C.

2.6 Metabolomic Sample Preparation and Analysis

2.6.1 Tissue Extraction

Individual cryovials containing samples from all three taxa were freeze dried for approximately 24 hours using an 18 L -50 °C FreeZone freeze dryer at approximately 0.05 mbar. Organisms were then transferred to new vials with grinding beads and were ground using a Precellys Evolution tissue homogenizer with a Cryolys Evolution cooling system. Following

grinding, 10 mg of tissue mass from each sample was weighed into 2 mL Eppendorf™ vials for further processing. Ground and weighed samples were stored at -80 ℃ when not in use.

To each 2 mL vial containing 10 mg of sample tissue, 0.60 mL of ice-cold methanol and 0.27 mL of ice-cold D_2O was added, then vortexed three times for 15 seconds, and centrifuged for 10 minutes at -2 ℃ and 14,000 rpm. The supernatant was then removed and placed in a new 2 mL vial to which 0.60 mL of ice-cold chloroform and 0.27 mL of ice-cold D_2O was added. The resulting final ratio was 2:2:1.8, methanol:chloroform:D_2O (sensu Viant, 2007). Samples were vortexed for 60 seconds to combine, then placed on ice for 10 minutes to partition. Partitioned samples were centrifuged for 10 minutes at -2 ℃ and 14,000 rpm to allow for separation of the layers. The upper methanol layer (polar metabolites) and the lower chloroform layer (non-polar metabolites) were removed into separate 2 mL vials, then placed in the Speedvac evapoconcentrator to dry both layers. Once no liquid remained, vials were removed from the Speedvac and stored at -80 ℃. Aquaria and taxa sample order were selected randomly for tissue extraction.

2.6.2 Nuclear Magnetic Resonance Spectroscopy Analysis

Polar metabolite samples were resuspended in 0.55 mL of 0.05 mM nuclear magnetic resonance (NMR) spectroscopy sodium phosphate buffer containing D_2O, 3 mM sodium azide, and 0.05 mM trimethylsilylpropanoic acid (TMSP, as the standard) at a pH of ~7.0. Samples were vortexed twice for 15 seconds, then centrifuged for 10 minutes at -2 ℃ and 14,000 rpm. Then, 0.52 mL of this resuspended solution was transferred to a 5 mm NMR glass precision tube for acquisition.

A Bruker Avance 500 MHz spectrometer at 500.17 MHz with a 5 mm TCI cryoprobe, was used to acquire all ^1H 1D NMR spectra. Prior to acquisition, samples were locked to D_2O, autotuned and matched, then shimmed. One-dimensional ^1H NMR spectra were recorded for each sample at 298˚K with a 60˚ pulse and 128 scans with excitation sculpting to maximize water peak suppression (Hwang & Shaka, 1995). Each sample was Fourier transformed for 1D NMR and calibrated to the TMSP standard (Viant et al., 2003). Order of taxa sample analysis was selected randomly for NMR analysis.

2.7 Data Analysis

General Linear Models (GLMs) were used to investigate the impact of NAFC exposure on the survival of each taxon and the number of egg masses laid by snails. Dependent variables were percent survival (percentage of total individuals that survived per aquaria), and for snails, the number of egg masses per individual per aquaria. NAFC exposure (fixed factor with 4 levels for ramshorn snails and the common whitetail, and 3 levels for the eastern pondhawk dragonfly nymphs) was the model factor. Change in mass was not analyzed for any taxa due to uncertainty surrounding variation in wet mass due to water retention after blotting. All GLMs were performed in R 4.2.2 with RStudio 2023.03.0+ 386 (R Core Team, 2022; RStudio Team, 2020) using the base function "lm" and an $\alpha = 0.1$. The R package "ggfortify" was used for model assumption checking to produce residuals vs fitted to detect non-linearity, normal Q-Q for normality, and scale-location for homoscedasticity (Horikoshi & Tang, 2018; Tang et al., 2016). Model assumptions were met for all model assumption testing. Tukey's pairwise comparisons ($\alpha = 0.1$) were performed within taxa when significant differences among NAFC treatment levels were found using the "emmeans" package (Lenth, 2023). The "ggplot2" package in R was used to produce all plots herein, unless indicated otherwise (Wickham, 2016).

Prior to metabolomic analyses, each spectrum was manually phased, and baseline corrected in the Bruker Topspin 3.6.5 software package. *Prometab*, in MATLAB (The Mathwork, Natick, MA), was used to bin and export data at 0.005 wide bins from 8.6025 – 0.6425 ppm (Viant, 2008). Bins from 7.7125 – 7.6175 ppm and 4.8525 – 4.6475 ppm were excluded due to a contaminant signal and the water peak, respectively.

MetaboAnalyst 5.0 was then used to filter the data, normalize (normalization by sum) and autoscale, and remove outliers using initial visualizations (Pang et al., 2021). Outlier removal was done by first using all binned data and statistically filtering it by interquantile range to remove variables which are constant across samples. Principal component analysis (PCA) of the binned data was used to visualize differences in organism metabolomes among NAFC concentration, and for the identification of outlying spectral data. The PCA plots (on PC axes 1, 2, and/or 3) were used to identify outliers by removing samples lying far outside the 95% confidence interval (CI) from the dataset. The PCA axes investigated were selected based on the PCA scree plots from MetaboAnalyst. After the exclusion of outliers (snails; 1 in 0 mg/L, 1 in 6

mg/L, 1 in 12 mg/L, 1 in 25 mg/L, common whitetail; 1 in 0 mg/L, and in the eastern pondhawk; 1 in 0 mg/L), data were refiltered, normalized and autoscaled.

Using the filtered, normalized, and autoscaled data, Euclidian distance matrices were calculated in R using the "vegan" package, and used to produce non-metric MultiDimensional Scaling (nMDS) plots with 20 iterations, 2-dimensional stress = 0.104 (Oksanen et al., 2022). In Primer-E (version 7.0 with PERMANOVA+, Primer-E Ltd., Plymouth), Euclidian distance matrices were calculated from the filtered, normalized, and autoscaled data using Primer-E (Clarke & Gorley, 2015). Separate PERmutational Mutivariate Analysis of Variance (PERMANOVA) analyses were run using Primer-E on the filtered, normalized, and autoscaled data to investigate differences among concentrations for each taxon, and pairwise comparisons were completed where significant ($p \leq 0.1$) NAFC concentration terms were observed (Anderson et al., 2008). Model factors were NAFC concentration (fixed factor; 4 levels for snails, and 3 levels for both dragonfly taxa as 12 mg/L did not have enough surviving individuals for the common whitetail) and aquaria (random factor).

2.7.1 Metabolite Identification

In cases where the PERMANOVA indicated that exposure to NAFCs resulted in significant differences in the metabolome, partial least squares discriminant analysis (PLS-DA) in MetaboAnalyst was used to determine the bins associated with differences amongst metabolomes in response to NAFC exposure (Pang et al., 2021). Bins with variable importance in projection (VIP) scores above 1.25 resulting from the PLS-DA were considered important for further investigation.

Chenomx NMR Suite software v.10.0 (Chenomx Inc., Edmonton, AB, Canada), was then used to profile metabolites. This was completed by fitting regions identified from bins with high VIP scores to metabolites indexed in Chenomx. When metabolite spectra contained multiple peaks, all regions were compared with the collected spectra to ensure metabolite fit.

In MetaboAnalyst 5.0, ANOVA and post-hoc analysis (FDR $\alpha = 0.1$; Tukey's pairwise comparisons) was used to identify direction of metabolite differences between the metabolomes of individuals exposed to different NAFC concentrations identified by VIP score (Pang et al.,

2021). To visualize differences between the NMR spectra of the NAFC exposure levels, average spectra plots were created where a significant exposure term was observed by plotting the average relative abundance for all bins, overlayed with identified and unidentified metabolites/bin regions in R 4.2.2 (R Core Team, 2022).

To understand which pathways may be impacted by changing metabolite levels, metabolites found to significantly differ among treatment levels were analyzed using pathway analysis in MetaboAnalyst. To understand the importance of identified metabolites upon different metabolic pathways, pathway impact measurement, and significance were generated by relative-betweenness centrality for topology analysis, and with Fisher's Exact Test as the enrichment method with an FDR $\alpha = 0.1$. As metabolites differ among organisms, a reference metabolic pathway library is used as it includes only measurable metabolites for the organism (Pang et al., 2021). The reference library used was the fruit fly (*Drosophila melanogaster*), as it presently is the only insect reference library, and thus would likely be more similar to the insects utilized in this study.

2.7.2 Taxa Metabolomic Comparisons

To assess differences in the metabolomes of the three taxa, metabolomic data from control samples of all three taxa were statistically filtered with interquantile range, normalized (normalization by sum) and autoscaled using MetaboAnalyst 5.0 (Pang et al., 2021). PCA was used to visualize differences and exclude metabolome samples lying far outside the 95% CI on PCA plots on PCA axes 1, 2, and/or 3. The number of PCA axes selected was based on scree plots from MetaboAnalyst. After the exclusion of outliers (snails; 1 individual, common whitetail; 1 individual, and in the eastern pondhawk; 1 individual), data were refiltered, normalized and autoscaled.

The metabolomes of control samples of all three taxa (snails, common whitetail, and eastern pondhawk) were analyzed together. This was completed using Primer-E to calculate Euclidian distance matrices from spectral bins. Then, PERMANOVA was used to investigate differences in the metabolomes of the taxa from the distance matrices. Model factors were taxa (fixed factor; 3 levels for the different taxa), and aquaria (random factor).

Where metabolomes were significantly different (i.e., $p \leq 0.1$) among control taxa, spectral bins driving differences in the metabolome were identified using a VIP score greater than 1.25 from a PLS-DA analysis. Chenomx NMR Suite software v.10.0 (Chenomx Inc., Edmonton, AB, Canada), was used to identify metabolites associated with spectral bins driving differences among the metabolomes. In MetaboAnalyst, ANOVA post-hoc analysis (FDR $\alpha = 0.1$; Tukey's pairwise comparisons) was used to compare between the relative abundance of identified metabolites among taxa. Pathway analysis in MetaboAnalyst was used to identify potentially altered pathways among the taxa. The Fisher's Exact Test was used for the enrichment method, relative-betweeness centrality for topology analysis, and an FDR $\alpha = 0.1$. Pathway analysis reference library was the fruit fly (*D. melanogaster*).

3.0 Results

3.1 Naphthenic Acid Fraction Component Concentration and Characterization

The measured concentration of the NAFC stock solution was 756 mg/L (previously measured by Marentette et al. (2015a) at 1243 mg/L), whereas the mean measured NAFC treatment concentrations were 4.20, 6.73, and 14.5 mg/L for the nominal treatment aquaria concentrations of 6, 12, and 25 mg/L, respectively (**Table 1**). Furthermore, a mean NAFC concentration of 0.935 mg/L was observed in the control samples. For NAFC concentrations, there was no consistent pattern of change over time, with decreases of up to 44%, 10%, and increases of up to 27%, and 18% for the 0, 6, 12, and 25 mg/L exposures, respectively (**Table 1**).

Characterization of NAFCs revealed a range of different compound classes (**Figure 3**). The NA stock solution contained >95% O_2 compounds. In the spiked aquaria (6, 12, and 25 mg/L), the relative amount of O_3 and O_4 compounds increased throughout the experiment. By day 7, O_3 compounds increased from less than 5% to over 30%, then to approximately 45% by day 14. While O_4 compounds increased to around 5% by day 7 and remained around 5% by day 14. Unlike the spiked aquaria, control aquaria contained >45% O_2 compounds, with no discernable pattern apparent over time. Other major compounds in the control aquaria included O_3, O_4, NO_3 and NO_6 compounds.

Table 1. Measured naphthenic acid fraction component (NAFC) concentrations taken on day 0, 7, and 14 for two ramshorn snail aquaria from each NAFC exposure level used in this experiment. Nominal concentrations were calculated based on dilutions of the 1243 mg/L stock solution.

Nominal Concentration	Day	Sample 1 (mg/L)	Sample 2 (mg/L)
0 mg/L	0	1.54	0.76
	7	1.00	0.67
	14	0.87	0.77
6 mg/L	0	3.90	4.67
	7	3.94	4.20
	14	4.06	4.48
12 mg/L	0	5.51	7.01
	7	7.42	6.08
	14	7.50	6.86
25 mg/L	0	13.79	12.31
	7	16.81	14.70
	14	14.44	14.89

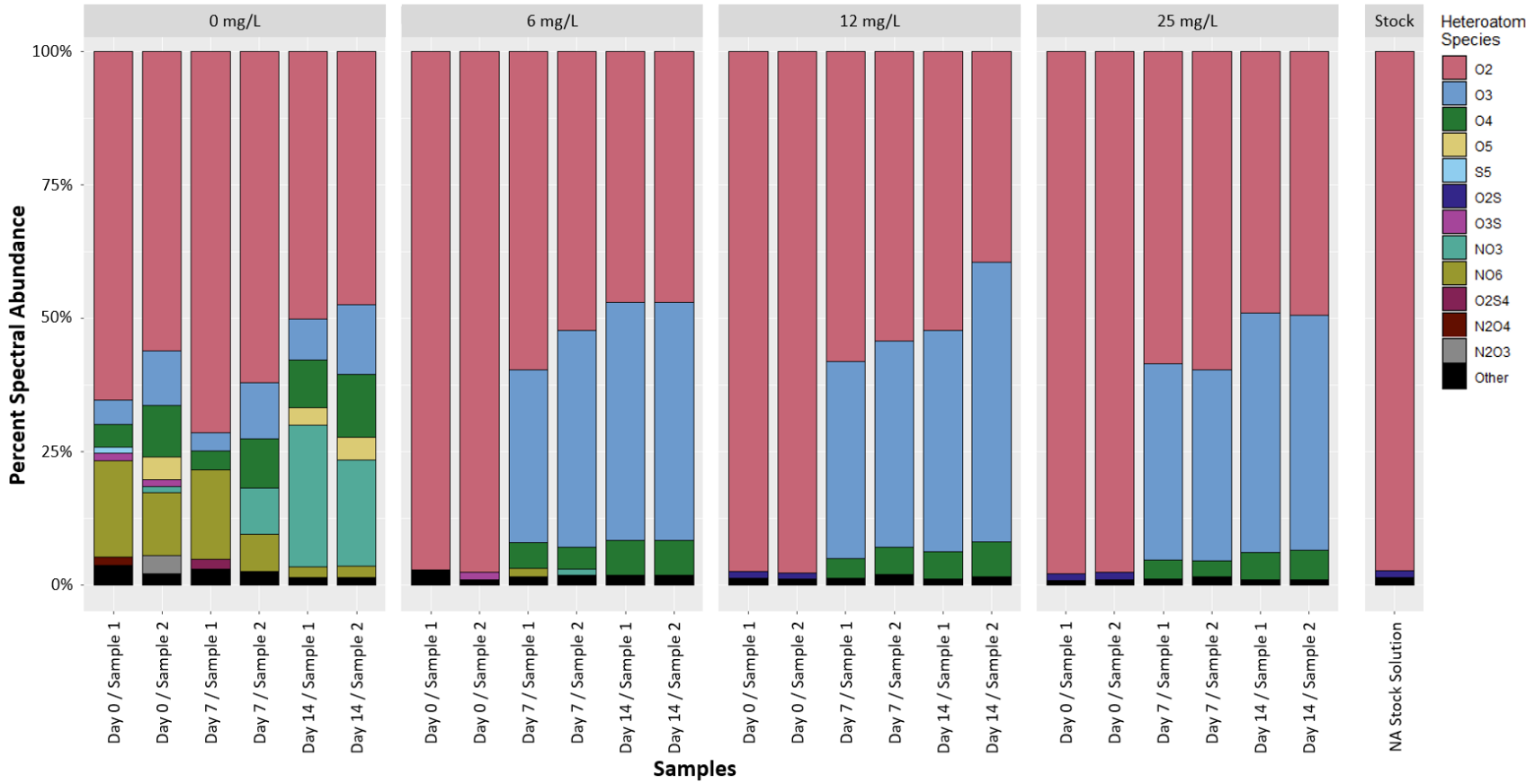

Figure 3: Naphthenic acid fraction component (NAFC) composition for the different treatment levels (0, 6, 12, 25 mg/L) for two ramshorn snail aquaria at the start of the experiment (day 0), after 7 days, and after 14 days using an Orbitrap LC-MS. Colours represent a broad range of heteroatom compound classes. Oxygenation state is represented by O_2, O_3, O_4, and O_5. S5 compounds contain sulfur, while O_2S, O_3S, and O_2S_4 compounds were oxygenated compounds containing sulfur. NO_3, NO_6, N_2O_4, and N_2O_3 compounds contain nitrogen and oxygen molecules.

3.2 Naphthenic Acid Fraction Components Toxicity Testing

3.2.1 Survival

Ramshorn snail survival did not change ($F_{3,19} = 1.61$, $p = 0.227$) with exposure to NAFCs with 97.5% mean survival overall (**Figure 4**). Mean survival of 97.1% (standard error, SE = 2.86) and 93.3% (SE = 4.08) for snails exposed to 0 and 6 mg/L NAFCs, respectively, whereas no deaths occurred in 12 and 25 mg/L NAFC treatment levels.

Survival for the common whitetail dragonfly changed with exposure to NAFCs ($F_{3,11} = 3.44$, and $p = 0.072$). Mean survival for the common whitetail was 91.7% (SE = 8.33), 83.3% (SE = 8.33), 16.7% (SE = 16.7), and 58.3% (SE = 30.0), for NAFC exposure of 0, 6, 12, and 25 mg/L, respectively. Only the survival of dragonflies in the 12 mg/L treatment level decreased compared to the control ($p = 0.075$).

No differences ($F_{2,8} = 0.125$, $p = 0.885$) in survival was found for the eastern pondhawk, although the survival in the 25 mg/L treatment level decreased by 33.3% in the 25 mg/L treatment level compared to the control ($p = 0.589$). Mean survival was 87.5% (SE = 22.0), 75.0% (SE = 0), and 66.7% (SE = 8.33), for NAFC treatment levels of 0, 12, and 25 mg/L, respectively.

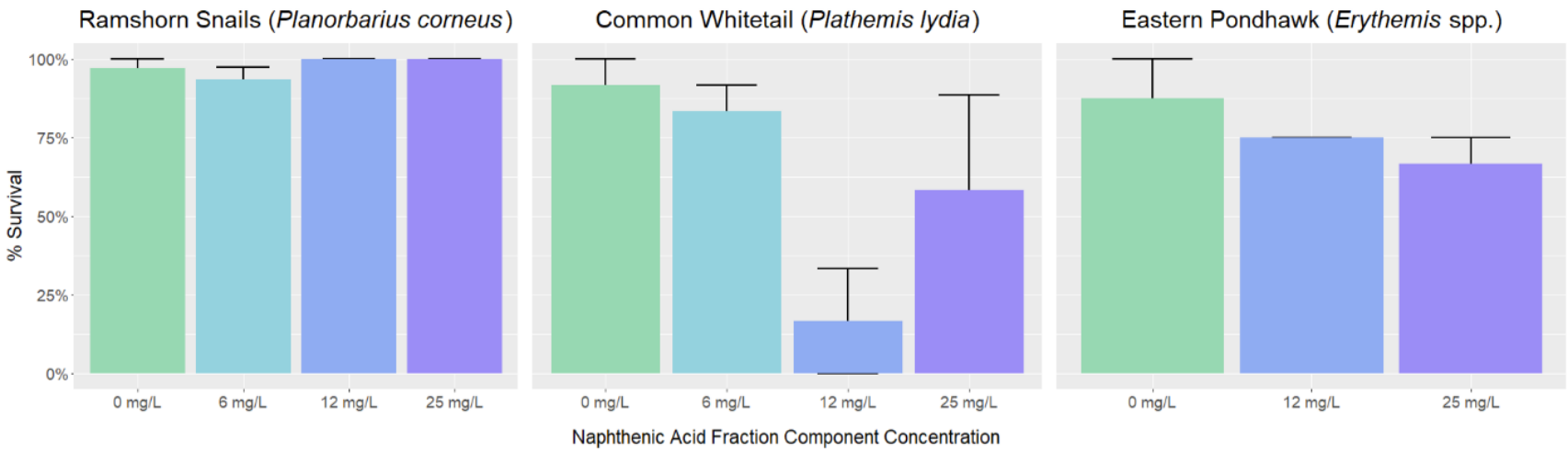

Figure 4: Mean survival of ramshorn snails, and the common whitetail and the eastern pondhawk dragonflies per aquaria for tested naphthenic acid fraction component treatment levels. Survival was calculated using the count of surviving individuals divided by the number of starting individuals. Error bars represent one standard error.

3.2.2 Egg Masses Laid

Snails exposed to 12 and 25 mg/L of NAFCs laid approximately double the egg masses of the control and 6 mg/L exposed individuals (**Figure 5**). However, differences in egg masses were not significant among treatment levels ($F_{3,19} = 1.88$, $p = 0.173$). Mean egg masses laid per snail were 0.84 (SE = 0.25), 0.69 (SE = 0.26), 1.6 (SE = 0.47), and 1.6 (SE = 0.39), for 0, 6, 12, 25 mg/L NAFCs, respectively.

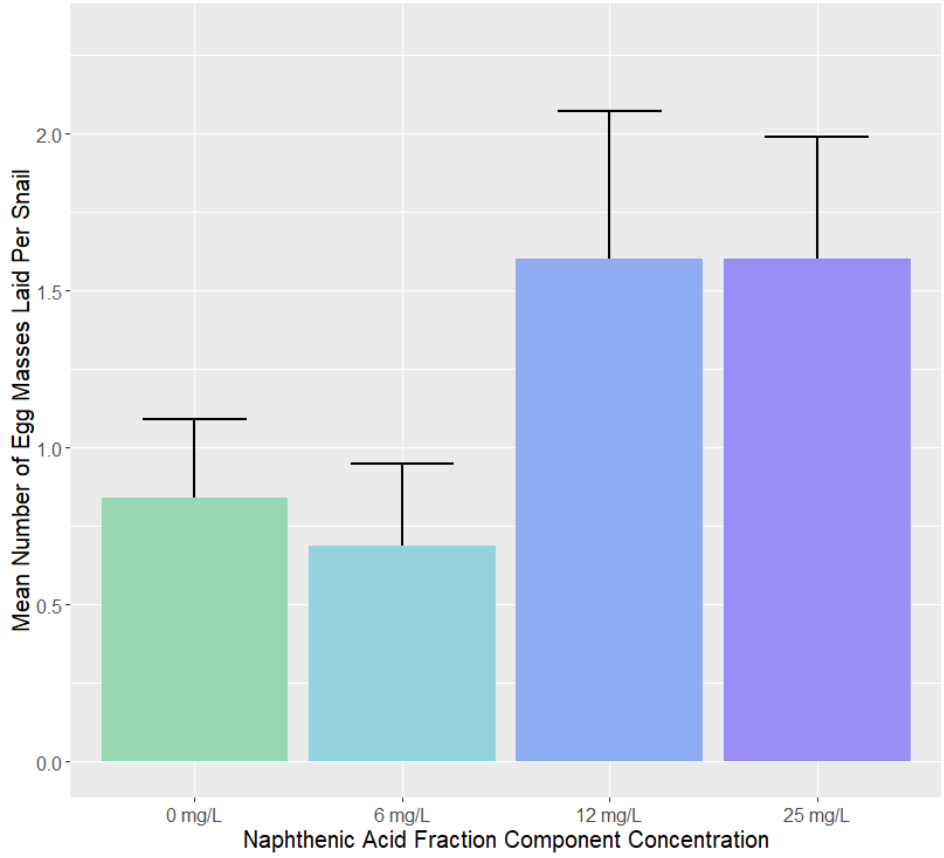

Figure 5: Mean egg masses laid per ramshorn snail (*Planorbarius corneus*) during the exposure period at four treatment levels of naphthenic acid fraction component concentrations. Error bars represent one standard error.

3.2.3 Metabolomic Response to Naphthenic Acid Fraction Components

The nMDS ordination of the metabolomes of ramshorn snails indicated no seperation between metabolomes exposed to the different NAFC concentrations (**Figure 6**)**.** Moreover, PERMANOVA analysis indicated that ramshorn snail metabolomes did not significantly differ with exposure to NAFCs ($F_{3,95} = 1.08$, $p = 0.379$).

For the common whitetail, the metabolomic response of the 12 mg/L exposure level could not be investigated due to an large number of deaths leaving only two individuals. The nMDS plot indicates that common whitetail individuals exposed to 6 and 25 mg/L NAFCs were more similar to each other than to the control. Investigating 0, 6, and 25 mg/L exposure levels for the common whitetail revealed that the metabolomes of individuals exposed to NAFCs were significantly different ($F_{2,19} = 1.996$, $p = 0.061$). Both 6 and 25 mg/L significantly differed from the control ($p = 0.065$ and $p = 0.037$, respectively), but not from each other ($p = 0.263$).

For the eastern pondhawk, the nMDS plot did not indicate any clear seperation among the NAFC treatment levels, but it appears that there may be two groups separated along the y-axis, separate from exposure levels. There were no significant differences in the metabolomes of the Eastern Pondawks exposed to NAFCs ($F_{2,15} = 0.745$, $p = 0.687$).

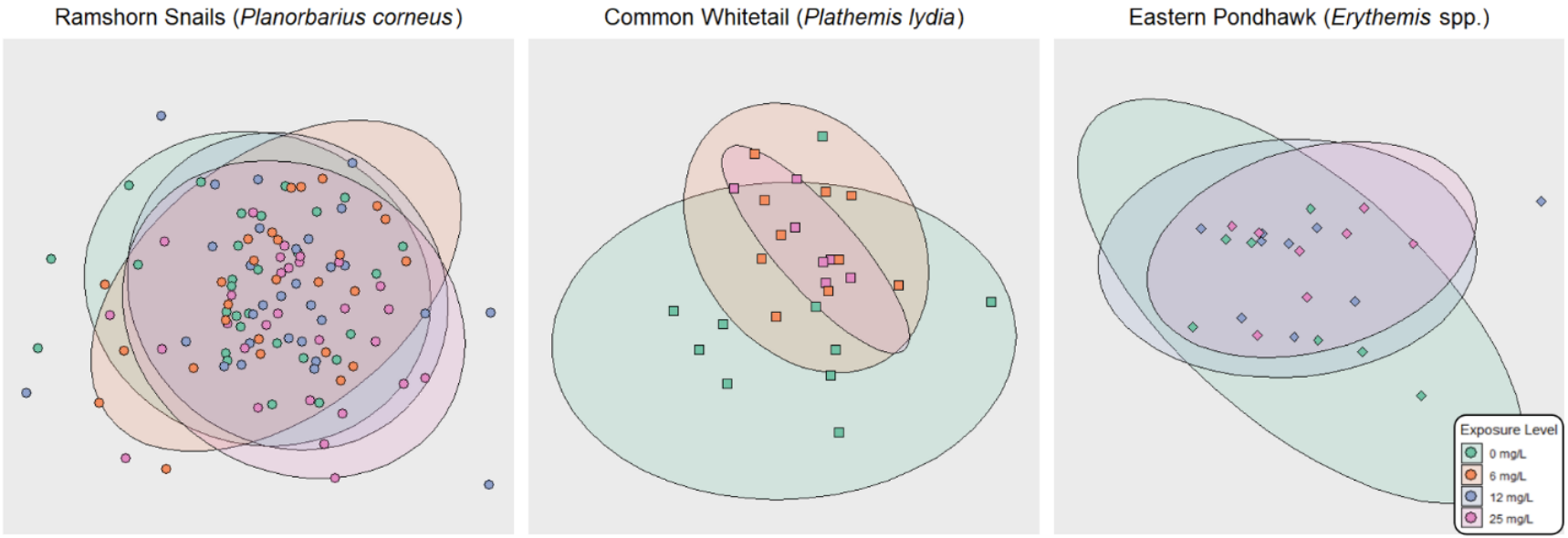

Figure 6 Non-metric multidimensional scaling plots showing the similarity of organism metabolomes exposed to naphthenic acid fraction components (NAFCs) for ramshorn snails, and the common whitetail and eastern pondhawk dragonflies. Each symbol represents an individual's metabolome, while colours represent NAFC concentrations. The NAFC concentrations were 0, 6, 12, and 25 mg/L for all taxa except for the eastern pondhawk with only 0, 12, and 25 mg/L NAFCs. Ellipses represent 95% confidence interval of the metabolomes at differing NAFC concentrations.

3.2.4 Metabolites Driving Differences in the Common Whitetail's Metabolomic Response

From the PLS-DA, there were 36 regions of the common whitetail spectra with VIP scores greater than 1.25. Of these, 23 were linked to specific metabolites (**Table 2**), while 13 were unknown metabolites (**Table A1**). Twelve metabolites (arginine, asparagine, agmatine, glutamate, alanine, galactonate, 3-hydroxyisobutyrate, glutamine, β-alanine, methionine, τ-methyhistidine, 4-aminobutyrate) were significantly higher at the control level compared to the exposed levels, whereas 6 metabolites (glucose-6-phosphate, adenosine, maltose, glycerol, lactate, and threonine) were significantly lower at the control level compared to the exposed individuals (**Figure 7**). There were two metabolites (tryptophan and lysine) that were higher at 6 mg/L than at 25 mg/L, and 3 metabolites (guanosine, histamine, and histidine) that were higher at 25 mg/L than at 6 mg/L.

Of identified metabolites, 9 were amino acids (alanine, arginine, asparagine, glutamine, histidine, lysine, methionine, threonine, and tryptophan), 2 were ribonucleosides (adenosine, and guanosine), 6 were compounds derived from amino acids or are involved in the metabolism of amino acids (agmatine, glutamate, 3-hydroxyisobutyrate, β-alanine, histamine, and τ-methyhistidine), 2 were sugars (glucose-6-phosphate, and maltose), 1 was a nonproteinogenic amino acid (4-aminobutyrate), 1 was an alcohol (glycerol), 1 was a metabolic product of glycolysis (lactate), and 1 was a sugar acid (galactonate).

Pathway analysis revealed that the identified metabolites were involved in 5 different metabolism pathways (**Table 3)**. Ten of the metabolites are involved in the aminoacyl-tRNA biosynthesis pathway. Among identified metabolites involved in the aminoacyl-tRNA biosynthesis pathway, all were lower with exposure to NAFCs, except for three metabolites. Tryptophan was higher in the control and 6 mg/L treatment individuals, while threonine was higher in NAFC treatment individuals than control, and histidine was higher in 25 mg/L NAFC treatment individuals. Among the four other pathways, all were associated with amino acid metabolism and the abundance of all identified metabolites involved in these pathways were lower in NAFC treatment individuals. Metabolites associated with alanine, aspartate and glutamate metabolism, arginine biosynthesis, and nitrogen metabolism all decreased in abundance relative to the controls.

Table 2. Metabolites characterized from spectral bins with a PLS-DA VIP score ≥ 1.25 indicating differences between the metabolome of the common whitetail exposed to 0, 6, and 25 mg/L NAFCs. Significant differences indicate metabolite abundance differences between exposure levels using Tukey's Honest Significant Difference (HSD) post-hoc analysis (FDR α = 0.1). Bins with a VIP score ≥ 1.25, where it was not possible to identify the metabolite, may be found in **Table A1**.

Metabolite	**VIP Score**	**f value**	**FDR**	**Significant Differences Between NAFC concentrations (Tukey's HSD α = 0.1)**
Arginine	2.04	18.95	0.004	0 > 6 = 25
Glucose-6-phosphate	1.91	17.13	0.005	0 < 6 = 25
Asparagine	1.84	11.55	0.011	0 > 6 = 25
Agmatine	1.83	13.39	0.010	0 > 6 = 25
Adenosine	1.81	12.27	0.010	0 < 6 = 25
Tryptophan	1.80	10.22	0.014	0 = 6 > 25
Maltose	1.75	14.24	0.009	0 < 6 = 25
Glutamate	1.72	10.80	0.012	0 > 6 = 25
Glycerol	1.71	10.36	0.014	0 < 6 = 25
Alanine	1.62	11.83	0.010	0 > 6 = 25
Lactate	1.60	8.25	0.024	0 < 6 = 25
Galactonate	1.58	9.37	0.016	0 > 6 = 25
3-hydroxyisobutyrate	1.57	19.71	0.004	0 > 6 = 25
Glutamine	1.57	19.71	0.004	0 > 6 = 25
β-alanine	1.57	6.70	0.036	0 > 6 = 25
Methionine	1.51	9.43	0.016	0 > 6 = 25
Guanosine	1.50	5.95	0.048	0 = 6 < 25
Lysine	1.48	5.50	0.056	0 = 6 > 25
Threonine	1.47	8.58	0.021	0 < 6 = 25
Histamine	1.47	5.27	0.061	0 = 6 < 25
τ-methyhistidine	1.39	5.42	0.058	0 > 6 = 25
4-aminobutyrate	1.37	5.06	0.066	0 > 6 = 25
Histidine	1.36	4.74	0.075	0 = 6 < 25

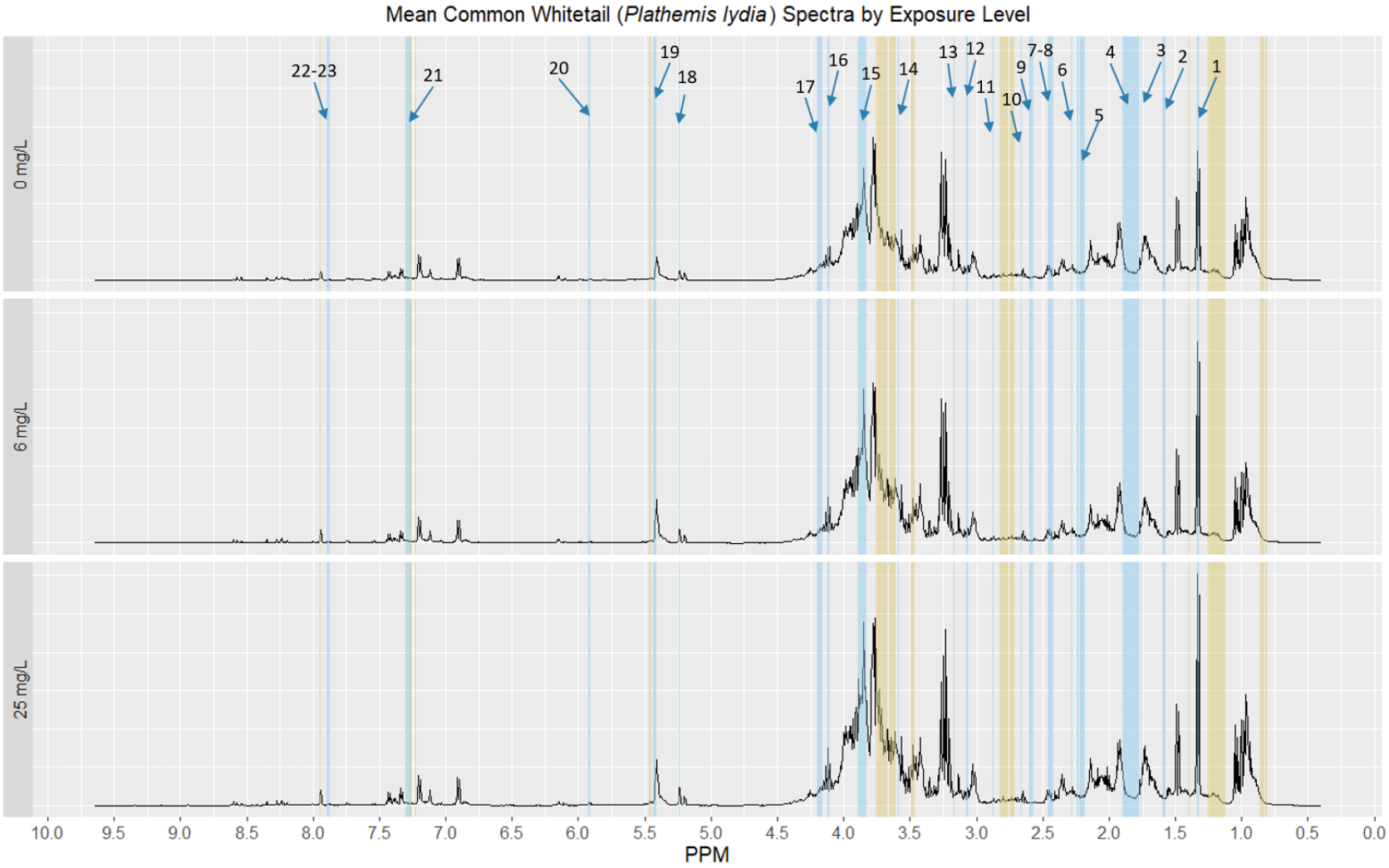

Figure 7. The common whitetail (*Plathemis lydia*) average spectra plots by exposure level annotated with metabolite regions associated with differences among treatment levels. Blue regions represent annotated metabolite range (metabolites may also have peaks in other regions) that differ among treatment levels. Yellow regions are unknown metabolites that differ among treatment levels

(**Table A1)**. Annotated metabolites: (1) threonine, (2) alanine, (3) agmatine, (4) arginine, (5) glutamate, (6) 4-aminobutyrate, (7) 3-hydroxyisobutyrate, (8) glutamine, (9) β-alanine, (10) methionine, (11) asparagine, (12) lysine, (13) τ-methyhistidine, (14) glycerol, (15) adenosine, (16) lactate, (17) galactonate, (18) glucose-6-phosphate, (19) maltose, (20) guanosine, (21) tryptophan, (22) histamine, (23) histidine.

Table 3. Impacted common whitetail pathways identified based on metabolites driving differences between the metabolomes of the common whitetail dragonfly exposed to 0, 6, and 25 mg/L NAFCs with an FDR $\alpha = 0.1$. The metabolites column refers to metabolites driving differences in the metabolome following naphthenic acid fraction component exposure, which are involved in this pathway (identified based on the list of metabolites).

Pathway	**Metabolites**	**p**	**-LOG$_{10}$(p)**	**FDR**	**Impact**
Aminoacyl-tRNA biosynthesis	alanine, arginine, asparagine, glutamate, glutamine, histidine, lysine, methionine, threonine, tryptophan	< 0.001	9.041	7.38E-08	0
Alanine, aspartate, and glutamate metabolism	4-aminobutyrate, alanine, asparagine, glutamate, glutamine	< 0.001	4.546	0.001	0.58
Arginine biosynthesis	arginine, glutamate, glutamine	0.001	3.017	0.026	0.46
Nitrogen metabolism	glutamine, glutamate	0.003	2.533	0.047	0
D-Glutamine and D-glutamate metabolism	glutamine, glutamate	0.003	2.533	0.047	1

3.3 Metabolomic Differences Among Taxa

The nMDS ordination shows separation of metabolomes of the control individuals of the three different taxa with the dragonflies clustering closer to each other than to snails (**Figure 8**). The PERMANOVA indicated differences among taxa metabolomes ($F_{2,44} = 11.551$, $p = 0.002$), such that all taxa were different (eastern pondhawk/common whitetail $p = 0.095$, eastern pondhawk/ramshorn snails $p = 0.021$, and common whitetail/ramshorn snails $p = 0.022$).

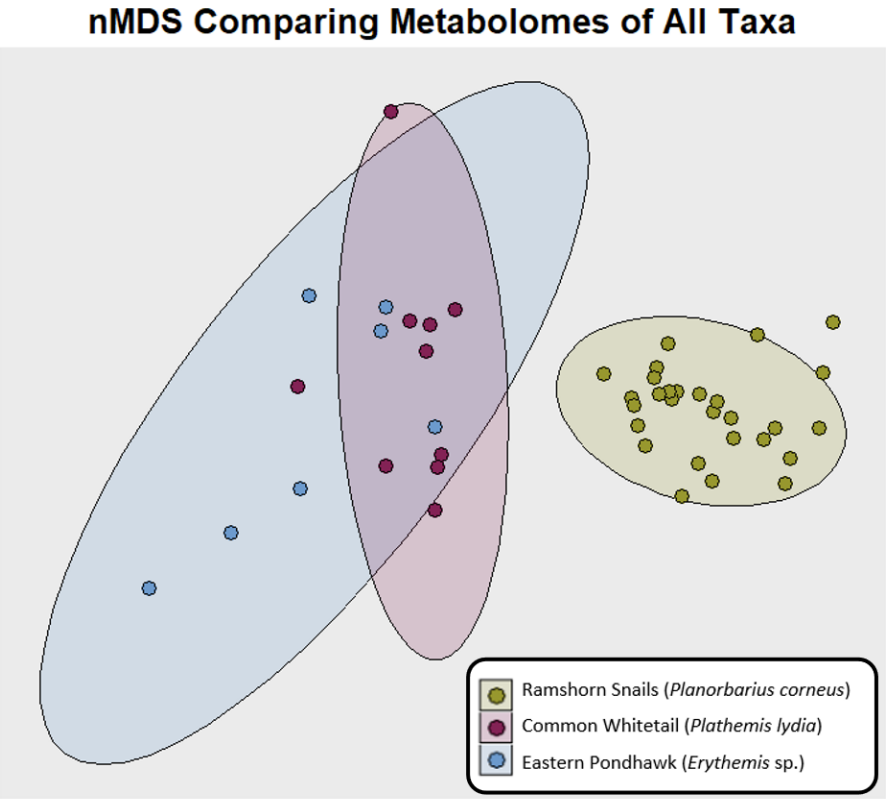

Figure 8: Non-metric multidimensional scaling plot comparing the metabolomes of control individuals of the ramshorn snail and the common whitetail and eastern pondhawk dragonflies. Circles on the plot represents an individual's metabolome and colours represent different taxa. Ellipsees represent the 95% CI for the metabolome of each taxon.

3.3.1 Comparing Metabolite Differences Among Taxa

From the PLS-DA, there were 47 regions of the spectra having VIP scores greater than 1.25. Of these, 25 were linked to specific metabolites (**Table 4**), while 22 were unknown metabolites (**Table A2**). Of the identified metabolites, 8 were amino acids (alanine, arginine, isoleucine, lysine, methionine, threonine, tyrosine, and valine), 7 were derived from amino acids (agmatine, glutarate, histamine, tyramine, phenylacetate, glutamate, and aspartate), 2 were dicarboxylic acids (malate and malonate), 2 were intermediates in the TCA cycle (citrate and succinate), 4-hydroxybutyrate is an organic acid, glutathione is an antioxidant, taurine is a β-amino acid, lactate is an α-hydroxy acid, inosine monophosphate is a nucleotide, and aspartate is an α-amino acid.

Metabolites identified as driving differences between the metabolomes of the three taxa were more similar between dragonflies than snails. The abundance of 20 metabolites (4-hydroxybutyrate, glutarate, arginine, lysine, glutathione, methionine, histamine, tyramine, phenylacetate, glutamate, succinate, malate, lactate, threonine, citrate, adenosine, tyrosine, alanine, inosine monophosphate, and aspartate) was higher in snails than dragonflies, while abundance of 5 metabolites (agmatine, taurine, malonate, valine, and isoleucine) were lower in snails than dragonflies. Between dragonflies, 3 (glutamate, lactate, and tyrosine) metabolites had higher abundance in the common whitetail, whereas the abundance of taurine, valine, and isoleucine was higher in the eastern pondhawk (**Figure 9)**.

Pathway analysis of identified metabolites that differed among taxa revealed 4 separate altered pathways (**Table 5)**. Ten of the metabolites were involved in aminoacyl-tRNA biosynthesis. Among identified metabolites involved in aminoacyl-tRNA biosynthesis, all were higher in snails than both dragonflies. However, valine and isoleucine were both highest in the eastern pondhawk, of intermediate abundance in the common whitetail, and lowest in the snail, while tyrosine was highest in the snail, intermediate in the common whitetail, and lowest in the eastern pondhawk. For valine, leucine and isoleucine biosynthesis, metabolite abundance was highest in the eastern pondhawk and lowest in the snails for both isoleucine and valine, while threonine was highest in snails and no difference between dragonflies. Alanine, aspartate and glutamate metabolism contained succinate, alanine, aspartate, and glutamate, which were all highest in snails, with no difference between dragonflies, except for glutamate, which was

highest in snails, but lowest in the eastern pondhawk. Finally, for the arginine biosynthesis pathway, arginine and aspartate were higher in snails than both dragonflies, while glutamate was highest in snails, but lowest in the eastern pondhawk.

Table 4. Metabolites characterized from spectral bins with a PLS-DA VIP score ≥ 1.25, indicating differences among the metabolomes of the different taxa: snails (S), common whitetail (CW), and eastern pondhawk (EP). Significant differences indicate metabolite abundance differences between taxa using Tukey's Honest Significant Difference (HSD) post-hoc analysis (FDR α = 0.1). Bins with a VIP score ≥ 1.25 of unknown metabolites are found in **Table A2**.

Metabolite	**VIP Score**	**f value**	**FDR**	**Significant Differences Between Taxa (Tukey's HSD α = 0.1)**
Agmatine	1.54	461.1	< 0.001	S < CW = EP
4-hydroxybutyrate	1.48	173.24	< 0.001	S > CW = EP
Glutarate	1.48	173.24	< 0.001	S > CW = EP
Arginine	1.45	141.66	< 0.001	S > CW = EP
Lysine	1.44	110.41	< 0.001	S > CW = EP
Glutathione	1.43	100.46	< 0.001	S > CW = EP
Methionine	1.41	104.63	< 0.001	S > CW = EP
Histamine	1.41	87.333	< 0.001	S > CW = EP
Tyramine	1.41	87.62	< 0.001	S > CW = EP
Phenylacetate	1.39	85.62	< 0.001	S > CW = EP
Taurine	1.38	107.72	< 0.001	S < CW < EP
Glutamate	1.38	95.92	< 0.001	S > CW > EP
Succinate	1.36	70.51	< 0.001	S > CW = EP
Malonate	1.36	73.06	< 0.001	S < CW = EP
Malate	1.36	62.58	< 0.001	S > CW = EP
Lactate	1.35	82.11	< 0.001	S > CW > EP
Threonine	1.35	62.73	< 0.001	S > CW = EP
Citrate	1.35	59.01	< 0.001	S > CW = EP
Valine	1.33	70.13	< 0.001	S < CW < EP
Adenosine	1.33	53.97	< 0.001	S > CW = EP
Tyrosine	1.30	58.49	< 0.001	S > CW > EP
Alanine	1.29	45.39	< 0.001	S > CW = EP
Isoleucine	1.29	103.78	< 0.001	S < CW < EP
Inosine monophosphate	1.29	45.96	< 0.001	S > CW = EP
Aspartate	1.25	37.06	< 0.001	S > CW = EP

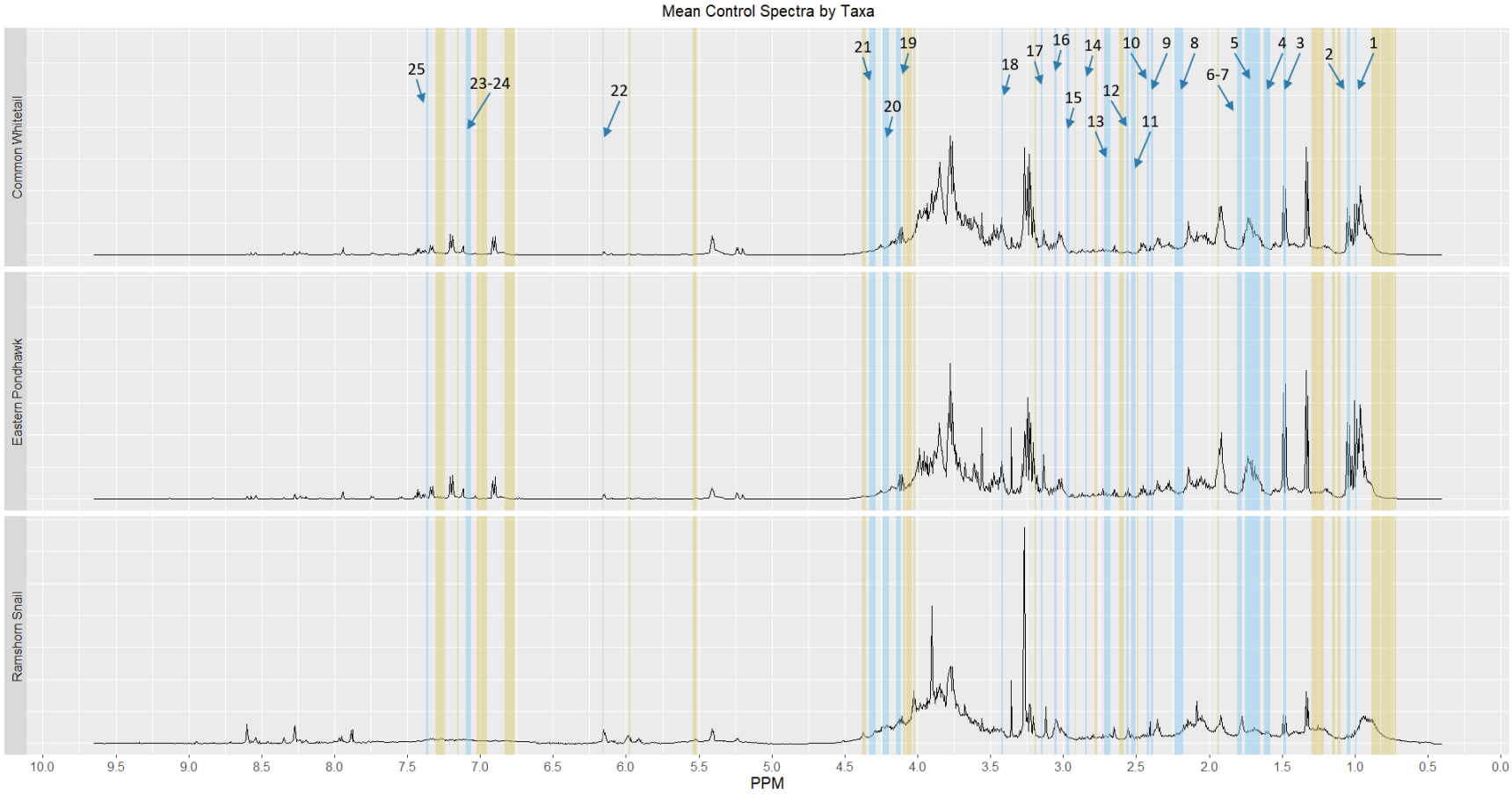

Figure 9. Average spectra plots of control common whitetail (*Plathemis lydia*), eastern pondhawk (*Erythemis* spp.), and ramshorn snail (*Planorbarius corneus*) invertebrates annotated with metabolite regions associated with differences among taxa. Blue regions represent annotated metabolite range (metabolites may also have peaks in other regions), while yellow regions are unknown metabolites (**Table A2)**. Annotated metabolites: (1) isoleucine, (2) valine, (3) alanine, (4) arginine, (5) agmatine, (6) 4-hydroxybutyrate, (7) glutarate, (8) methionine, (9) glutamate, (10) succinate, (11) glutathione, (12) citrate, (13) malate, (14) aspartate,

(15)histamine, (16) lysine, (17) malonate, (18) taurine, (19) lactate, (20) threonine, (21) adenosine, (22) inosine monophosphate, (23) tyramine, (24) tyrosine, (25) phenylacetate.

Table 5. Biochemical pathways with that differed (FDR $\alpha = 0.1$) among taxa identified based on changing relative abundances of metabolites among three different taxa (the ramshorn snail, the common whitetail dragonfly, and the eastern pondhawk dragonfly). The identified metabolites that differed among the metabolomes are listed with their respective altered pathways.

Pathway	**Metabolites**	**p**	**-LOG$_{10}$(p)**	**FDR**	**Impact**
Aminoacyl-tRNA biosynthesis	alanine, arginine, aspartate, glutamate, isoleucine, lysine, methionine, threonine, tyrosine, valine	< 0.001	8.239	< 0.01	0
Valine, leucine and isoleucine biosynthesis	threonine, valine, isoleucine	< 0.001	3.395	0.02	0
Alanine, aspartate and glutamate metabolism	aspartate, alanine, glutamate, succinate	< 0.001	3.027	0.03	0.43
Arginine biosynthesis	arginine, aspartate, glutamate	0.002	2.824	0.03	0.46

4.0 Discussion

4.1 Metabolomic Impacts of Naphthenic Acid Fraction Component Exposure

The common whitetail dragonfly was the only taxa exhibiting a metabolomic response to NAFC exposure. Moreover, the common whitetail showed metabolomic effects at both 6 and 25 mg/L of NAFCs. Thus, compared to the eastern pondhawk and the ramshorn snail, the common whitetail appears to be most sensitive to NAFC exposure. Moreover, my findings for the common whitetail adds to existing evidence from past studies that exposure to environmentally relevant concentrations of NAFCs can result in sublethal stress in some aquatic taxa. For example, previous sublethal toxicity studies have detected sublethal stress from NA exposure as low as 0.5 mg/L by detecting genetic signs for endocrine disruption in zebrafish embryos (Wang et al., 2015a). Other examples include transcriptome changes detected in fish larvae exposed to 1.25 mg/L NAs (Loughery et al., 2019), or genetic signs of stress in the Walleye (*Sander vitreus*) at 4.2 mg/L NAFCs (Marentette et al., 2017). The only other metabolomics study involved the exposure of *Hexagenia* spp. mayflies to commercial NAs from 0.0001 – 100 µg/L and observed a correlation between a shift in the metabolome and increasing NA concentrations (Pomfret et al., 2021). The metabolome of the common whitetail could potentially act as a good bioindicator species for NAFC exposure as its metabolism appears to detect NAFCs at relatively low concentrations, while also able to withstand higher concentrations, allowing its use in bioassays across a range of conditions currently observed in the Athabasca Oilsands Region.

The majority of metabolites driving differences in the common whitetail metabolome when exposed to NAFCs were amino acids or related to amino acid metabolism. Differences in amino acid abundances indicates that amino acid metabolism is impacted, and likely affects closely tied metabolic processes, such as oxidative stress and energy metabolism (Labine et al., 2023). There are other potential implications, such as alterations in protein synthesis/degradation or nitrogen metabolism (Labine et al., 2023). Three of the metabolites differing most with NAFC exposure (arginine, asparagine, and agmatine) all decreased with NAFC exposure. These three amino acids are involved in amino acid biosynthesis pathways

and related pathways, such as energy metabolism and energy storage (Bai et al., 2022; National Center for Biotechnology Information, 2023; Viant et al., 2001; Yina et al., 2016). Thus, there may be downregulation of amino acids with NAFC exposure, however, other amino acids increased in abundance. As amino acids are a diverse group of metabolites involved in numerous metabolic processes, it is perhaps unsurprising that there is no clear overall trend in amino acid levels. A study by Jia et al. (2023), observed a similar finding, that alterations in amino acid levels occurred in the grass *Phragmites australis* with NA exposure. In the future, it would be beneficial to investigate amino acids using targeted metabolomics to quantify differences and potentially to develop amino acid biomarkers for NAFC stress. Furthermore, there are likely other implications of alterations in amino acid metabolism, such as with energy metabolism or oxidative stress.

Energy metabolism appeared to be downregulated in the common whitetail with exposure to NAFCs. Through the pathway analysis of the identified metabolites, three pathways related to energy metabolism were indicated as altered in response to NAFCs. Furthermore, all the metabolites associated with these pathways decreased in abundance with NAFC exposure, suggesting the downregulation of energy metabolism. These results agree with past studies investigating NA exposure to aquatic organisms, where altered energy metabolism has been suggested as a potential mode of toxicity for NAFC exposure (Melvin et al., 2013; Zhang et al., 2023). Altered energy production is linked to multiple deleterious effects, such as decreased survival, altered reproduction, or altered growth (Goodchild et al., 2019), indicating that the observed pathway effects in the common whitetail could ultimately cause fitness effects and lead to population decline.

Oxidative stress may also contribute to toxicity from NAFCs in the common whitetail as my results indicated that metabolites related to protection from oxidative stress were altered with exposure to NAFCs. Oxidative stress occurs when the equilibrium between reactive oxygen species and their detoxification, generated through aerobic respiration in the mitochondria, is altered, resulting in damage to molecules within the organism (Lubawy et al., 2022). My findings align with prior studies observing that exposure to NAs may result in

oxidative stress and/or inhibit protection from oxidative stress (Gagné et al., 2012; He et al., 2012; Wang et al., 2015b). The metabolites related to oxidative stress driving differences among NAFC exposure levels were involved in pathways, such as alanine, aspartate, and glutamate metabolism, and are related to the metabolism of glutathione, an important antioxidant in oxidative stress (Liu et al., 2016). Furthermore, the metabolites tryptophan, and histidine were both lower in abundance among individuals exposed to the highest concertation of NAFCs. In previous studies, tryptophan has been found to be a biomarker for oxidative stress, while histidine is part of β-alanine metabolism contributing to protection from oxidative stress (Bala et al., 2021; Moro et al., 2020). However, further investigating the role of oxidative stress in NA toxicity, such as using genomics to monitor changes related to genes known to be associated with oxidative stress, would improve our understanding of NA toxicity, including impacts, such as sublethal or chronic stress.

The eastern pondhawk appeared to be insensitive to NAFC exposure at the investigated concentrations. There did not appear to be a clear trend in survival or the metabolome following exposure to NAFCs in the eastern pondhawk. However, the nMDS plot did appear to potentially show two groups, that were unrelated to NAFC exposure (**Figure 6**). This separation could indicate that different species were assessed as this organism was only identified to the genus level and it has previously been documented that metabolomics is sensitive enough to distinguish between closely related taxa (Burlikowska et al., 2020; Zelentsova et al., 2022). Alternatively, it is possible that other factors, such as sex, could contribute to differences in the metabolome, and could potentially explain this separation (Zhang et al., 2021). To investigate this grouping in future studies, it may be beneficial to utilize other techniques, such as genomics, to identify if there were multiple taxa grouped together or to identify other potential contributing factors. Alternatively, it is possible that exposing organisms to higher concentrations may overwhelm these other effects and thus reveal changes occurring with NAFC exposure.

4.2 Assessing Naphthenic Acid Fraction Component Toxicity Through Traditional Toxicity Endpoints

There was approximately a 2-fold increase in egg laying for ramshorn snails exposed to the higher concentrations of NAFCs. Consequently, the increased egg laying may be a stress response. Increased reproduction with contaminant exposure is consistent with ecological theory, where organisms under stress reallocate resources to ensure reproduction by investing more resources into a lower number of offspring or increasing the number of offspring, such as by increased egg laying (Cassill, 2019). Previous research has also observed increased egg laying with exposure to contaminants, such as that of Das & Khangarot (2011), where an increase in egg laying with copper exposure was observed, or that of Tripathi & Singh (2004) where *Lymnaea acuminata* snails exposed to pesticides (cypermethrin and alphametrin) had increased egg laying with higher concentrations of exposure. However, other previous findings suggest that egg laying increases at low levels of contaminant exposure and decreases at high levels of exposure (Czech et al., 2001; Seeland et al., 2013). Only one study has investigated the impacts of NAs to snail (*Lymnaea stagnalis*) egg laying, finding that for commercial NAs, exposure above 25 mg/L resulted in a decrease in egg masses laid, as well as a decrease in hatching (Johnston, 2015). Thus, prior research suggests that egg laying as a stress response may depend on factors, such as taxa, concentration, and the contaminant itself (Das & Khangarot, 2011; Seeland et al., 2013; Tripathi & Singh, 2004). Some of the aforementioned studies also observed decreased viability despite the increase in egg laying (Das & Khangarot, 2011; Seeland et al., 2013). Egg viability was not assessed in this study, and thus, the fitness implications are currently unknown. If the experiment were to be repeated, it would be useful to assess the viability of egg masses, as well as investigating a wider range of NAFC concentrations, to assess the effects of both chronic and acute NAFC toxicity on egg mass laying.

Although the increase in egg laying suggests that sublethal stress may be occurring, the metabolomes of the assessed ramshorn snails did not change in response to NAFC exposure. This is perhaps surprising as metabolomic changes are considered precursors to

changes in traditional fitness measures, such as fecundity (Dumas et al., 2022; Lankadurai et al., 2013). The lack of correspondence between egg laying and the metabolome may be because only polar metabolites were assessed in my study. Non-polar metabolites, such as fatty acids and membrane lipids, which are important metabolites for reproduction (Kirkwood et al., 2013), were not assessed in my study. For snails, lipoproteins constitute a large proportion of egg composition and previous studies have demonstrated that exposure to pesticides can reduce survival and egg laying, as well as altering the lipid and fatty acid profiles of snails (Bakry et al., 2016; Garin et al., 1996). Moreover, it is known that NA exposure can alter the lipid bilayer resulting in cell death through narcosis (Frank et al., 2008). It is therefore possible that by investigating non-polar metabolites a metabolomic response may be observed in ramshorn snails at the tested NAFC concentrations. Indeed, a previous study by Pomfret et al. (2021) observed that *Hexagenia* spp. mayflies exposed to low concentrations of commercial NAs displayed alterations in the lipid metabolome. Thus, assessing the non-polar metabolites would be a useful next step to understand the increase in egg-laying observed in this study.

Survival of common whitetail decreased with exposure to NAFCs, however, there was no clear trend across exposure levels. While NAFC concentrations were selected to limit impacts to organism survival to allow for metabolomic analysis, the common whitetail displayed a survival response at medium concentration only. Considering toxicity dose-response curves, one would expect that with increasing contaminant concentrations, an increased response would be observed (Hartung, 1987). In contrast, I found no clear difference in survival was observed at the highest concentration. Therefore, it is possible that other factors, such as intraspecies competition led to deaths during the experiment, as opposed to NAFC exposure.

4.3 Comparing the Base Metabolomes Among Taxa

The amount of metabolomic variation among taxa was associated with taxonomic relatedness. While perhaps unsurprising, the metabolomes of both dragonflies were more similar to each other than to snails. However, differences still existed between dragonflies, despite being more closely related (i.e., same family). My findings indicate that metabolomics is sensitive enough to distinguish between even related taxa; a finding consistent with prior work that used metabolomics to distinguish between species or even strains of the same taxon (Burlikowska et al., 2020; Zelentsova et al., 2022). This is an important consideration for metabolomics in toxicity testing as this suggests that there are differences in the metabolome even between related taxa. Thus, it may be more beneficial to investigate specific metabolites driving differences than to compare compare the response of the whole metabolome of different taxa.

Metabolites driving differences between taxa were primarily amino acids or related to amino acid metabolism. As amino acids are related to numerous metabolic pathways, this is perhaps unsurprising. My finding agrees with prior research, that amino acid levels were more similar among related taxa, and interestingly, that some differences can be attributed to different functional feeding groups (FFGs) or could potentially be related to dietary amino acids (Bundy et al., 2008; Thera et al., 2020). Importantly, as amino acids appeared to be the main metabolites impacted by NAFC exposure, it is possible that differences in the metabolomic response of the different taxa (or lack thereof) are in part due to differences in the base metabolite levels of organisms. It is well known that metabolites that are low in abundance are more difficult to detect and identify, and the metabolomes of different organisms may respond differently to the same contaminant (Lankadurai et al., 2013; Lee et al., 2007). Thus, when comparing different taxa it is important to note that there may be alterations in some metabolites that are not captured in the analysis. By targeting specific metabolites or biomarkers for contaminant stress using techniques, such as targeted metabolomics with mass spectrometry, it is possible to reveal additional information or effects (Lee et al., 2007). There may be a benefit to assessing specific metabolites of interest

as it is possible that certain important metabolites may be missed in the analysis due to differences in abundance. It is possible that by analyzing the levels of metabolites altered with NAFC exposure in the common whitetail in the other investigated taxa, could reveal otherwise undetected effects in the snail or the eastern pondhawk.

4.4 Naphthenic Acid Fraction Component Composition

Over the course of the experiment, NAFC composition shifted towards more oxygenated compounds. During the experiment there was a shift from the less oxygenated O_2 compounds to O_3 and O_4 compounds. More oxygenated compounds are thought to be less toxic than O_2 species (Yue et al., 2015; Yue et al., 2016), and thus, there may have been a shift to lower toxicity over the course of exposure in my study. While it is well documented that factors, such as aging or degradation due to temperature or light exposure, can affect NA composition, changes to NA composition from aging typically takes years to decades to complete (Johnson et al., 2011). It is therefore unlikely that aging or light/temperature exposure shifted the composition in my 14-day experimental period. Therefore, other factors likely contributed to this change in composition, such as constant air bubbling resulting in free oxygen molecules reacting with the NAs. However, it is difficult to determine the reason for this change in composition without repeating the experiment with additional controls to better investigate the composition. These controls could include having some aquaria without bubblers, without light exposure, or without organisms in some aquaria, to test if these factors may have contributed. It may also be useful to sample the water more frequently to follow the changes more closely and to experiment with a larger range of NAFC concentrations. Regardless of the reason for this change in composition, it is possible that the shift towards less toxic compounds led to a decrease in toxicity to the investigated organisms. While low mortality was expected given the concentrations investigated, measured concentrations were lower than anticipated and this shift towards less toxic forms of NAs in the NAFC solutions may have further reduced toxicity in my study. For the common whitetail, a metabolomic effect was detected at the lowest investigated exposure level,

suggesting that the metabolome of this taxa may be extremely sensitive to NAFC exposure. This may also suggest that snail egg laying is highly sensitive to NAFC exposure, however, further investigation would be required to understand why this increase was observed.

4.5 Implications

When utilizing SSDs to assess the risks of NAs, it must be noted that sublethal responses may vary even among related taxa. Using SSDs is common in risk assessment for contaminants, where the responses of a wide range of taxa are assessed to statistically represent the greater ecosystem (Fox et al., 2021). In my study, the common whitetail was sensitive to NAFCs, while the eastern pondhawk, a species of the same family, did not appear to respond. This suggests that for the use of SSDs, many species must be investigated as using only a small number of taxa may not be indicative of the potential effects of NAFCs. This agrees with prior work suggesting that the usefulness of SSDs increases with the number of investigated taxa, as more sensitive organisms are likely to be assessed, allowing for the protection of a wider range of organisms (Fox et al., 2021; Raimondo et al., 2008). There is thus a remaining need for additional NAFC toxicity studies in a wide range of taxa to support the development of SSDs for NAFC toxicity.

Metabolomics appears to be useful for revealing information about affected metabolites and pathways, sublethal toxicity, and the potential environmental consequences of exposure to NAs. Through my metabolomic analysis, NAFC exposure appeared to alter amino acid abundance, energy metabolism, and protection from oxidative stress, demonstrating that metabolomics can be used to reveal information about NAFC toxicity. In the future, there is a remaining opportunity to reveal additional information about NAFC toxicity, such as by investigating energetically important metabolites in a targeted metabolomic analysis to better understand these alterations. It is certainly possible that the metabolome of the common whitetail is highly sensitive to NAFC exposure, and therefore may be a useful indicator species. However, further metabolomic analyses of NAFCs would

help to reveal organism sensitivity to these contaminants. While some prior studies have indicated sublethal or chronic effects of NAFCs, the mode of action, and impacts of these effects are relatively unknown (Arens et al., 2015; Melvin et al., 2013; Robinson et al., 2023). In snails, the amount of egg laying altered suggested that NAFC exposure is causing sublethal stress, however, no metabolomic response was observed. The non-polar metabolome may be useful for assessing metabolites related to egg laying (Kirkwood et al., 2013), and therefore, future studies should assess the non-polar metabolome in the ramshorn snail.

4.6 Limitations and Future Directions

Additional studies involving environmental metabolomics applied to NAFCs should be conducted to understand potential sublethal impacts of NAFCs. This current study is useful in helping to strengthen the knowledge base for NAFC toxicity, but there are still remaining knowledge gaps. Other studies have observed sublethal effects, however, the implications of these effects are not well understood and could potentially have long lasting effects, such as resulting in multigenerational changes over time (Cardoso et al., 2020; Robinson et al., 2023). Thus, a useful next step would be to perform sublethal-multigenerational studies to better understand the impacts of long term NAFC exposure. For example, utilizing experimental ponds with differing levels of NAFCs conducted over a long time period. Furthermore, most NAFC and NA toxicity research has focused on a small number of taxa (Reynolds et al., 2022). It was observed in this study that some taxa (such as the common whitetail) may be highly sensitive or some taxa (such as the eastern pondhawk) may be insensitive to NAFC exposure at the investigated concentrations. Therefore, further research to assess NAFC and NA toxicity in a wide range of different taxa would both improve the knowledge base of NAs, and aid in the development of toxicity standards through SSDs. In particular, assessing NAFC toxicity across numerous FFGs would potentially help to reveal which groups of organisms are of the highest risk.

Further studies investigating snail egg laying in response to NAFCs should be conducted to understand egg laying as a toxicity endpoint for NAFCs. While other studies have observed similar increases in egg laying at low levels of exposure to certain contaminants, generally, decreased fecundity is also observed (Das & Khangarot, 2011; Johnston, 2015; Seeland et al., 2013; Tripathi & Singh, 2004). As I did not assess egg viability, in the future it would be useful to assess this reproductive fitness component in the ramshorn snail with NAFC exposure. It is also possible that over a long exposure period, egg laying may not be consistent (Das & Khangarot, 2011). Thus, it would be useful to frequently (i.e., daily) count egg masses during the exposure period to understand how egg mass laying could change over time. It is also possible that at higher NAFC concentrations than investigated in this study, a decrease in egg masses would be observed, which would align with other studies where increased egg laying occurs at low levels of contaminant exposure, but decreases occur at high levels (Czech et al., 2001; Seeland et al., 2013). Thus, future studies should assess the effects of NAFC exposure to snail egg laying at a wider range of NAFC concentrations.

Investigating fitness indicators, such as growth, may improve the usefulness of the metabolome in bioassessment. While growth was measured in this experiment through mean mass per aquaria, the large number of deaths among certain taxa and error from water remaining on the organisms contributing to the mass made interpretation of these results unreliable (**Figure A1, Figure A2**). Previous studies, such as that of Robinson et al. (2023) and Melvin et al. (2013), indicated that growth is altered with exposure to NAFCs. Furthermore, altered growth is thought to have population-level effects and altered community structure, and is a common endpoint in toxicity as growth is considered a sensitive indicator (Cairns et al., 1993; Sprague, 1971). As a fitness indicator, growth has also been linked to metabolite changes and may be useful in demonstrating linkages between organism level changes , and metabolomic changes (Hines et al., 2010; Pomfret et al., 2021). Furthermore, growth is known to be linked to energy metabolism and as energy metabolism appeared to be altered with NAFC exposure in the common whitetail, it is possible that a

growth effect may occur (Sancho et al., 2009). Thus, it would be useful to include growth as an additional useful endpoint in the future. As there is a high level of uncertainty with mass due to water on the body of organisms (especially with small organisms), measuring organism length may be a useful substitute. By measuring organism length, length-mass relationships can be used to effectively estimate organism mass (Sabo et al., 2002). Thus, if this experiment were to be repeated, it may be useful to assess growth utilizing length-mass relationships to estimate organism mass in tandem with other endpoints, such as metabolomics, survival, or fecundity.

5.0 Conclusion and Summary

Naphthenic acids are known to be toxic to aquatic organisms (Hughes et al., 2017), but the potential sublethal effects are largely unknown. In this study, the common whitetail dragonfly showed promise of being a sensitive indicator of NAFC exposure as it displayed a metabolomic response even at low concentrations, while the metabolomes of other investigated taxa did not appear to respond. In addition, the number of eggs masses laid by the ramshorn snail were approximately double at higher levels of NAFC exposure. Despite this change in the amount of egg laying, no metabolomic effect was observed, and thus, further study should be conducted to better understand this mismatch. Variation in the metabolomes of control individuals was taxa specific, as the dragonflies were more similar to each other than to the snail. This suggests that it may be difficult to directly compare the response of the metabolome, and instead, be better to compare changes in the abundance of specific metabolites as slight changes in the abundance of metabolites may be metabolically important. Overall, there appeared to be sublethal effects of NAFCs in the common whitetail at the investigated concentrations. Therefore, the common whitetail may be a useful indicator for NAFC toxicity when developing standards. Furthermore, metabolomic response varied by taxa, and thus, when using metabolomics in toxicity testing many different organisms must be investigated. There remains a need for additional toxicity data to support the development of toxicity standards for NAFCs, and therefore investigating a wide range of NAFC concentrations (including sublethal levels) in a wide range of taxa would be useful to better understand the potential ecological impacts of NAs. In toxicity testing, metabolomics appears to be useful when used with other toxicity endpoints to detect potential stress and reveal information about toxicity in sublethal and chronic studies to evaluate the mode of action of the contaminant. However, further work must be completed to further develop the usefulness metabolomics in toxicity testing.

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Appendix

Supplemental Information

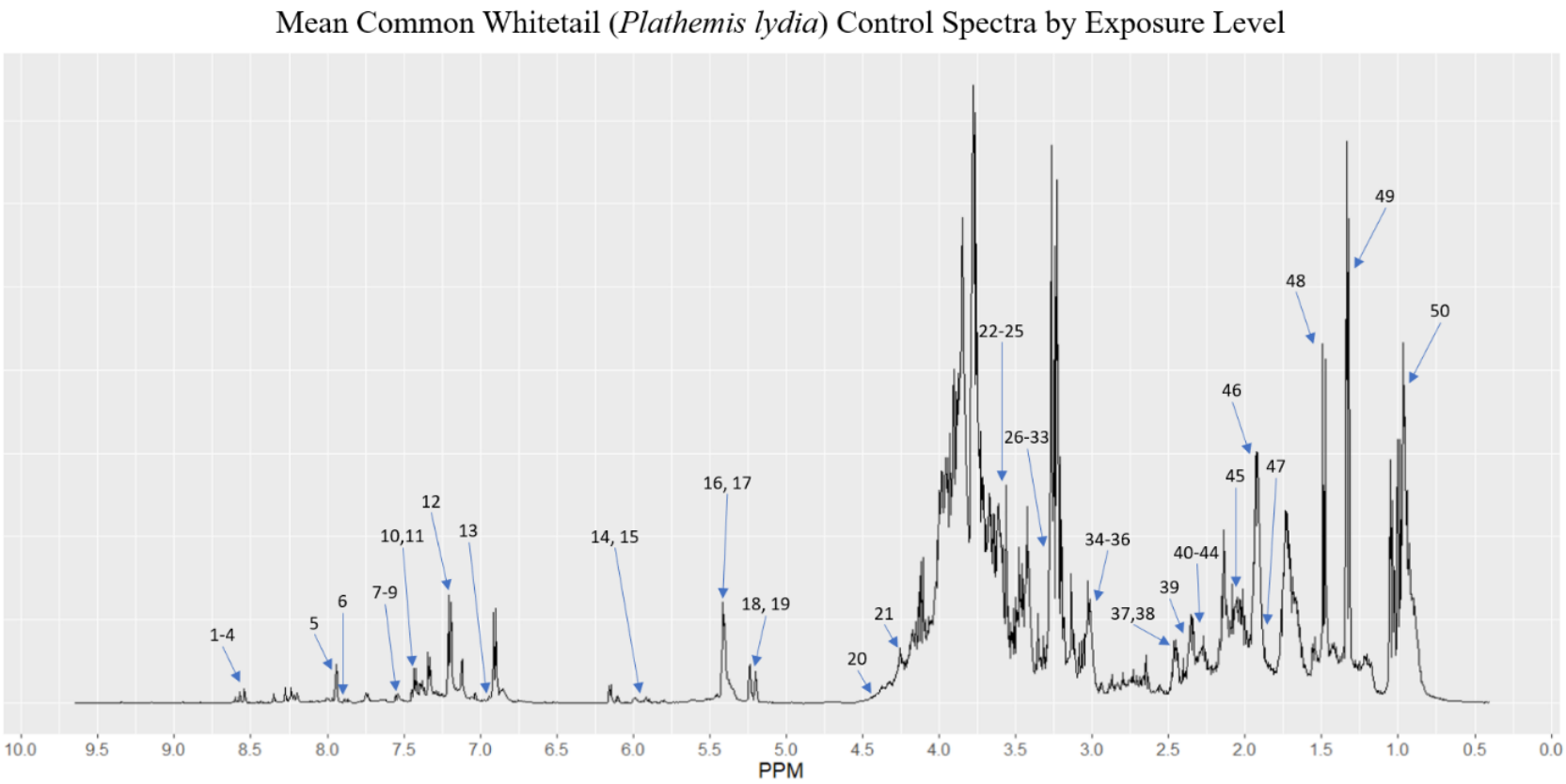

Figure A1 Overall mean Common Whitetail (*Plathemis lydia*) spectrum with 50 metabolites (regardless of VIP score) labelled based on metabolite peaks. Annotated metabolites: (1) AMP, (2) IMP, (3) ATP, (4) NADH, (5) riboflavin, (6) histamine, (7) τ-methylhistidine, (8) asparagine, (9) uracil, (10) melatonin, (11) phenylalanine, (12) tyrosine, (13) tyramine, (14) guanosine, (15) uridine, (16) sucrose, (17) maltose, (18) xylose, (19) trehalose, (20) adenosine, (21) threonine, (22) galactonate, (23) glycerol, (24)

choline, (25) glucose-6-phosphate, (26) carnitine, (27) tryptophan, (28) betaine, (29) cellobiose, (30) glucose, (31) lactose, (32) taurine, (33) trimethylamine n-oxide, (34) histidine, (35) 4-aminobutyrate, (36) agmatine, (37) 3-hydroxyisobutyrate, (38) glutamine, (39) 3-hydroxy-3-methylglutarate, (40) succinate, (41) pyruvate, (42) isobutyrate, (43) proline, (44) glutamate, (45) methionine, (46) lysine, (47) arginine, (48) alanine, (49) lactate, (50) valine.

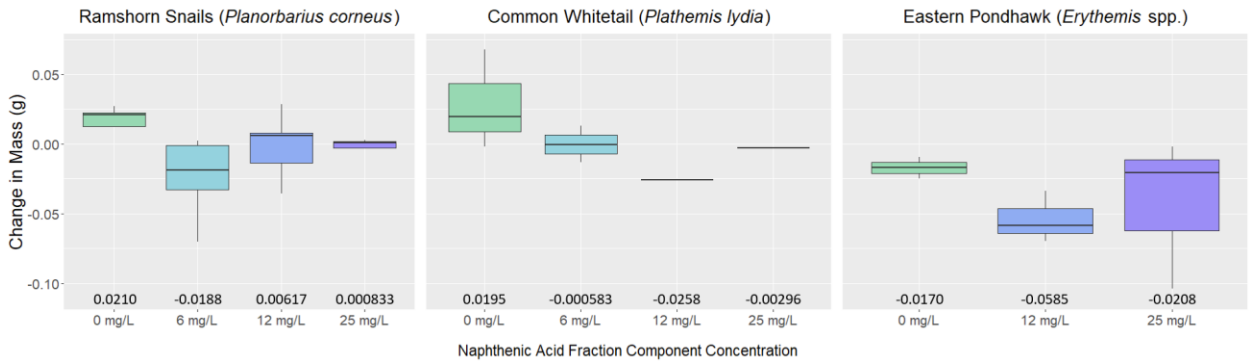

Figure A2 Box plot showing the change in mass (g) of taxa exposed to NAFCs. Due to excessive deaths for one treatment level in the common whitetail and likely error from remaining water in wet mass measurement, interpretation of data was not possible. Median change in mass shown at the bottom of plot.

Table A1. Bins identified with a VIP score > 1.25 for common whitetail dragonflies that could not be identified. Significant differences indicate differences in the identified spectral region's abundance between exposure levels (0, 6, and 25 mg/L) using Tukey's Honest Significant Difference (HSD) post-hoc analysis (FDR $\alpha = 0.1$).

Bin PPM	VIP Score	f value	p value	FDR	Significant Differences Between Exposure Levels (Tukey's HSD $\alpha = 0.1$)
3.6725	1.93	16.40	< 0.001	0.005	0 > 6 = 25
1.1175	1.84	15.11	< 0.001	0.008	0 > 6 = 25
3.6025	1.74	14.82	< 0.001	0.008	0 > 6 = 25
2.7125	1.68	9.14	0.001	0.017	0 > 6 = 25
2.7625	1.60	7.50	0.003	0.028	0 > 6 = 25
5.4525	1.54	6.63	0.005	0.037	0 > 6 = 25
3.4625	1.50	5.87	0.008	0.048	0 = 6 < 25
7.9425	1.35	4.42	0.023	0.087	0 = 6 < 25
0.8275	1.34	6.70	0.005	0.036	0 > 6 = 25
7.2225	1.34	4.43	0.023	0.087	0 = 6 > 25
1.3875	1.32	4.21	0.027	0.095	0 = 6 > 25
7.2575	1.29	5.38	0.012	0.059	0 < 6 = 25
0.8025	1.27	4.49	0.022	0.085	0 > 6 = 25

Table A2. Bins identified when analyzing differences in the metabolomes of the ramshorn snail (s), and the common whitetail (CW) and the eastern pondhawk (EP) dragonflies with a VIP score > 1.25 which could not be identified. Significant differences indicate the relative abundance among taxa in the identified spectral region using Tukey's Honest Significant Difference (HSD) post-hoc analysis ($\alpha = 0.1$).

Bin PPM	VIP Score	f value	p value	FDR	Significant Differences Between Taxa (Tukey's HSD $\alpha = 0.1$)
0.8325	1.47	164.14	< 0.001	< 0.001	S > CW > EP
0.8175	1.45	128.48	< 0.001	< 0.001	S > CW = EP
1.2525	1.45	127.06	< 0.001	< 0.001	S > CW = EP
4.3725	1.45	121.04	< 0.001	< 0.001	S > CW = EP
7.2625	1.42	103.74	< 0.001	< 0.001	S < CW = EP
4.0475	1.41	116.59	< 0.001	< 0.001	S > CW > EP
6.8225	1.40	83.62	< 0.001	< 0.001	S > CW = EP
6.9575	1.39	76.18	< 0.001	< 0.001	S > CW = EP
1.9325	1.38	86.57	< 0.001	< 0.001	S < CW = EP
1.1075	1.37	66.75	< 0.001	< 0.001	S > CW = EP
2.4875	1.36	62.37	< 0.001	< 0.001	S > CW = EP
2.6075	1.36	63.26	< 0.001	< 0.001	S > CW = EP
5.5175	1.356	61.94	< 0.001	< 0.001	S > CW = EP
4.0875	1.35	69.39	< 0.001	< 0.001	S > CW > EP
2.7775	1.34	63.59	< 0.001	< 0.001	S > CW = EP
1.1575	1.32	58.20	< 0.001	< 0.001	S > CW = EP
2.9125	1.30	47.57	< 0.001	< 0.001	S > CW = EP
3.1875	1.30	62.19	< 0.001	< 0.001	S < CW < EP
7.1475	1.30	49.53	< 0.001	< 0.001	S > CW = EP

5.9825	1.28	42.87	< 0.001	< 0.001	S > CW = EP
0.7175	1.28	44.79	< 0.001	< 0.001	S > CW = EP
4.0175	1.28	41.75	< 0.001	< 0.001	S > CW = EP