

Abstract

The suitability of the crayfish metabolome as a bioindicator of stream conditions was investigated because there is a need for fast responding, sensitive, diagnostic, and ecologically relevant bioindicators. A laboratory study assessed *Procambarus clarkii* metabolome sensitivity to six different treatments of food or DO stress. A field study in subcatchments of the Red River Valley, Manitoba, Canada assessed the effects of land-use type (reference, agriculture, wastewater effluent) on the *Orconectes virilis* metabolome. Crayfish were sacrificed and hepatopancreas, gill, and tail tissues were dissected. Analyses found the metabolome was sensitive enough to potentially diagnose food and DO stress in *P. clarkii* tail muscle. *Orconectes virilis* gill metabolomes were the most sensitive to land-use treatments associated with substantive differences in the stressor environments. Although this study shows promise in using the crayfish metabolome in bioassay monitoring, further metabolomics studies are needed to assess the predictability and ecological relevance of crayfish metabolic changes.

Keywords: crayfish, bioindicator, metabolomics, *Procambarus clarkii*, *Orconectes virilis*, food, dissolved oxygen, agriculture, wastewater effluent

Co-Authorship Statement

This thesis contains two manuscripts. Natalie M. Izral, will be the first author of both manuscripts because of her pivotal role in identifying the research objective, implementing the research design, and analyzing and interpreting the collected data. Robert B. Brua will be second author for both manuscripts as he co-advised all aspects of the research and provided funding. For their advice and provision of funding, Joseph M. Culp and Patricia A. Chambers will be co-authors in both manuscripts. The last author will be Dr. Adam Yates who acted as the principal advisor during the studies and provided the majority of the funding.

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List of Abbreviations

^1H NMR – proton nuclear magnetic resonance

AGR – agriculture

ATP – adenosine triphosphate

CoA – coenzyme A

CV – coefficient of variance

DO – dissolved oxygen

GDH – glutamate dehydrogenase

GLM – general linear model

LOD – level of detection

LOOCV – leave one out cross validation

MANOVA – multivariate analysis of variance

NADH – nicotinamide adenine dinucleotide

NH_3 – ammonia

NMR – nuclear magnetic resonance

$\text{NO}_2 + \text{NO}_3$ – nitrate + nitrite

PCA – principal component analysis

PLS-DA – partial least squares discriminant analysis

PPCP – pharmaceutical and personal care product

REF – reference

SD – standard deviation

SFG – scope for growth

SRP – soluble reactive phosphorous

TCA – tricarboxylic acid

TDP – total dissolved phosphorous

TMSP – trimethylsilyl propanoic acid

TN – total nitrogen

TP – total phosphorous

TSS – total suspended solids

VIP – variable importance in projection

WWT – wastewater treatment

Chapter 1: General Introduction

1.1 Literature Review

Aquatic biomonitoring is the ongoing evaluation and assessment of aquatic ecosystem responses to external or anthropogenic stressors (Li et al., 2010).

Traditionally, aquatic biomonitoring programs have used water quality measurements and bioindicators to monitor system responses to in-stream stressors. Hunsaker and Carpenter (1990) define a bioindicator as a “measurable, environmental characteristic that quantifies magnitude of stress, degree of exposure to the stressor, and degree of ecological response to the stressor.” Desirable characteristics of bioindicators of environmental or water quality have been described in numerous papers (*sensu* Hammons, 1981; Suter, 1989; Hunsaker and Carpenter, 1990; Kerr, 1990; Cairns et al., 1993). They include the following: 1) measurability; 2) sensitivity to stressors; 3) broadly applicable to other stressors and regions; 4) diagnostic of the stressor causing the problem; 5) predictable or anticipatory of future conditions; 6) biologically relevant, i.e., important in maintaining ecosystem integrity; and 7) socially relevant, i.e., addresses concerns of stakeholders.

A common type of bioindicator is a sentinel species, defined as species whose abundance and relative well-being indicate the overall environmental health of an ecosystem (Johnson et al., 1993). It is important that biomonitoring programs utilize sentinel species as indicators of aquatic stress because physiological changes and bioaccumulation of pollutants are often the earliest warnings of adverse environmental conditions (Johnson et al., 1993). Desirable characteristics of sentinel species chosen as indicators of water quality have been described (*sensu* Rosenberg and Wiens, 1976;

Ryder and Edwards, 1985; Hellawell, 1986; Johnson et al., 1993) and include the following: 1) taxonomic soundness, i.e., species are clearly distinguishable; 2) a cosmopolitan distribution; 3) numerical abundance; 4) large body size; 5) limited mobility; 6) relatively long lifespan; and 7) low genetic variability. Sentinel species should also have narrow and specific physical, nutritional, or chemical tolerances. Thus, when environmental variables exceed a species' tolerance threshold, changes in, and regulation of, population dynamics can occur, resulting in meaningful and measurable changes at higher levels of biological organization (Johnson et al., 1993). In aquatic biomonitoring, selection of unique bioindicators and use of sentinel organisms is important in evaluating and monitoring an aquatic system before more permanent and socially impactful damage at the ecosystem level occurs.

Biomonitoring tools traditionally fall under one of two approaches (Munkittrick and McCarty, 1995). The first approach is toxicological in nature and utilizes a bottom up methodology that examines the relationships between stressors and specific biological responses at one or two lower levels of biological organization (i.e., cell or tissue levels). Responses to stress, including biochemical and physiological responses of individual organisms, occur quickly at these lower levels of biological organization (Kingett, 1985; Mitin, 1985; Graney and Giesy, 1986). Thus, changes in amino acid and protein content, ion regulation, and respiration rate can act as biomarkers and provide early warnings of adverse effects of aquatic stressors (Johnson et al., 1993). Although biomarkers provide early warning of aquatic stressors, it is difficult to extrapolate biochemical and physiological responses to higher levels of biological organization (Munkittrick and McCarty, 1995; Adams and Greeley, 2000). Stress results in alteration of biochemical or

physiological processes but may not result in whole organismal change. Therefore, only ecologically relevant changes (i.e., reproduction or growth) are reflected at population or community levels (Cairns et al., 1993; Munkittrick and McCarty, 1995). Moreover, laboratory studies most often only measure biochemical or physiological responses to environmental stress that may not be representative of in-stream, natural conditions (Munkittrick and McCarty, 1995; Adams and Greeley, 2000).

The development of toxicological biomonitoring tools has led to the identification of a wide range of biomarkers (Martinez-Haro et al., 2015). Well established biomarkers include the induction of metallothioneins (MT) and metallothionein-like proteins when benthic invertebrates are exposed to heavy metals (Amiard et al., 2006; Martinez-Haro et al., 2015). A change in crayfish gill protein carbonyl concentration is a biomarker of copper and beta-cypermethrin contamination (Wei and Yang, 2015). Matozzo et al. (2008) found that vitellogenin levels change in aquatic invertebrates exposed to estrogenic stress. Changes in amino acid levels (e.g., methionine) and DNA damage are biomarkers of wastewater effluent in fish and molluscs (Wepener et al., 2005). The above biomarkers are only a miniscule fraction of discovered biomarkers of environmental stress in aquatic fish and invertebrates. These biomarkers are important in quickly assessing damage at cellular, tissue, and organ levels, but more research needs to be done to identify their relevance at higher levels of biological organization.

The second approach to biomonitoring is more ecological in nature and uses a top down methodology to studying environmental impacts (Munkittrick and McCarty, 1995). In the top down approach, changes at higher levels of biological organization (i.e., the population and community levels) are determined and related to an underlying cause at

lower levels of biological organization using exposure indicators. Changes at the community and population levels provide a holistic view of the ecosystem and are, therefore, more ecologically relevant than lower levels of biological organization (Adams and Greeley, 2000). However, ecological studies conduct field experiments where anthropogenic and natural stressors interact to create complex systems; thus, there can be uncertainty in linking cause and effect at the higher levels of biological organization when multiple stressors are present (Munkittrick and McCarty, 1995; Adams and Greeley, 2000). Changes at the community or population level may not link back to causative agents at the biochemical level or the underlying aquatic stressor (Munkittrick and McCarty, 1995).

The loss of specificity for the gain of ecological relevance in the top down approach has been documented. For example, Johnson et al. (2006) found that major community assemblages (i.e., benthic diatoms, fish, macroinvertebrates, and macrophytes) are sensitive to different stressors, but there is a high percentage of error for all individual metrics. Likewise, Clapcott et al. (2012) investigated the suitability of multiple structural and functional metrics (e.g., nitrate + nitrite, macroinvertebrate community index) for assessing land-use effects on ecological integrity of New Zealand streams and found not all indicators were sensitive enough to link back to an underlying stressor. Allan (2004) argued that although land use and other anthropogenic stressors affect stream ecosystems, anthropogenic and natural factors are confounded and difficult to bridge to corresponding effects. Thus, exposure indicators are important in evaluating factors affecting ecosystem health, but it is difficult to connect a specific effect to a specific stressor in a complex system with cumulative effects.

In order to better identify causal relationships between a specific stressor and an ecologically relevant response in systems with multiple stressors, early warning biomarkers (e.g., biochemical responses such as an acid change) and slower responding but ecologically important exposure indicators (e.g., community responses such as presence or absence of a species) of stress need to be used in tandem (Adams and Greeley, 2000; Adams, 2001). Using indicators across levels of biological organization helps mitigate problems associated with bottom up or top down approaches. Many studies have acknowledged the need to use a combination of indicators across levels, but few studies have actually investigated the relationship between biomarkers and exposure indicators. Those studies that have been done have shown promise in extrapolating early warning and diagnostic responses across levels of biological organization. Adams et al. (2000) found that biomarkers (e.g., MFO enzymes, organ dysfunction) and exposure indicators (i.e., age structure and growth of male sunfish, relative population size, Index of Biotic Integrity) responded similarly when fish were exposed to point source discharge of various contaminants. Martinez-Haro et al. (2015) reviewed European Water Framework Directive (WFD) studies and found that some studies were able to correlate biochemical and cellular biomarkers with exposure indicators (e.g., community assemblages and metrics) in fish and aquatic invertebrates exposed to varying contaminants. Moreover, the most promise in establishing causal relationships between biomarkers and exposure indicators has been in studies that have used a combination of laboratory and field experiments. Clements et al. (2002) used field and lab experiments to validate a cause-effect relationship between heavy metal exposure and reduced species richness. Triebskorn et al. (2001) not only found that cellular changes were related to

effects at the fish population and invertebrate community levels in organisms exposed to agriculture and wastewater, but was also able to identify the effects of specific stressors (chemical pollutants) driven by the varying land usages. Because of the different sensitivity of biomarkers and ecological indicators, an integrative bioassessment approach is needed to establish indicators that are early warning, diagnostic, and ecologically relevant.

Many studies that use multiple bioindicators still cannot establish definite relationships between ecologically relevant endpoints (e.g., individual survival, growth and reproduction) and stressors. These studies lack information on what specific metabolic pathway disruptions or interactions lead to these ecologically important endpoints or biological responses (Lin et al., 2006). By identifying the effects of specific stressors on metabolic pathways, researchers can manage stressors before the reversible metabolic disruptions cause irreversible damage to community or population dynamics. Thus, in order to more quickly, holistically, and definitively predict the influence of aquatic stressors on higher levels of biological organization, normal and abnormal cellular regulatory processes (i.e., metabolic pathways) of individuals need to be known. The field of metabolomics has shown promise in creating stronger relationships between biomarkers and higher level indicators.

Metabolomics is the study of metabolite profiles of cells, tissues, fluids, and other biological samples. Metabolites are small, low molecular weight compounds (i.e., sugars, amino acids, lipids) that serve as substrates and products in metabolic pathways (Lin et al., 2006; Miller, 2007; Viant, 2008). The entire compilation of metabolites is called the metabolome and is essentially a metabolic fingerprint or snapshot of an organism's

condition (Lin et al., 2006; Jones et al., 2013; Lankadurai, 2013). The metabolic fingerprint can be altered by environmental changes, and through metabolomics these changes are recorded and associated with biological functions/cellular regulatory pathways (Lin et al., 2006). One analytical tool in metabolomics is Nuclear Magnetic Resonance (NMR) spectroscopy. Through NMR the metabolic fingerprint is recorded with well-resolved peaks that correspond to individual metabolites as well as cellular regulatory pathways (Lin et al., 2006; Miller, 2007). NMR uses pattern recognizing algorithms to distinguish normal metabolite profiles/pathways from abnormal perturbations (Miller, 2007). Thus, NMR will detect when a change of peak height acts as a biomarker of some adverse or abnormal environmental condition; relationships can be established between metabolite levels/cellular response and chemical/nutritional stimuli or other stressors (Miller, 2007). For example, extensive studies on mice and rats have detected damage to liver, lung, and kidney function in response to toxicological stress (Coen et al., 2004; Azmi et al., 2005; Bollard et al., 2005; Waters et al., 2006). Studies assessing effects of temperature elevation and anoxia on fish embryos have identified metabolic variations related to development and accumulation of large amounts of γ -aminobutyrate that protects neural function (Podrabsky et al., 2007; Turner et al., 2007). The application of metabolomics to environmental assessment is still in its infancy, but the past decade has seen an increase in the number of ecologists and toxicologists utilizing this type of analysis (Lankadurai, 2013).

Viant (2008) defines 'environmental metabolomics' as the metabolic characterization of the interactions of organisms with their environment, while Miller (2007) describes 'environmental metabolomics' as any large metabolic data set that

describes the effects of chemicals and other stressors in the environment on a biological system. The majority of current literature on environmental metabolomics falls under three categories: 1) toxicant exposure and risk assessment; 2) response to environmental stressors; and 3) disease monitoring (Lin et al., 2006). These studies include the following: 1) identifying indicators of metal bioaccumulation in aquatic and terrestrial invertebrates; 2) monitoring metabolomic changes in human development due to diseases; and 3) assessing chemical changes in biofluids due to anthropogenic toxicants (Tomanek, 2011; Campos et al., 2012).

The potential for metabolomics to identify valid biomarkers and establish linkages between biomarkers and higher levels of biological organization has resulted in studies testing metabolomics as indicators of ecosystem health. About half of all environmental metabolomics research has been conducted with earthworms as potential indicators or monitors of soil ecosystem health (e.g., Brown et al., 2009; Rochfort et al., 2009; Simpson and McKelvie, 2009; McKelvie et al., 2011). Rochfort et al. (2009) found that earthworms exposed to conventional soil treatment experienced elevated sugar and triglyceride levels, changes that have been seen previously in earthworms affected by environmental stressors (e.g., toxins). Additionally, earthworms exposed to an array of organic compounds (i.e., pesticides, pharmaceuticals, organohalogenes, industrial compounds) experienced contaminant-specific metabolic responses (McKelvie et al., 2011). The extensive metabolomics study of earthworms has identified metabolic biomarkers of soil stress, which may make the earthworm metabolome a suitable indicator of soil conditions. Exploration of the potential of metabolomics as an indicator of ecosystem health in aquatic environments is more limited than in terrestrial

environments. However, research on aquatic organisms has shown promise.

A number of laboratory studies have identified and described metabolic changes in freshwater and marine organisms exposed to dose-dependent toxicological stress (e.g., heavy or trace metals, pesticides, pharmaceutical products) (Samuelsson et al., 2006; Viant et al., 2006; Ekman et al., 2008; Katsiadaki et al., 2010; Hines et al., 2010; Liu et al., 2011; Samuelsson et al., 2011; Southam et al., 2011; Ji et al., 2015; Li et al., 2015). For example, rainbow trout exhibited noticeable changes in vitellogenin (VTG), alanine, phospholipids, and cholesterol levels in blood plasma after exposure to 10 ng/L of synthetic estrogen 17 α -ethynylestradiol (EE2) (Samuelsson et al., 2006). Eyed eggs and alevins of Chinook salmon exposed to different concentrations of the pesticides diazinon, dinoseb, and esfenvalerate experienced decreases in adenosine triphosphate (ATP) and phosphocreatine (Viant et al., 2006). Ekman et al. (2008) exposed male and female fathead minnows to two levels of EE2 with the most noticeable changes occurring in metabolites associated with energy metabolism (e.g., glycogen, lactate) and liver toxicity (e.g., bile acids); males produced more amino acids associated with VTG synthesis. Hepatopancreas of clams exposed to mercury expressed changes in metabolites associated with osmoregulation (e.g., betaine, glycine) and the nervous system (i.e., glutamate) (Liu et al., 2011). Southam et al. (2011) found that aquatic roaches exposed to toxic doses of fenitrothion experienced changes to the hepatic phosphagen system and phenylalanine system in liver and testes. Li et al. (2015) found that levels of metabolites involved in amino acid metabolism, energy metabolism (e.g., lactate), and osmoregulation (e.g., betaine) changed in goldfish (brain, kidney, liver, plasma) exposed to avermectin (AVM). Therefore, laboratory studies have identified dose-dependent

biomarkers diagnostic of a variety of toxicological stressors that are commonly found in polluted aquatic ecosystems; the altered metabolites are involved with a variety of metabolic pathways that may have implications at the organismal level.

Additionally, aquatic field studies have detected metabolic changes associated with anthropogenic stressors and management actions (Fasulo et al., 2012; Kwon et al., 2012; Davis et al., 2013; Skelton et al., 2014; Campillo et al., 2015; Watanabe et al., 2015). Hepatopancreas of caged mussels in coastal waters polluted with mercury and polycyclic aromatic hydrocarbons (PAHs) showed an increase in amino acids and altered levels energy metabolites (e.g., lactate, acetoacetate) (Fasulo et al., 2012). Livers of caged male fathead minnows showed diverse metabolic changes after exposure to pulp and paper mill (PPM) effluent (Davis et al., 2013). Skelton et al. (2014) placed caged fathead minnows in rivers exposed to varying types of land use (i.e., wastewater treatment plant (WWTP), agriculture, control); hepatic metabolite analysis indicated significant differences among the three different sites. Campillo et al. (2015) found that hepatopancreas of caged clams in a lagoon with anthropogenic influence showed a change in metabolites involved in energy metabolism, osmoregulation, and lipid metabolism. Field studies have identified metabolites sensitive to in-stream anthropogenic conditions, which has greater applicability to bioassessment programs and management.

Metabolomics in aquatic studies is relatively new, but past studies have shown that: 1) metabolomics can diagnose dose-dependent toxicological pollutants; 2) the metabolome is sensitive to changes in anthropogenic conditions *in situ*; 3) metabolomics is applicable to a wide range of organisms, tissues, and regions; and 4) the metabolome is

measurable. However, the above studies did not assess the ecological significance or predictability of metabolomics. Studies did not measure whether the changed metabolite levels were predictive of metabolic pathway disruption and organismal change that can lead to altered higher levels of biological organization (*sensu* Southam et al., 2011). A laboratory study by Hines et al. (2010) demonstrated the utility in using metabolomics to identify metabolic biomarkers that can predict physiological toxicity at the organism level. Mussels were exposed to copper and pentachlorophenol (PCP), and physiological responses established scope for growth (SFG) as a measurement of organism energetic fitness. Biomarkers predictive of SFG (e.g., methionine, allantoin) were discovered and predicted reduced fitness of mussels exposed to contaminated waters. The study by Hines et al. (2010) is highly significant in that the discovered biomarkers were relevant to organismal responses (e.g., growth), which increases its value in aquatic biomonitoring. If a large enough percentage of organisms are affected by the aquatic stressor, including copper and PCP contaminants, the response of these stressors may be subsequently measured at higher levels of biological organization (e.g., changes in population size, alterations in community structure) (Adams, 2003). Therefore, the study by Hines et al. (2010) discovered metabolic biomarkers that are ideal bioindicators; they measure rapidly changing effects at the biochemical level, but they are also able to create causal relationships between the copper and PCP stressors and higher levels of biological organization through SFG measurements.

Research on metabolomics in aquatic bioassessment is making progress; however, a large knowledge gap remains. Metabolomics is sensitive to gender, reproduction, species, and tissue (Viant, 2007; Eckman et al., 2008; Liu et al., 2011; Jones et al., 2013;

Shen et al., 2014; Ji et al., 2015), and many studies have only addressed a few of these differences in a handful of organisms. For example, many benthic invertebrates, such as the crayfish, have not been used before in metabolomics studies. Also, while organ-specific metabolic responses have been reported, not all metabolomics studies analyze multiple tissues. Rosenblum et al. (2005) found that food limitation caused a decrease in metabolite levels in shellfish foot muscle, but the metabolite levels remained constant or increased in the digestive gland. Abductor muscle and mantle of mussels were both capable of identifying metabolic responses to copper and PCP, but abductor muscle was more predictive in a study by Hines et al. (2010). Hence, individual organs within a sentinel organism need to be investigated for sensitivity as well. Moreover, few lab studies have assessed metabolite sensitivity to, and diagnosis of, altered natural in-stream conditions (e.g., dissolved oxygen (DO), food availability, salinity, temperature; Tuffnail et al., 2009; Wagner et al., 2015). Few field studies have assessed the impact of land-use activities on the metabolome of organisms, and those that have been done cannot definitively attribute metabolite changes to a specific stressor (Davis et al., 2013; Skelton et al., 2014). Thus, a laboratory study on in-stream conditions should be conducted in conjunction with a field study on land use. The main objective of this thesis was to conduct lab and field studies to investigate the suitability of the crayfish metabolome as an indicator of stream conditions. The secondary goal was to evaluate the relative metabolic response of hepatopancreas, gill, and tail tissues to aquatic stress.

1.2 Study Goals and Objectives

The overall goal of this thesis was to assess the suitability of the crayfish metabolome as a bioindicator of stream conditions. This thesis consisted of two parts: a laboratory study to identify crayfish metabolome sensitivity to food or dissolved oxygen (DO) stress (chapter 2) and a field study to assess the response of the crayfish metabolome to the removal (recovery) or addition (impact) of land-use associated stressors (chapter 3). Each component had specific goals and objectives.

Part 1. Laboratory study

Goal:

To assess the suitability of the crayfish metabolome as an indicator of food or DO stress through controlled laboratory experiments

Objectives:

1. identify crayfish metabolites sensitive to changes in food availability
2. identify crayfish metabolites sensitive to changes in DO concentrations
3. compare significant crayfish metabolites between the food and DO experiments
4. compare the sensitivity of three different tissues (i.e., hepatopancreas, gill, tail) to food availability
5. compare the sensitivity of three different tissues (i.e., hepatopancreas, gill, tail) to DO stress

Predictions:

1. the metabolome of starved crayfish will be significantly different from the metabolome of fed crayfish

2. the metabolome of crayfish exposed to low DO concentrations will be significantly different from the metabolome of crayfish exposed to high DO concentrations
3. the crayfish metabolome will be significantly different among hepatopancreas, gill, and tail tissues in the food study
4. the crayfish metabolome will be significantly different among hepatopancreas, gill, and tail tissues in the DO study

Part 2. Field study

Goal:

To comparatively assess the effects of three land-use types (natural vegetation (reference), agriculture, wastewater) within a catchment on the crayfish metabolome

Objectives:

1. measure water physicochemical characteristics (i.e., temperature, nutrients, dissolved oxygen and pharmaceutical and personal care products (PPCPs)) at natural, agricultural, and wastewater sites
2. identify specific crayfish metabolites sensitive to land-use recovery treatments
3. identify specific crayfish metabolites sensitive to land-use impact treatments
4. compare the sensitivity of three different tissues (i.e., hepatopancreas, gill, tail) to recovery treatments
5. compare the sensitivity of three different tissues (i.e., hepatopancreas, gill, tail) to impact treatments

Predictions:

1. the metabolome of crayfish transferred from an agricultural site to a reference site will be significantly different from the metabolome of crayfish exposed to agriculture
2. the metabolome of crayfish transferred from a wastewater site to an agricultural site will be significantly different from the metabolome of crayfish exposed to wastewater effluent
3. the metabolome of crayfish transferred from a wastewater site to a reference site will be significantly different from the metabolome of crayfish exposed to wastewater effluent
4. the metabolome of crayfish transferred from a reference site to an agricultural site will be significantly different from the metabolome of crayfish exposed to natural vegetation
5. the metabolome of crayfish transferred from an agricultural site to a wastewater site will be significantly different from the metabolome of crayfish exposed to agriculture
6. the metabolome of crayfish transferred from a reference site to a wastewater site will be significantly different from the metabolome of crayfish exposed to natural vegetation
7. the crayfish metabolome will be significantly different among hepatopancreas, gill, and tail tissues in the recovery treatments
8. the crayfish metabolome will be significantly different among hepatopancreas, gill, and tail tissues in the impact treatments

1.3 References

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Chapter 2: An Assessment of the Sensitivity of the Crayfish Metabolome to Food and Dissolved Oxygen Stress

2.1. Introduction

Anthropogenic activities drive in-stream conditions that cumulatively contribute to adverse biological impacts (Adams, 2001; Malmqvist and Rundle, 2002; Adams, 2003; Allan, 2004). Aquatic assessment and monitoring programs have generally relied upon bioindicators based on community or population responses to evaluate causal relationships between in-stream conditions and biological responses (Culp and Baird, 2006). Although exposure indicators at higher levels of biological organization are ecologically significant (e.g., Allan, 2004; Johnson et al., 2006; Resh, 2008; Gallardo et al., 2011, Clapcott et al., 2012), they are slow responding and difficult to relate back to a specific in-stream stressor (Cairns et al., 1993; Munkittrick and McCarty, 1995; Adams and Greeley, 2000). In contrast, organismal indicators, such as biomarkers, better attribute a specific dose dependent stressor to a biochemical or physiological response in an organism (e.g., Johnson et al., 1993; Wepener et al., 2005; Amiard et al., 2006; Matozzo et al., 2008), but these lower level changes may not predict ecologically relevant changes (Cairns et al., 1993; Peakall, 1994; Munkittrick and McCarty, 1995; Adams and Greeley, 2000; Bartell, 2006; Koop et al., 2011). In order to better establish and validate causal relationships between a specific stressor and an ecologically relevant response, biomarkers and exposure indicators need to be used in tandem (Adams and Greeley, 2000; Adams et al., 2001). Initial studies, using a combination of early warning, diagnostic biomarkers and ecologically significant indicators, have shown promise in extrapolating cause and effect among multiple levels of biological organization (Adams

et al., 2000; Triebskorn et al., 2001; Martinez-Haro et al., 2015). Therefore, there is a need to establish indicators in multi-stressed aquatic systems that are not only early warning and diagnostic of a specific stressor but are also ecologically significant.

Metabolomics has been used to assess biological impacts of aquatic stressors by simultaneously measuring hundreds of low molecular weight metabolites (e.g., amino acids, lipids, osmolytes, sugars) within cells, tissues, or biological fluids of individual organisms (Lin et al., 2006; Miller, 2007; Viant, 2007). Environmental metabolomics studies the impacts of anthropogenic and natural stressors on an organism's metabolite levels. Ideally, metabolite changes can be used as biomarkers of a specific stressor (Lin et al., 2006; Miller, 2007; Viant, 2007), and the altered metabolic pathways can be predictive of organism fitness (Lin et al., 2006; Miller, 2007; Hines et al., 2010; Southam et al., 2011). The ability to predict changes at the individual level would be a step closer to predicting potential changes at ecologically significant levels of biological organization. Several studies on freshwater and marine organisms described impacts of chemical pollutants, land-use activities, and wastewater effluent on metabolites involved in a variety of metabolic processes, including energy metabolism, lipid metabolism, osmoregulation, and reproduction (e.g., Hines et al., 2010; Liu et al., 2011; Samuelsson et al., 2011; Southam et al., 2011; Fasulo et al., 2012; Davies et al., 2013; Skelton et al., 2014; Campillo et al., 2015; Ji et al., 2015). Controlled laboratory studies have predominantly assessed metabolite sensitivity to dose-dependent toxicological stress (e.g., heavy or trace metals, pesticides, pharmaceutical products; Hines et al., 2010; Liu et al., 2011; Samuelsson et al., 2011; Southam et al., 2011; Ji et al., 2015). Fewer laboratory studies examined metabolite sensitivity to variations in natural in-stream conditions, such

as dissolved oxygen (DO), food availability, pH, or salinity (Tuffnail et al., 2009; Wagner et al., 2015). Field studies have demonstrated the effectiveness of metabolomics in assessing metabolite changes of organisms exposed *in situ* to anthropogenic stressors, but the changes could not definitively be attributed to a specific stressor (Fasulo et al., 2012; Davies et al., 2013; Skelton et al., 2014; Campillo et al., 2015). Laboratory studies measuring metabolic impacts of specific altered in-stream conditions may lead to a better understanding of metabolic impacts of cumulative effects *in situ*.

It is well known that food availability and DO are two parameters that are altered by anthropogenic activities. The biological responses to these variations include physiological changes, shifts in fish and invertebrate community composition and/or richness, changes to trophic structure, and impaired water quality for human use and consumption (Carpenter et al., 1998; Correll, 1998; Miltner and Rankin, 1998). The most common organisms used as indicator species of food and DO stress are macroinvertebrates, including crayfish who have many characteristics of sentinel organisms (e.g., large body size, wide distribution, sensitive to environmental change; *sensu* Rosenberg and Wiens, 1976; Ryder and Edwards, 1985; Hellawell, 1986; Johnson et al., 1993). Although crayfish have not been used in metabolomics studies to date, different biomarker studies have reported physiological responses of crayfish to food or DO stress (Shirf et al., 1987; Okama and Abe, 1998; Fujimori and Abe, 2002; Nystrom, 2002; Bonvillain et al., 2012). Food stress causes changes in alanine, ATP/ADP, carbohydrates, free amino acids, glutamate, and lipid concentrations (Shirf et al., 1987; Okama and Abe, 1998), whereas DO stress causes changes in alanine, glucose, lactate, and protein concentrations (Fujimori and Abe, 2002; Nystrom, 2002; Bonvillain et al.,

2012). These studies showed that some biomarkers of food or DO stress are the same, whereas, others may be stressor-specific. Because metabolomics simultaneously measures hundreds of metabolites, it may be useful in identifying food-specific and DO-specific metabolic changes that could potentially affect organism fitness.

The primary goal of this study was to determine the suitability of the crayfish metabolome as an indicator of food or DO stress through controlled laboratory studies. The first objective was to identify metabolites sensitive to changes in food availability and to separately identify metabolites sensitive to changes in DO concentrations. The second objective was to determine if there were specific metabolites that are diagnostic of food or DO stress. The results of this study will provide information useful to disentangling cumulative effects in future field studies.

2.2 Materials and Methods

2.2.1 Laboratory Experiments

Forty-eight adult, female crayfish, *Procambarus clarkii*, were purchased and transported dry to the National Hydrology Research Center in Saskatoon, Saskatchewan, Canada. Upon arrival, crayfish were randomly grouped and placed into one of seven 10 L aquaria for four days of acclimation to laboratory conditions. Plastic pots were added as refuges. Aerated, dechlorinated tap water was maintained at a temperature of 17 ± 1 °C with a pH of approximately 7 and cleaned daily. The photoperiod was held at 12:12 (L:D). Crayfish were fed a commercial crayfish pellet diet *ad libitum* during the acclimation period.

After acclimation, weight (to the hundredth decimal) and total length of crayfish were recorded using a conventional analytical balance and Vernier caliper. In order to

avoid conspecific interactions during the experiment, crayfish were housed in individual 1.4 L aquaria with a plastic pot provided as refuge. Crayfish were randomly placed into one of six groups (eight crayfish per group). Each group was then randomly assigned a separate food or DO treatment for a fourteen day exposure period. While studies have shown that significant physiological changes occur in starved *P. clarkii* (Schirf et al., 1987; Okama and Abe, 1998), studies on the physiological impacts of over-consumption were not found. Therefore, the feeding levels were determined by observing crayfish feeding behavior during the acclimation period. The feeding treatments were as followed: approximately 0, 1, or 2 g of crayfish diet for the starved, normal or control, and elevated feeding groups. Crayfish that received food were fed daily. Food treatment aquaria were kept at full DO saturation. Furthermore, previous studies have found *P. clarkii* tolerate low levels of DO with significant physiological changes occurring < 2 mg/L (Fujimori and Abe, 2002; Nystrom, 2002; Bonvillain et al., 2012). Three DO concentrations were used: high or the control (approximately 8 to 9 mg/L), medium (4 to 5 mg/L), and low (1 to 2 mg/L). Aquaria exposed to high and medium concentration were kept at desired DO levels by bubbling in air with full and half inflow rates, respectively. Aquaria exposed to the low concentration were maintained by bubbling in a nitrogen:air (60:40) mixture. Crayfish in DO treatments were fed approximately 1 g of a commercial crayfish pellet daily. Food treatment aquaria were kept at full DO saturation. For all 6 treatment aquaria, water parameters were the same as in the acclimation period. Temperature, DO, and pH were monitored daily with a handheld multiparameter YSI (YSI Pro Plus, Yellow Springs, Ohio). Aquaria water was cleaned every three days. Crayfish behavior and occurrence of ecdysis were recorded. After the fourteen day exposure period, crayfish

were removed, weighed, measured, and sacrificed. Hepatopancreas, gill, and tail muscle tissues were dissected and immediately frozen in liquid nitrogen and stored in a -80°C freezer.

2.2.2 Preparation of Samples for ^1H NMR

Tissue samples were weighed into subsamples (hepatopancreas = 80 mg wet, gill = 10 mg dry, tail = 15 mg dry). Metabolites were extracted in random order from hepatopancreas, gill, and tail tissues using Viant's (2007) dual-phase methanol:chloroform:water procedure with a final solvent ratio of 2:2:1.8. Polar samples were lyophilized overnight and re-suspended in 650 μl of a 100 mM NMR sodium phosphate buffer (pH = 7.0) in 90% H_2O and 10% D_2O , containing 1 nM sodium 3-trimethylsilyl-2,2,3,3- d_4 -propionate (TMSP) and 3 mM sodium azide. TMSP served as an internal chemical shift standard. Once re-suspended, samples were immediately transferred to 5 mm NMR glass precision tubes for NMR analysis.

2.2.3 ^1H NMR Spectroscopy

All ^1H NMR spectra were analyzed on a Bruker Avance 600 MHz spectrometer operated at 600.17 MHz using a 5mm broadband probe. Tuning and gradient shimming was done for each sample. Suppression of the large water resonance in the spectra data was accomplished using excitation sculpting (Bollard et al., 2005). One dimensional (1D) ^1H NMR data were obtained at 298°K using a 60° pulse, a 7183.91Hz spectral width, a 2 second relaxation delay, and 128 scans. The experimental time for each sample was approximately 8 minutes. Resulting spectra were phase- and baseline-corrected, and TMSP was calibrated at 0.0 ppm using TopSpin software (Bruker). *Prometab* within MATLAB (The MathWorks, Natick, MA, USA) was used to convert spectra data into a

working format for statistical analysis (Viant, 2003). The 1D spectra were reduced from 0.50-10.00 ppm wide bins to 0.005 ppm wide bins (Aich et al., 2007). The total spectral area of the bins was integrated and normalized to the TMSP peak area (Viant, 2003).

2.2.4 Statistical Analysis

Results for the food and DO experiments were analyzed separately with data being normalized and auto-scaled using a web-based metabolomics data processing tool called MetaboAnalyst 2.0 (Xia et al., 2012). Using MetaboAnalyst, principle component analysis (PCA) of the 1D data was conducted to visually compare treatment trends, groupings, and outliers. Samples that fell outside Hotelling's T^2 eclipse were identified as outliers and removed. PCA scores (PC1 and PC2) for spectral bins were imported to SYSTAT 13 (Systat Software, San Jose, CA, USA) where differences among treatment groups for each tissue were analyzed using a General Linear Model (GLM) followed by Tukey HSD post-hoc analysis. Only the tissue(s) that showed significant differences ($\alpha = 0.10$) among treatment groups were used in subsequent data analyses. Random Forest was used to calculate classification errors of treatments, and new treatment groups were created if warranted. Projections to latent structures discriminant analysis (PLS-DA) models within MetaboAnalyst were used to identify treatment separating groups and to identify the spectral bins most responsible for the identified treatment differences. Leave one out cross validation (LOOCV) was used to assess model predictability. Using PLS-DA models, variable importance in projection (VIP) scores for each spectral bin were calculated and importance of each bin estimated. Bins with a VIP score greater than 1 were considered important contributors to treatment differences. Due to the large number of spectral bins, only those with VIP scores greater than 1 across 5 principal components

were included as significant bins. Metabolite peaks within the significant bins were identified using Chenomx NMR suite 8.1 (Chenomx, Inc.) and through previously published metabolite chemical shift values (Fan, 1996; Tuffnail et al., 2009; Fasulo et al., 2012). Significant metabolite peaks were validated by applying a t-test (SYSTAT 13) to the average spectrum within each significant bin. Significant metabolite level changes (i.e., increase or decrease) were calculated by subtracting the average spectrum of experimental treatment groups (i.e., low or medium DO saturation, starved or elevated feeding groups) from the average spectrum of their respective control treatment group (i.e., high DO saturation or normal feeding group). Once the significant metabolite changes were identified among treatments for food or DO stress, the metabolites were compared across studies (food vs. DO stress) in order to identify possible stress-specific metabolites.

2.3. Results

Procambarus clarkii total lengths did not differ significantly among DO treatments ($p = 0.13$; control/high = 95.60 ± 3.9 mm, medium = 91.66 ± 5.1 mm, low = 90.93 ± 3.8 mm). Measurements of crayfish mass at the beginning of the DO experiment (control/high = 22.94 ± 3.3 g, medium = 23.55 ± 4.5 g, low = 23.76 ± 3.7 g) were not significantly different ($p = 0.97$) for any of the DO treatments. Percent mass change of crayfish did not differ significantly ($p = 0.92$) among DO treatments after two weeks of exposure.

Procambarus clarkii total lengths did not differ significantly among food treatments ($p = 0.69$; control = 91.91 ± 3.7 mm, elevated = 91.47 ± 5.2 mm, starved = 92.33 ± 5.6 mm). Measurements of crayfish mass at the beginning of the food experiment

(control = 22.66 ± 3.5 g, elevated = 21.58 ± 3.4 g, starved = 23.51 ± 2.5 g) were not significantly different ($p = 0.36$) among treatments. However, starved crayfish had significantly less percent mass change (23.64 ± 2.5 %) than those in the control ($p = 0.026$, 23.96 ± 3.2 %) and elevated ($p = 0.0003$, 24.16 ± 3.3 %) food treatments after two weeks of exposure.

2.3.1 Feeding Experiment

The PCA scores for gill showed no statistically significant separation among any of the food treatments along PC1 ($p = 0.98$, explaining 18.3 percent of variance) or PC2 ($p = 0.72$, explaining 16.5 percent of variance) (Fig. 2.1A). PCA scores for hepatopancreas also showed no statistically significant separation of any of the treatments along PC1 ($p = 0.95$, explaining 45.9 percent of variance) or PC2 ($p = 0.63$, explaining 29.2 percent of variance) (Fig. 2.1B). PCA scores were significantly separated ($p = 0.086$) between the starved and control tail treatments along PC1 (explaining 21.3 percent of variance), but the starved and control tail treatments were not significantly different ($p = 0.33$) along PC2 (explaining 20.4 percent of variance) (Fig. 2.1C). There was no significant difference between the control and elevated tail treatments along PC1 ($p = 0.70$) or PC2 ($p = 0.94$), and the starved and elevated tail treatments were not significantly different along PC1 ($p = 0.38$) or PC2 ($p = 0.22$). Based on Random Forest analysis of the PCA scores, the control and elevated tail treatments had classification errors greater than sixty percent with the majority of their samples being classified as the control or elevated; thus, suggesting that the control and elevated tail treatments are indistinguishable. The starved tail treatment possessed a classification error of about ten percent with all but one of its samples being classified as starved. A two sample t-test

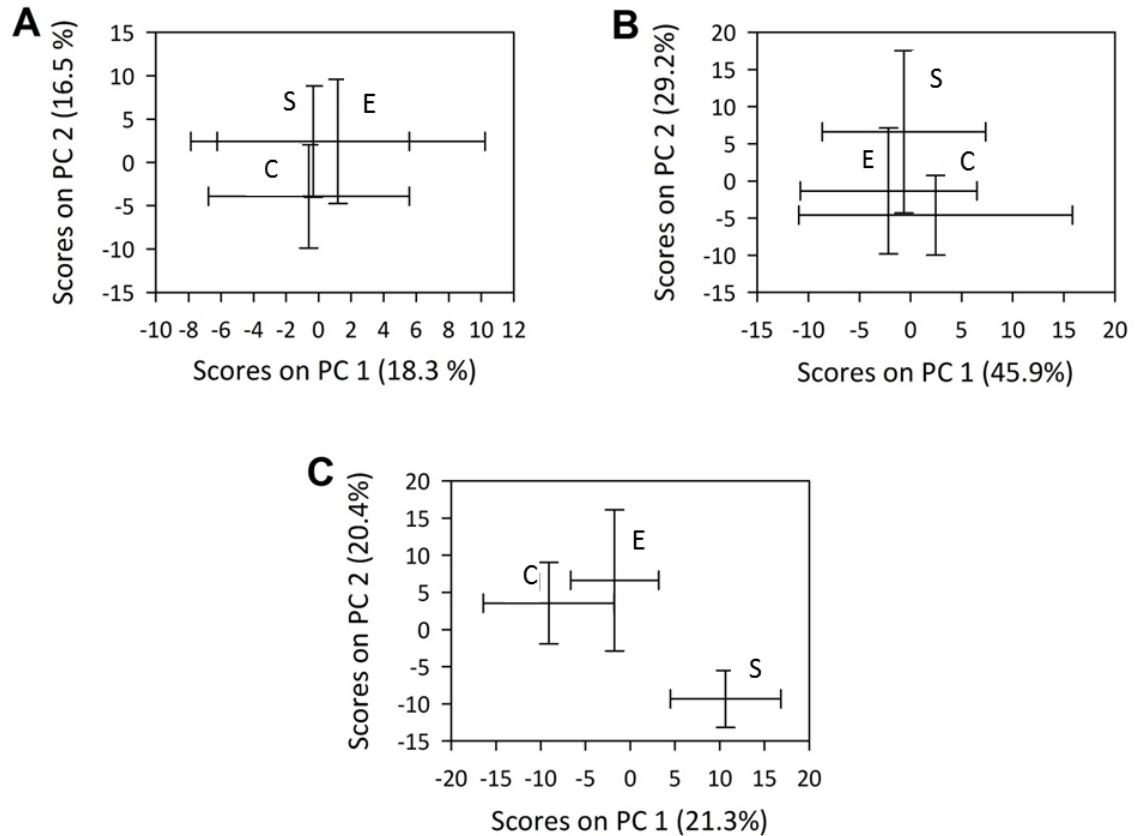


Figure 2.1. PCA score plots of (A) gill, (B) hepatopancreas, and (C) tail muscle from red swamp crayfish (*Procambarus clarkii*) after exposure to the three food treatment concentrations. Classes are labeled as (E) elevated food intake, (C) control (normal) food intake, and (S) starvation. The bars represent the mean of the score for each tissue and its corresponding standard error.

between the starved and control tail treatments confirmed the original statistically significant separation along PC1. Moreover, a two sample t-test between the starved and elevated tail treatments showed a statistically significant separation ($p = 0.051$) along PC2, but a two sample t-test showed no significant difference between control and elevated tail treatments along PC1 ($p = 0.34$) or PC2 ($p = 0.91$). These findings supported the random forest classification errors. Thus, the control and elevated tail samples were combined into one fed tail treatment for further analyses. Separation between the starved and fed tail treatments was statistically significant along PC1 ($p = 0.05$) and PC2 ($p = 0.036$). Subsequent metabolic profiling used the starved and fed tail muscle samples.

Changes in the metabolic profiles of *P. clarkii* tail muscle were found to be in amino acid and osmolyte levels based on the calculated PLS-DA VIP scores (Table 2.1). Significant levels ($p < 0.1$) of glutamine (Gln), glycine (Gly), isoleucine (Ile), leucine (Leu), lysine (Lys), tyrosine (Tyr), valine (Val), and betaine (Bet) decreased in tail muscle of starved crayfish (Fig. 2.2). Some significant peaks need further exploration.

2.3.2 DO Experiment

Similar to the food experiment, no significant separation of gill PCA scores was found for DO treatments along PC1 ($p = 0.61$, explaining 28.1 percent of variance) or PC2 ($p = 0.39$, explaining 20.9 percent of variance) (Fig. 2.3A). There was also no significant separation of hepatopancreas treatments along PC1 ($p = 0.77$, explaining 60.5 percent of variance) or PC2 ($p = 0.46$, explaining 21.7 percent of variance) (Fig. 2.3B). However, the PCA scores of tail muscle (Fig. 2.3C) differed among the three DO treatments along PC1 (explaining 19.8 percent of variation). The high and low DO tail treatments were found to be significantly separated ($p = 0.080$) along PC1, but not

Table 2.1. Metabolites in *Procambarus clarkii* tail muscle identified by PCA and PLS-DA analyses presented with the significance ($p < 0.10$) of a selected peak for each of the metabolites. Key: *s* singlet, *d* doublet, *t* triplet, *dd* double doublet, *m* multiplet.

Metabolites	Chemical shift [ppm, (multiplicity)]	P-value	
		Food	DO
<i>Amino acids</i>			
Alanine	1.48 (d), 3.78 (m)		0.045, 0.036
Glutamate	2.05 (m), 2.36 (m), 3.75 (t)		0.016, 0.015, 0.071
Glutamine	2.14 (m), 2.44 (m)	0.012, 0.013	
Glycine	3.56 (s)	0.011	0.007
Isoleucine	0.92 (t), 1.00 (d)	0.007, 0.076	
Leucine	0.94 (d), 0.96 (d)	0.009, 0.031	
Lysine	3.01 (t)	0.029	
Tyrosine	7.18 (d)	0.012, 0.032	0.085
Valine	0.98 (d), 1.04 (d)	0.044, 0.057	
<i>Energy metabolites</i>			
Acetoacetate	3.42 (s)		0.015
Succinate	2.39 (s)		0.091
<i>Miscellaneous</i>			
Betaine	3.91(s)	0.063	
Trehalose	3.44 (t), 3.64 (dd), 5.19 (d)		0.015, 0.011, 0.011

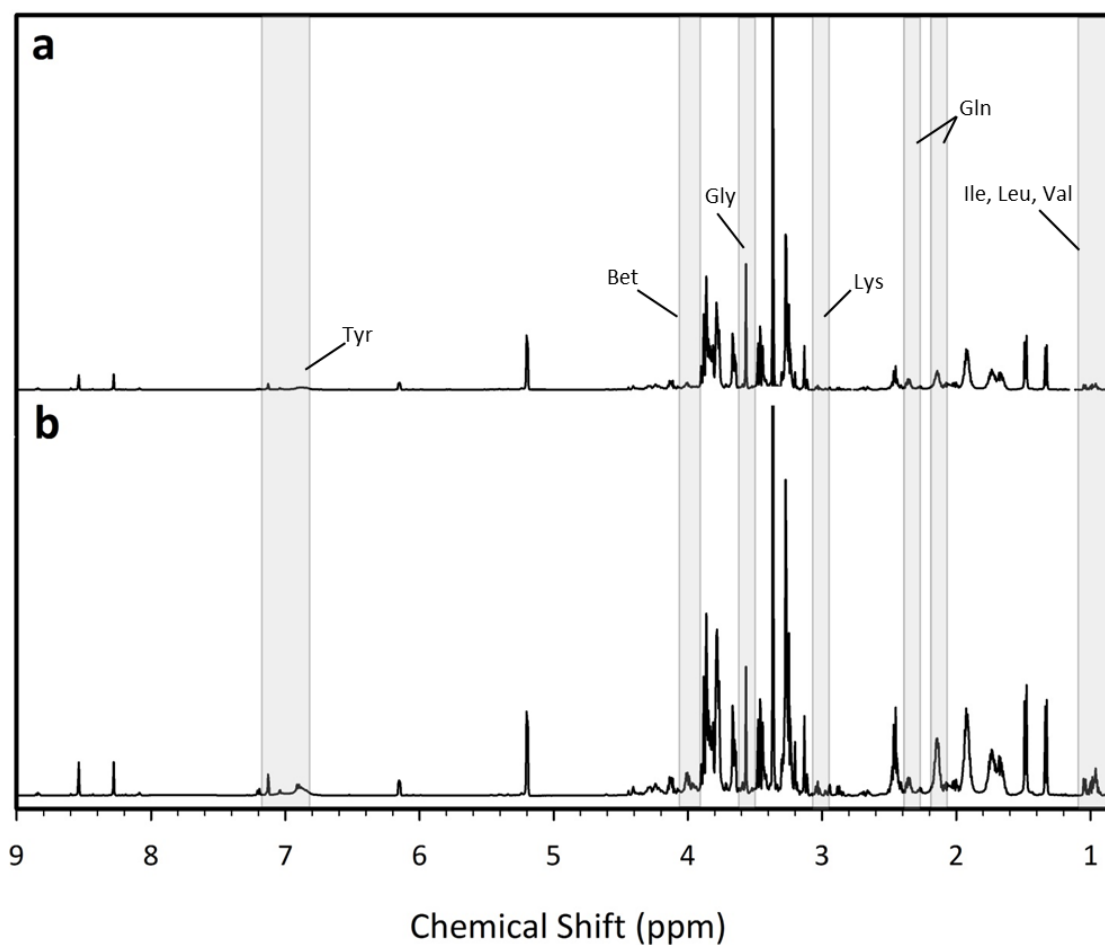


Figure 2.2. Average 1-D 600 MHz ^1H NMR spectra from the tail muscle of (a) starved and (b) fed (elevated and control groups combined) red swamp crayfish (*Procambarus clarkii*). The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Bet*- betaine, *Gln*- glutamine, *Gly*- glycine, *Ile*- isoleucine, *Leu*- leucine, *Lys*- lysine, *Tyr*- tyrosine, *Val*- valine.

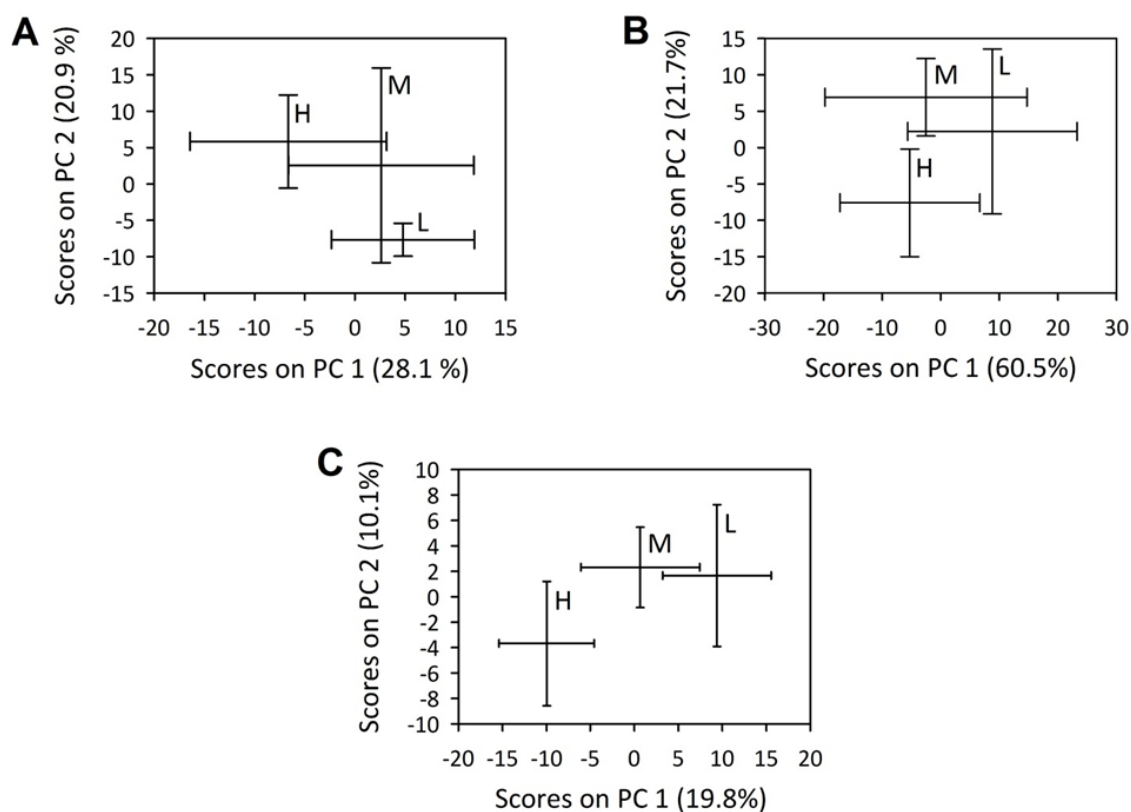


Figure 2.3. PCA score plots of (A) gill, (B) hepatopancreas, and (C) tail muscle from red swamp crayfish (*Procambarus clarkii*) after exposure to the three DO treatment concentrations. Classes are labeled as DO concentrations of high (H) 75-100 %, medium (M) 45-60 %, and low (L) 15-35 %. The bars represent the mean of the score for each tissue and its corresponding standard error.

significantly separated ($p = 0.70$) along PC2. The significant difference was confirmed ($p = 0.047$) with a two sample t-test. There was no significant separation between high and medium DO tail treatments along PC1 ($p = 0.46$) or PC2 ($p = 0.66$) and no significant separation between low and medium tail treatments along the PC1 ($p = 0.59$) or PC2 ($p = 0.99$) axes. Because of the central positioning of the medium tail treatment on the PCA scores plot (Fig. 2.3C), Random Forest calculated a class error of one hundred percent for the medium treatment. Half the samples were classified as low and the other half as high. Thus, the medium tail samples were removed, and subsequent metabolic tail analyses only used the high and low tail treatments.

Significant changes ($p < 0.1$) in amino acid, energy metabolite, and trehalose levels were found in tail muscles exposed to different DO treatments using VIP PLS-DA scores (Table 2.1). The average ^1H NMR spectra for tail muscle exposed to high and low DO treatments (Fig. 2.4) showed a decrease in some amino acids (Gly, Tyr) and acetoacetate and an increase in other amino acids (Alanine (Ala), Glutamate (Glu)), succinate, and trehalose in crayfish exposed to low concentrations of DO. Other significant metabolite peaks could not be assigned and need further exploration

2.4. Discussion

2.4.1 Organ Sensitivity

Traditional biomarker studies have found that food and DO stress elicit different metabolic responses in crayfish and crab organs (Albert and Ellington, 1985; Shirf et al., 1987), indicating the necessity to evaluate organs individually in response to a stressor. However, in the present study, tail muscle was the only tissue to show metabolic changes between the different treatment groups in both experiments. Although gill and

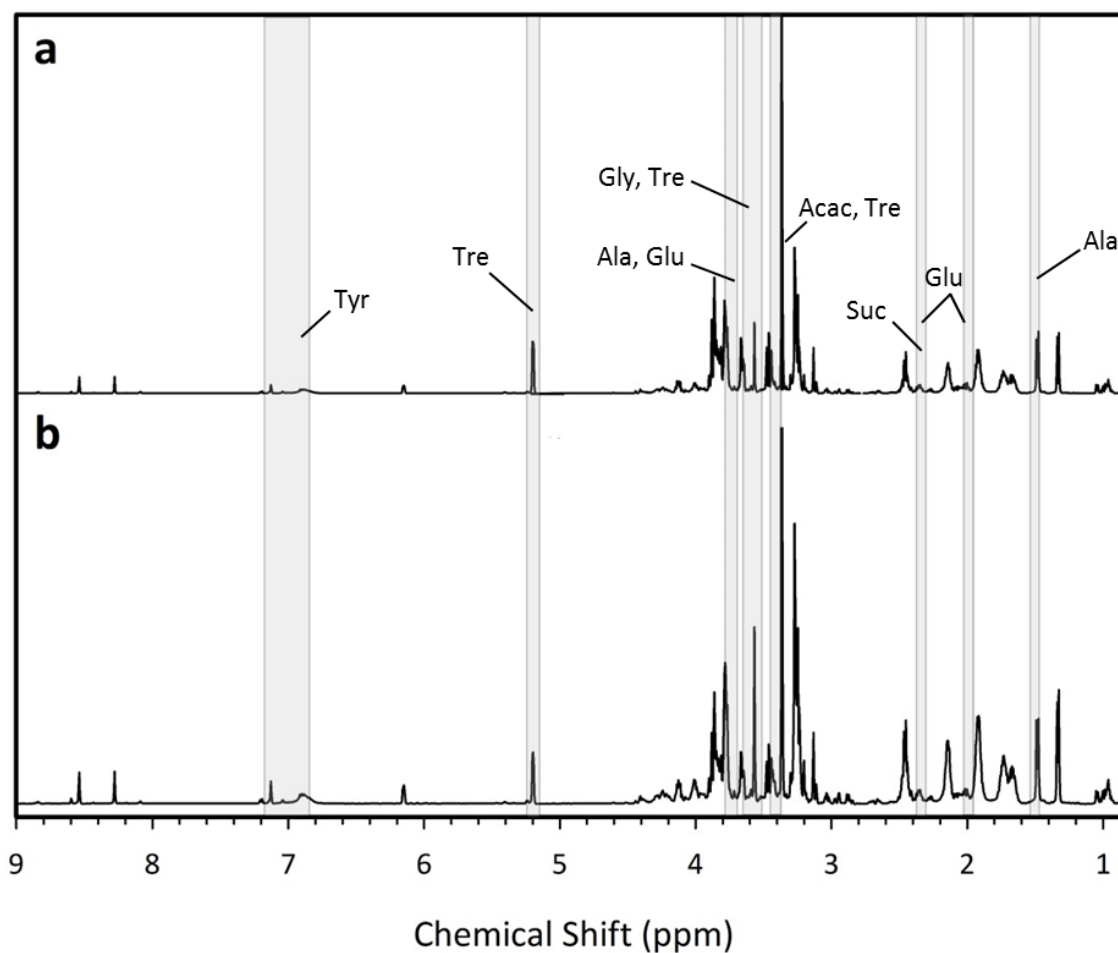


Figure 2.4. Average 1-D 600 MHz ^1H NMR spectra from the tail muscle of red swamp crayfish (*Procambarus clarkii*) exposed to (a) low and (b) high DO levels. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Ala*- alanine, *Acac*- acetoacetate, *Glu*- glutamate, *Gly*- glycine, *Suc*- succinate, *Tre*- trehalose, *Tyr*- tyrosine.

hepatopancreas tissues did not show metabolic changes to food or DO stress in the present study, previous aquatic invertebrate studies have found that these two tissues showed metabolic changes after exposure to chemical and metal pollutants (Liu et al., 2011; Fasulo et al., 2012, Campillo et al., 2015; Ji et al., 2015; Wei and Yang, 2015). The gill's lipophilicity and contact with water and the digestive gland's role in detoxification result in greater metabolic sensitivity to toxicological stress (Alcorlo et al., 2006; Fasulo et al., 2012; Wei and Yang, 2015). Moreover, both food deprivation and DO stress alter the cellular energy state in tissues. When food is unavailable, protein in muscle breaks down into free amino acids (FAA) for conversion to glucose or ketone bodies (Dall and Smith, 1987; Reddy and Sailaja, 1996; Fasulo et al., 2012; Wagner et al., 2015). Ketone bodies are alternative sources of energy derived from fatty acids during fasting or carbohydrate restriction. When exposed to chronic hypoxia, *P. clarkii*'s protein concentrations decreased, indicative of their use in energy pathways (Bonvillain et al., 2012). In the present study, tail muscle may have been more sensitive to changes in food availability and DO concentrations because of its use as an alternative energy source; thus, tail muscle of crayfish should be used as a target tissue for studying the metabolic effects of altered natural in stream conditions that induce food or DO stress.

2.4.2 Interpretation of Metabolomics Analysis

2.4.2.1 Food Metabolic Profiling

The metabolic differences between fed and starved crayfish were primarily associated with amounts of amino acids (glutamine, glycine, isoleucine, leucine, lysine, tyrosine, valine) and betaine in the tail muscle. Amounts of all amino acids decreased in starved crayfish. Energy sources are needed for cellular maintenance, but energy storage

only occurs when enough food is present (Koop et al., 2011). In the absence of food, proteins break down and release amino acids to provide energy for maintenance (Dall and Smith, 1987; Reddy and Sailaja, 1996; Jones et al., 2008b; Wagner et al., 2015). In the present study, ketogenic amino acids, such as isoleucine, leucine, lysine, and tyrosine, may have been converted into acetyl-coenzyme A (acetyl-CoA) for synthesis into a ketone body for energy in starved tail muscle (Fasulo et al., 2012). The glucogenic amino acids glutamine, glycine, isoleucine, tyrosine, and valine may have been used in gluconeogenesis to produce glucose in the starved tail muscle (Wagner et al., 2015). Note that isoleucine and tyrosine are both ketogenic and glucogenic. *P. clarkii* exposed to food limitation in other biomarker studies exhibited similar metabolic changes to the present study (Shirf et al., 1987; Okama and Abe, 1998). Therefore, changes to ketogenic and glucogenic amino acids in the present study were probably due to their use to replenish energy sources in the starved crayfish. Furthermore, betaine levels decreased in the starved crayfish. Betaine is an antioxidant that can be consumed to maintain methionine levels, an essential amino acid (Ekman et al., 2008). A decrease in betaine suggests the starved crayfish underwent oxidative stress. Overall, the decrease in amino acid and betaine levels in starved crayfish tail muscle is indicative of an energy crisis and a shift to an alternative energy supply.

2.4.2.2 DO Metabolic Profiling

Significant differences among levels of amino acids between crayfish tail muscle exposed to high DO vs low DO concentrations were observed. Levels of alanine and glutamate increased, but levels of glycine and tyrosine decreased in crayfish exposed to low DO concentrations. Glutamate is used in many metabolic pathways. Glutamate

dehydrogenase (GDH) converts glutamate to α -ketoglutarate, an intermediate and fuel in the tricarboxylic acid (TCA) cycle (Dawson and Storey, 2012). Glutamate dehydrogenase was less active in crayfish exposed to hypoxic conditions because oxygen needed to drive adenosine triphosphate (ATP) production in the TCA cycle was unavailable (Dawson and Storey, 2012). In the present study, reduction in TCA cycle activity may have suppressed GDH, increasing glutamate levels in the tail muscle. Thus, an increase in glutamate may suggest reduced energy production in crayfish tail muscle. Likewise, alanine has been identified as an end product of anaerobic respiration in both clams and crustaceans (Stokes and Awapara, 1968; Albert and Ellington, 1985; Fujimori and Abe, 2002; Lee et al., 2008). An increase in alanine levels of crayfish exposed to low DO concentrations indicates a switch from the TCA cycle to glycolysis in the crayfish exposed to hypoxic conditions. Similarly to the food experiment, glycine and tyrosine levels decreased in crayfish exposed to low DO concentrations. Glutamate also participates in amino acid synthesis through transamination (Okama and Abe, 1998; Dawson and Storey, 2012). An increase in glutamate indicates a reduction in ATP expensive amino acid synthesis. Thus, production of glycine and tyrosine may have decreased with an insufficient oxygen supply, or they may have been used as alternative sources of energy. An increase in alanine and glutamate and a decrease in glycine and tyrosine in crayfish tail muscle exposed to low DO concentrations indicate a disruption in energy metabolism and enhancement of anaerobic respiration.

A decrease in acetoacetate and an increase in succinate and trehalose in tail muscle were also observed in crayfish exposed to low DO concentrations. Acetoacetate, a ketone, is synthesized from three molecules of acetyl-CoA (Fasulo et al., 2012). Pyruvate

produced during glycolysis is converted to acetyl-CoA to fuel the TCA cycle (Li et al., 2015). An enhancement of anaerobic respiration would slow the TCA cycle, converting Acetyl-CoA to acetoacetate for use as alternative fuel. Thus, a reduction in acetoacetate suggests energy or lipid metabolism were altered in crayfish exposed to low DO concentrations. Succinate is an end product of anaerobic respiration in clams but has not been found to be an anaerobic end product in crustaceans (Stokes and Awapara, 1968; Lee et al., 2008). Because succinate is an intermediate in the TCA cycle, an increase in succinate suggests an inhibited TCA cycle in stressed crayfish. Trehalose levels also increased in crayfish exposed to low DO concentrations. Studies on trehalose use in crayfish are limited, but insects use it as an energy storage molecule, later utilized for bursts of speed (Schwoch, 1972). Increases in trehalose could be expected in crayfish exposed to low DO concentrations because energy consuming processes like movement would decrease with hypoxia. Movement decrease was supported by visual observations (Izral, personal observation). Thus, a poor oxygen supply appears to have reduced TCA cycle activity and energy consuming activities in crayfish tail muscle.

A change in lactate and glucose levels was not observed between the two DO treatment groups of crayfish in the present study. Although some crayfish species like *P. clarkii* are able to tolerate lower oxygen concentrations, lactate and glucose levels have been shown to increase in these crayfish exposed to DO concentrations below their tolerance threshold (Fujimori and Abe, 2002; Nystrom, 2002; Bonvillain et al., 2012). There are two hypotheses as to why lactate and glucose levels showed no significant differences between the groups in the present study. First, crayfish in past studies only produced end products of anaerobic respiration when exposed to DO concentrations less

than 2 mg/L for shorter exposure periods. In the present study, the low DO treatment crayfish were only exposed to approximately 2 mg/L DO; thus, DO stress may have been great enough to cause changes in energy metabolism but not enough to initiate anaerobic respiration. If this hypothesis is correct, the observed increase in alanine in crayfish exposed to low DO concentrations can be explained by cellular energy decline, not anaerobic respiration (*sensu* Peukurinen et al., 1984). Secondly, crayfish exposed to high DO concentrations molted (Izral, personal observation) shortly before being sacrificed. Physiological changes occur across organs in molting crustaceans, and molting crustaceans use more oxygen (Chang, 1995). The molting crayfish in the high DO treatment may have experienced stress that resulted in metabolic changes similar to the crayfish exposed to the lower oxygen concentrations. While known metabolic changes associated with hypoxia were not observed in the present study, changes in levels of amino acids, betaine, energy metabolites, and trehalose suggest alterations to the energy metabolism of crayfish exposed to low DO concentrations.

2.4.3 Metabolic Differences Between Food and DO Experiments

Results of the present study suggest that several identified metabolites may be able to diagnose food and oxygen stress in *P. clarkii* tail muscle. There was clear separation between the metabolite composition of *P. clarkii* exposed to food or dissolved oxygen stress. Metabolites that may be sensitive enough to diagnose food stress in *P. clarkii* tail muscle include glutamine, isoleucine, leucine, lysine, valine, and betaine. Similarly, alanine, glutamate, acetoacetate, succinate, and trehalose may be sensitive enough to diagnose DO stress in *P. clarkii* tail muscle. Levels of two metabolites, glycine and tyrosine, decreased in the experimental treatment groups (starved and low DO)

during both the food and DO experiments. Thus, these two metabolites cannot discern between food and DO stress. Although potential biomarkers diagnostic of food or DO stress were discovered, the effects of these altered metabolites on organism fitness was not measured. A good bioindicator must be ecologically relevant. Hines et al. (2010) was able to link metabolite changes to individual fitness and implications at higher levels of biological organization. Further study is required to determine whether the diagnostic metabolites in the present study can alter individual fitness.

2.4.4 Potential Limitations

Although the present study identified metabolites potentially diagnostic of food or DO stress, it also found that interpretation of metabolic responses can be difficult. The metabolic response of an organism is sensitive to gene regulation of individual organisms and tissues, sex, season/reproduction, and environmental conditions. (Viant, 2007; Ekman et al., 2008; Liu et al., 2011; Jones et al., 2013; Shen et al., 2014, Ji et al., 2015). For example, in the present study, half of the medium DO treatment group responded similarly to the control/high treatment group, while the other half responded similarly to the low DO treatment group. This suggests that individual crayfish may respond differently to the same stressor based on genetics. Additionally, the present study found that only tail muscle showed metabolic changes from food and DO stress, emphasizing the need to analyze multiple tissues. The potential stress-specific metabolites found in this experiment also only apply to female *P. clarkii* intermolt. Overall, the food and DO experiments identified metabolite sensitive factors that need to be accounted for when using metabolites as biomarkers of food or DO stress in crayfish.

2.4.5 Summary and Conclusions

The present study provides a starting point for identifying potential food or DO stress specific metabolites in crayfish. When metabolite sensitive factors (e.g., sex, organ, season) are accounted for, metabolomics may be used to identify significant energy related metabolite changes between fed and starved food treatments or high and low DO concentration treatments. Metabolomics also showed promise in identifying biomarkers (significant metabolites) diagnostic of food or DO stress. Further metabolomics investigations are needed to relate metabolic effects of food and DO stress to the individual level of crayfish.

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Chapter 3: An Assessment of the Effects of Land-Use Activities on the Crayfish Metabolome

3.1. Introduction

Land-use activities, including agricultural production (AGR) and wastewater treatment (WWT), are well documented in altering in-stream natural conditions and introducing a wide range of pollutants into aquatic ecosystems (Carpenter et al., 1998; Correll, 1998; Allan, 2004; Foley et al., 2005). Compounds and chemicals in wastewater effluent are not always degraded or removed before the effluent is released (Daughton and Ternes, 1999; Heberer et al., 2002; Kolpin et al., 2002; Fono et al., 2006; Schwarzenbach, 2006). The effects of wastewater release may persist for kilometers downstream of the point source, interacting with non-point agricultural inputs (Fono et al., 2006). Agricultural runoff and wastewater effluent can act cumulatively to cause sedimentation, nutrient enrichment (e.g., manure, fertilizers), pesticide and chemical contamination (e.g., pharmaceutical and personal care products (PPCPs), industrial and agro chemicals), and changes in dissolved oxygen (DO) concentrations (Carpenter et al., 1998; Correll, 1998; Miltner and Rankin, 1998; Bourne et al., 2002). The biotic implications of these stressors include loss of aquatic habitat, changes in primary productivity and food trophic structure, shifts in fish and invertebrate community composition and/or richness, and degradation of overall water quality (Carpenter et al., 1998; Correll, 1998; Miltner and Rankin, 1998; Bourne et al., 2002).

Causal relationships between land-use and biological responses in streams are often examined using exposure indicators. An exposure indicator is a measurable environmental characteristic that quantifies degree of exposure and response to a stressor

(Adams and Greeley, 2000). Exposure indicators at higher levels of biological organization (i.e., changes at the community or population level) provide an ecologically relevant and holistic view of an aquatic system, but they are slow responding and difficult to link back to a specific, underlying stressor (Cairns et al., 1993; Munkittrick and McCarty, 1995; Adams and Greeley, 2000). For example, aquatic studies have evaluated the sensitivity of benthic invertebrate and fish population metrics, size diversity, nutrient constituents (e.g., nitrate + nitrite), chlorophyll-a, stable isotope tracers, and stream metabolism to varying land usages (e.g., Allan, 2004; Johnson et al., 2006; Resh, 2008; Gallardo et al., 2011, Clapcott et al., 2012, Yates et al., 2014). Although these structural and functional indicators have been associated with different land uses, it is often difficult to relate the observed ecological changes to a specific in-stream stressor (e.g., nutrient enrichment, change in DO concentrations) (Munkittrick and McCarty, 1995; Adams and Greeley, 2000; Adams et al., 2001). Many of these in-stream stressors are driven by both a variety of natural and anthropogenic factors. Alternatively, early warning indicators, such as biomarkers, are better able to attribute biochemical and physiological responses to a specific aquatic stressor, but their lack of ecological relevance prevents their widespread use in land-use studies (Cairns et al., 1993; Peakall, 1994; Munkittrick and McCarty, 1995; Adams and Greeley, 2000; Bartell, 2006; Koop et al., 2011). Thus, ecologically relevant exposure indicators should be used in combination with diagnostic biomarkers to better establish causal relationships between a specific stressor and ecological response (Adams and Greeley, 2000; Adams et al., 2001). While this approach is not common in land-use studies, Prat et al. (2013) found that biomarkers were able to detect the effects of wastewater on macroinvertebrate assemblages when used in tandem

with structural indicators. Therefore, indicators that are early warning, diagnostic, and ecologically relevant need to be established in order to better assess multi-stressed aquatic systems.

Metabolomics is a relatively new field of study that has been used in recent years to evaluate the effect of stressors on aquatic organisms. Metabolomics, which measures hundreds of endogenous, low molecular weight metabolites (e.g., amino acids, lipids, osmolytes, sugars), is attractive for use in environmental studies because metabolite concentrations change when exposed to a variety of anthropogenic and natural stressors (Lin et al., 2006; Miller, 2007; Viant, 2007). Thus, they have biomarker potential. Because metabolites are substrates and products in metabolic pathways, their concentration changes may have implications for organism fitness and ultimately affect higher levels of biological organization (Lin et al., 2006; Miller, 2007; Hines et al., 2010; Southam et al., 2011). Although laboratory studies assessing the metabolic impact of a variety of dose-dependent toxicological stressors (e.g., heavy or trace metals, pesticides, pharmaceutical products) are relatively commonplace (e.g., Samuelsson et al., 2006; Viant et al., 2006; Ekman et al., 2008; Katsiadaki et al., 2010; Hines et al., 2010; Liu et al., 2011; Samuelsson et al., 2011; Southam et al., 2011; Ji et al., 2015; Li et al., 2015), fewer field studies have been conducted (e.g., Fasulo et al., 2012; Kwon et al., 2012; Davis et al., 2013; Campillo et al., 2015), and fewer yet have analyzed the metabolic impact of land-use and wastewater treatment effluent on aquatic organisms (but see Skelton et al., 2014). Skelton et al. (2014) found that the hepatic metabolome of lab raised, male fathead minnows was sensitive to different wastewater treatment effluents, locations within sites (i.e., upstream, downstream, effluent), and overall land use (i.e.,

agriculture, urbanization), suggesting metabolomics has promise in identifying land use-sensitive metabolites that may affect organism fitness. However, no metabolomics study has conducted a reciprocal transfer experiment, assessing the metabolic response of multiple organismal tissues to removed (recovery) and added (impact) land-use stressors.

The overall goal of this study was to determine the suitability of the crayfish metabolome as an indicator of stream conditions associated with three types of land use (i.e., natural vegetation (aka reference), agriculture, and urban wastewater effluent). More specifically, the main objective of this study was to assess the response of the crayfish metabolome to recovery and impact land-use treatments. The results of this study will provide much needed information on using the crayfish metabolome to disentangle cumulative effects in land-use studies.

3.2. Methods

3.2.1 Study Sites

This study was conducted within Red River Valley subcatchments of Lake Winnipeg in southern Manitoba, Canada (Fig. 3.1). The sites were chosen based on a study by Yates et al. (2012). The sites varied in agricultural land cover (percentage of area within subcatchment) and/or wastewater lagoon activity. The Brokenhead River catchment (49.925884°W, -96.38316°N) was the reference (REF) site with minimal agricultural production (< 5% agricultural land cover) and no wastewater lagoon activity. Shannon Creek (49.278703°W, -98.024131°N) was the agricultural (AGR) site with greater than 65% agricultural land cover and no wastewater lagoon activity. Dead Horse Creek (49.236809°W, -98.001699°N) had agricultural production similar to Shannon Creek (> 65%) but received effluent from a sewage lagoon during the summer. Thus,

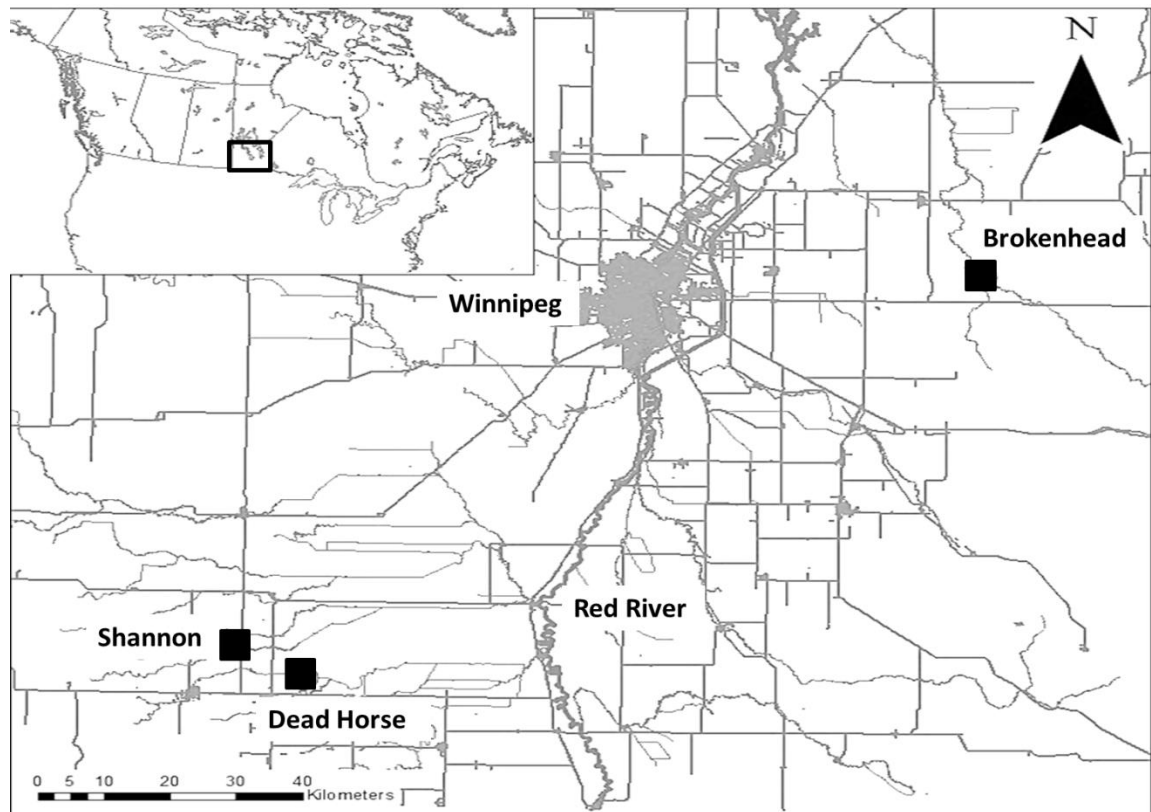


Figure 3.1. Location of the three study sites in July 2014 near the city of Winnipeg in the Red River Valley of the Lake Winnipeg Basin in southern Manitoba, Canada. Key: *Brokenhead*- reference (REF), *Shannon*- agriculture (AGR), *Dead Horse*- wastewater treatment (WWT).

Dead Horse Creek was the wastewater treatment (WWT) site. Although there was minimal variation in the physical environment, Brokenhead River was characterized by cobble substrate while the other sites were characterized by fine to very fine soil substrate.

3.2.2 Field Collections and Experiment

The northern crayfish *Orconectes virilis* was chosen because it possesses many qualities of a good sentinel species: 1) cosmopolitan distribution; 2) large body size for tissue extraction; 3) numerical abundance; and 4) sensitive to environmental stressors (*sensu* Rosenberg and Wiens, 1976; Ryder and Edwards, 1985; Hellowell, 1986; Johnson et al., 1993). Crayfish also have the ability to accumulate metals from surrounding waters, and past studies showed that physiological changes occurred when crayfish were exposed to varying aquatic stressors (Shirf et al., 1987; Johnson et al., 1993; Okama and Abe, 1998; Fujimori and Abe, 2002; Nystrom, 2002; Alcorlo et al., 2006; Faria et al., 2010; Bonvillain et al., 2012; Wei and Yang, 2015). In this study, crayfish were abundant in the three study sites' fine or cobble substrates, common burrowing habitats of crayfish. Lastly, only females were used because metabolic changes can be sex specific (Viant, 2007).

Field collections of adult female *O. virilis* began on July 6 2014, three days before effluent from the Winkler, MB, Canada sewage lagoon cells was discharged into Dead Horse Creek (the WWT site) on July 9 (Table 3.1). The crayfish needed to be housed in their cages at the WWT site before or close to the start of the release in order to measure the effects of the effluent and maximum nutrient concentrations on the crayfish metabolome. Due to time restraints, distance between sites, and difficulty in collecting a

Table 3.2. Chronological table of events for the collection, reciprocal transfer, and sacrifice of *O. virilis* at the reference, agriculture, and wastewater treatment study sites of the Red River Valley, Manitoba, Canada in July 2014.

Date	Experimental Event
July 6	Minnow traps deployed at REF site
July 8	Field collection and reciprocal transfer of crayfish caught at REF site
July 9	Cells 5, 7, 8, & 9 of Winkler, MB lagoon released into WWT creek Minnow traps deployed at AGR site
July 10	Field collection and reciprocal transfer of crayfish caught at AGR site Minnow traps deployed at WWT site
July 12	Field collection and reciprocal transfer of crayfish caught at WWT site
July 16	Week 1 sacrifice of crayfish captured at REF site
July 17	Week1 sacrifice of crayfish captured at AGR site
July 19	Week 1 sacrifice of crayfish captured at WWT site
July 23	Week 2 sacrifice of crayfish captured at REF site
July 24	Week 2 sacrifice of crayfish captured at AGR site
July 25	Winkler, MB lagoon release ends
July 26	Week 2 sacrifice of crayfish captured at WWT site

sufficient number of same-sex crayfish at each site, field collections took place between July 6 and July 12 (Table 3.1). Thus, crayfish collections and reciprocal transfers at the REF, AGR, and WWT sites occurred on separate days. Crayfish were randomly assigned exposure lengths of one or two weeks, and the exact day of sacrifice was based on capture location (Table 3.1). Exposure times were based on findings of past studies that found significant metabolic effects from exposure to adverse physicochemical conditions in crayfish for one and two week exposure periods (McWhinnie and O'Connor, 1967; Claussen, 1980; Armitage and Wall, 1982).

Fifty-three female *O. virilis* were captured at each site using 35 to 50 standard minnow traps (6.4 mm mesh, 2.54 cm opening, 42 cm long) baited with commercial dog food pellets. Minnow traps were deployed at each site for one or two days. Only female crayfish between 55 and 80 mm in total length were used in order to mitigate sex and size influence on metabolic response and reduce the likelihood of crayfish escape. Once crayfish were removed from the minnow traps, they were placed together into 19 L utility buckets filled with ambient temperature stream water. The crayfish were weighed (nearest 10 mg) and measured using a conventional analytical balance and Vernier caliper, respectively. At each site (week 0), 5 *O. virilis* were randomly selected and dissected to remove hepatopancreas, tail muscle, and gill tissues. The tissues were immediately frozen in liquid nitrogen and stored on dry ice. The remaining 48 crayfish captured at each site were randomly assigned an exposure site and used in the reciprocal transfer ($n = 16$ to each site). A random reciprocal transfer was conducted in order to assess the response of the crayfish metabolome to recovery and impact treatments. The samples were labeled with the first letter indicating capture site and the second letter

indicating exposure site (Table 3.2). Three recovery treatments were created where crayfish were moved from a presumably more impacted stream to a presumably less impacted stream (i.e., AGR to REF, WWT to AGR, or WWT to REF). Three impact treatments were also created where crayfish from a less impacted stream were moved to a more impacted stream (i.e., REF to AGR, AGR to WWT, REF to WWT). Each of the six treatments contained the following sets of crayfish samples: capture site control, experimental (transferred), and exposure site control (Table 3.3). After being assigned an exposure site, all crayfish regardless of whether they remained at their site of capture were placed in individual containers filled with stream water in coolers containing ice. This was done to ensure that all crayfish experienced similar degrees of stress associated with transfer and to increase the chance of survival during transfer. Following the reciprocal transfer, each crayfish was individually housed (to prevent cannibalism) in a wire cage (21 cm L x 18 cm W x 10 cm H) covered in 1 cm mesh. Each cage was attached to a PVC sheet (18 cm x 140 cm) in rows of 4 per sheet. Cages contained a plastic pot where crayfish could seek refuge from antagonistic behavior of neighboring crayfish and predators in the stream. The sheets were anchored perpendicular to stream flow along the bank of the streams in habitat similar to where the crayfish were collected. The 4 cages per sheet held crayfish originating from the same capture site. The sheets were alternatively placed between 1 and 3 m apart based on capture site. Thus, crayfish from all three capture sites experienced similar conditions across the exposure stream section. Crayfish were not fed a supplementary diet. At the conclusion of the one or two week exposure periods, crayfish were removed, weighed, and measured. The crayfish were dissected to remove hepatopancreas, tail muscle, and gill tissues, and the samples

Table 3.3. Sample key for crayfish used in the reciprocal transfer experiments based on capture site (first letter) and exposure site (second letter) in the Red River Valley, Manitoba, Canada.

Sample	Classification	Capture Site	Exposure Site
AA	Control	AGR	AGR
AR	Experimental	AGR	REF
AW	Experimental	AGR	WWT
RR	Control	REF	REF
RA	Experimental	REF	AGR
RW	Experimental	REF	WWT
WW	Control	WWT	WWT
WA	Experimental	WWT	AGR
WR	Experimental	WWT	REF

Table 3.4. Recovery and impact treatments for crayfish in three study subcatchments in the Red River Valley, Manitoba, Canada. Every treatment contained two sets of control samples and one set of experimental samples.

Scenario	Treatment	Samples
Recovery	AGR to REF	AA, AR, RR
	WWT to AGR	WW, WA, AA
	WWT to REF	WW, WR, RR
Impact	REF to AGR	RR, RA, AA
	AGR to WWT	AA, AW, WW
	REF to WWT	RR, RW, WW

were immediately flash frozen in liquid nitrogen and stored on dry ice. These samples as well as the 5 originally sacrificed at each capture site were shipped on dry ice overnight to the National Hydrology Research Center in Saskatoon, Saskatchewan, Canada.

At each site, water quality parameters were measured, and nutrient and pharmaceutical samples collected. A YSI 6600 Multi Parameter Water Quality Meter Sonde (YSI Inc.) was deployed at Shannon Creek (AGR), whereas a D-Opto Logger (Zebra-tech Ltd) was deployed at both Brokenhead River (REF) and Dead Horse Creek (WWT) on the first day of field collections (July 8) in well-mixed areas of each stream reach. Loggers at each site measured dissolved oxygen (DO) concentrations and temperature (°C) every 15 minutes throughout the course of the study. Grab samples measuring total suspended solids (TSS) were taken on July 8, 14, 21, and 28 at all three sites using 1 L polyethylene sample bottles. These analyses were performed in house at Western University, Ontario, Canada. Moreover, 250 mL polyethylene sampling bottles were used to take grab samples for nutrient analyses on July 14, 21, and 28. Samples were analyzed for ammonia (NH_3), nitrite + nitrate ($\text{NO}_2 + \text{NO}_3$), total nitrogen (TN), soluble reactive phosphorous (SRP), total dissolved phosphorous (TDP), and total phosphorous (TP). Nutrient samples were shipped overnight to the Biogeochemical Analytical Service Lab, University of Alberta (Alberta, Canada). Lastly, grab samples for pharmaceutical and personal care product (PPCP) analyses were obtained at all sites on July 15. An additional PPCP sample was collected at Dead Horse Creek on July 28 after effluent release ended. Two 500 mL polyethylene bottles were used for each sample (sample volume = 1 L). These samples were shipped overnight to AXYS Analytical Services Ltd. (British Columbia, Canada) where concentrations of a total of 58

compounds found in consumer products, medicine, or agricultural/livestock production (Appendix A, Table A.1) were measured.

3.2.3 Preparation of Samples for ^1H NMR

A dual-phase methanol:chloroform:water (2:2:1.8) procedure was used to extract polar and nonpolar metabolites from subsamples of the hepatopancreas (80 mg wet), gill (10 mg dry), and tail (15 mg dry) tissues (*sensu* Viant, 2007). Only the polar metabolites were subsequently lyophilized overnight. Before samples were ready for NMR analysis, the lyophilized samples were re-suspended in 650 μl of a 100 mM NMR sodium phosphate buffer (pH = 7.0) in 90% H_2O and 10% D_2O , containing 1 mM sodium 3-trimethylsilyl-2,2,3,3- d_4 -propionate (TMSP) and 3 Mm sodium azide. TMSP was the internal chemical shift standard. Immediately prior to NMR analysis, re-suspended samples were transferred to 5 mm NMR glass precision tubes. The preparation order and NMR analysis were random for all samples.

3.2.4 ^1H NMR Spectroscopy

Hepatopancreas, gill, and tail extracts were analyzed on a Bruker Avance 600 MHz spectrometer using a spin speed of 600.17 MHz (at 298°K). After tuning and gradient shimming, one dimensional (1D) ^1H NMR spectra were recorded using a 60° pulse, a 7183.91Hz spectral width, a 2 second relaxation delay, and 128 scans with pre-excitation sculpting for water suppression. This required an 8 minute acquisition time. All ^1H NMR spectra were manually phase- and baseline-corrected and calibrated (TMSP at 0.0 ppm) (TopSpin, Bruker). Data were exported to *Prometab* within MATLAB (The MathWorks, Natick, MA, USA) and converted to a format for multivariate statistical analysis (Viant, 2007). Each spectrum was segmented into 0.005 ppm wide bins (Aich et

al., 2007), and the total spectral area of each spectrum was integrated and normalized under the TMS peak.

3.2.5 Statistical Analysis

Data were normalized and auto-scaled using a web-based metabolomics data processing tool called MetaboAnalyst 2.0 (Xia et al., 2012). The data was first separated into the six recovery and impact treatments in Table 3.3. Data was also separated by tissue with each tissue being analyzed separately. 1D-¹H NMR spectra were first analyzed using principle component analysis (PCA) to visually compare treatment trends, groupings, and outliers. Outliers that fell outside Hotelling's T^2 ellipse were identified and removed. PCA scores (PC1 and PC2) for each treatment's spectral bins were imported to SYSTAT 13 (Systat Software, San Jose, CA, USA) where differences among samples and between weeks for each treatment were analyzed with a multivariate analysis of variance (MANOVA). If there was a significant difference ($\alpha = 0.05$) by week within a treatment, week 1 and week 2 samples were subsequently analyzed separately. If there was no significant difference ($\alpha = 0.05$) by week, weeks were combined into a single treatment group. PCA scores were generated for the new recovery and impact treatments (week 1, week 2, or combined) using MetaboAnalyst and imported to SYSTAT 13. Differences among samples within treatments were analyzed using a general linear model (GLM) followed by Tukey HSD post-hoc analysis. Only the impact or recovery treatments where samples were significantly different ($\alpha = 0.05$) were used in subsequent data analyses. Projections to latent structures discriminant analysis (PLS-DA) models within MetaboAnalyst were used to identify the spectral bins most responsible for differences among samples in each treatment. Leave one out cross validation (LOOCV)

was conducted, and treatments with poor Q^2 predictive capabilities (e.g., negative Q^2 values) were removed from further analyses. PLS-DA cross validation helped determine how many principal components to use for metabolite identification. For cross validated treatment groups, variable importance in projection (VIP) scores for each spectral bin were calculated and the importance of each bin estimated. A bin with a VIP score greater than 1 was considered an important contributor to metabolic differences. Metabolite peaks within the significant bins were identified through visual inspection using Chenomx NMR suite 8.1 (Chenomx Inc.) and previously published metabolite chemical shift values (Fan, 1996; Tuffnail et al., 2009; Fasulo et al., 2012). Significant metabolite level changes (i.e., increase or decrease) were determined by comparing averaged spectra of each set of samples within recovery or impact treatments. Weight differences among sets of samples within each recovery or impact treatment were analyzed by week using a GLM and Tukey's HSD. The weight difference was measured as the percentage change in weight between start and end weight measurements and was defined as growth rate in this study.

3.3. Results

3.3.1 Physicochemical Parameters

Measured water temperatures ranged between 14.35 °C and 24.90 °C across the three sample sites (Table 3.4). Daily variations were consistent and comparable among sites. Measured concentrations of dissolved oxygen (DO) ranged from 0.0 mg/L to 23.64 mg/L, although minimal variation was observed at the REF (CV = 0.13) and AGR (CV = 0.09) sites with both sites averaging approximately 8 ± 1 mg/L for the duration of the study period. Greater within site variation (CV = 0.66) was seen in the WWT site, being

Table 3.4. Descriptive statistics of water physico-chemical parameters and nutrients sampled in three study catchments of the Red River Valley, Manitoba, Canada in July 2014.

	Brokenhead (REF ¹)			Shannon (AGR ²)			Dead Horse (WWT ³)		
	Mean \pm SD	Max	Min	Mean \pm SD	Max	Min	Mean \pm SD	Max	Min
DO (%)	92.21 \pm 12.74	124.83	73.38	88.11 \pm 6.8	102.17	65.32	66.36 \pm 44.0	283.04	0.00
DO (mg/L)	8.25 \pm 1.1	11.20	6.68	8.01 \pm 0.7	9.86	6.11	5.83 \pm 3.8	23.64	0.00
Temperature (°C)	20.77 \pm 1.9	24.95	16.35	19.91 \pm 2.0	24.06	14.35	22.17 \pm 1.6	24.90	18.01
NH ₃ (N mg/L)	0.004 \pm 0.003	0.006	<LOD ⁴	0.04 \pm 0.1	0.12	<LOD	4.56 \pm 4.4	8.75	<LOD
NO ₂ +NO ₃ (N mg/L)	<LOD	<LOD	<LOD	0.004 \pm 0.01	0.010	<LOD	3.42 \pm 3.4	7.35	1.24
TN (N mg/L)	1.02 \pm 0.03	1.05	0.99	1.47 \pm 0.02	1.48	1.45	9.02 \pm 3.3	12.40	5.92
SRP (P mg/L)	0.02 \pm 0.001	0.02	0.01	0.02 \pm 0.01	0.03	0.01	1.89 \pm 0.5	2.31	1.32
TP (P mg/L)	0.02 \pm 0.01	0.03	0.02	0.11 \pm 0.01	0.13	0.10	2.34 \pm 0.2	2.44	2.17
TDP (P mg/L)	0.02 \pm 0.004	0.02	0.01	0.03 \pm 0.004	0.03	0.02	2.22 \pm 0.2	2.48	2.05
TSS (mg/L)	9.49 \pm 14.8	31.70	1.86	62.49 \pm 34.3	98.67	19.25	17.40 \pm 8.4	28.00	10.00

¹ REF = reference site; < 5% agricultural land cover and no wastewater lagoon activity

² AGR = agricultural site; > 65% agricultural land cover and no wastewater lagoon activity

³ WWT = wastewater treatment site; > 65% agricultural land cover and wastewater lagoon activity

⁴ <LOD = nutrient concentrations were below level of detection

8.44 ± 1.1 mg/L during the first week of effluent release but dropping to 3.54 ± 4.0 mg/L during the second week. The average DO for the entire period was 5.83 ± 3.8 mg/L. The concentrations of total suspended solids (TSS) varied across sites (Table 3.4), measuring lowest for the REF site ($\bar{x} = 9.49 \pm 14.8$ mg/L) and highest for the AGR site ($\bar{x} = 62.49 \pm 34.3$ mg/L).

The concentration of total nitrogen (TN) was greatest in the WWT site where TN averaged 9.02 ± 3.3 mg/L (Table 3.4). Average TN concentrations of the REF and AGR sites were 1.02 ± 0.03 mg/L and 1.47 ± 0.02 mg/L, respectively. The greater TN concentration at the WWT site was due to the greater concentrations of ammonia (NH_3) and nitrite + nitrate ($\text{NO}_2 + \text{NO}_3$) measured during the lagoon discharge at the WWT site. Measured concentrations of total phosphorous (TP) followed the same trend as TN with the greatest concentration present at the WWT site ($\bar{x} = 2.34 \pm 0.2$ mg/L) (Table 3.4). Average TP was 5 times greater at the AGR site ($\bar{x} = 0.11 \pm 0.01$ mg/L) than at the REF site ($\bar{x} = 0.02 \pm 0.01$ mg/L). Soluble reactive phosphorous (SRP) and total dissolved phosphorous (TDP) concentrations were also greatest at the WWT site.

Fifteen of the 58 pharmaceutical and personal care compounds analyzed for were detected at one or more sites (Table 3.5). The compounds included human-use pharmaceuticals or psycho-pharmaceuticals, such as caffeine, gemfibrozil, and ibuprofen. Other compounds detected were agricultural fungicides (i.e., thiabendazole) and veterinary or human antibiotics (i.e., carbadox, erythromycin- H_2O , and trimethoprim). None of the compounds were detected at reporting limits in the REF site, and only erythromycin- H_2O , an antibiotic, was detected in the AGR site. All fifteen compounds were detected in the samples collected from the WWT site during the lagoon discharge.

Table 3.5. Pharmaceutical and personal care products (PPCPs) detected in at least one of the study catchments in the Red River Valley, Manitoba, Canada in July 2014. Note that Dead Horse Creek was sampled once during the release and once post release. Key: *REF*-reference site (< 5% agricultural land cover and no wastewater lagoon activity), *AGR*-agricultural site (> 65% agricultural land cover and no wastewater lagoon activity), *WWT*-wastewater site (> 65% agricultural land cover and wastewater lagoon activity), U-not detected at reporting limit.

Compound (ng/L)	Brokenhead (REF)	Shannon (AGR)	Dead Horse July 15 (WWT)	Dead Horse July 28 (WWT)
Caffeine ¹	U	U	1350.00	863.00
Carbadox ²	U	U	35.00	72.00
Carbamazepine ³	U	U	114.00	114.00
Clarithromycin ⁴	U	U	1.94	1.42
Dehydronifedipine ⁵	U	U	2.68	U
Diphenhydramine ⁶	U	U	1.59	0.61
Erythromycin-H ₂ O ⁷	U	2.74	15.60	6.24
Gemfibrozil ⁸	U	U	5.80	4.54
2-Hydroxy-ibuprofen ⁹	U	U	7690.00	5540.00
Ibuprofen ¹⁰	U	U	266.00	211.00
Naproxen ¹¹	U	U	15.60	U
Thiabendazole ¹²	U	U	5.98	8.19
Trimethoprim ¹³	U	U	34.00	U
Virginiamycin M1 ¹⁴	U	U	132.00	U
1,7-Dimethylxanthine ¹⁵	U	U	1080.00	460.00

¹ psychoactive drug

² swine growth-promoting and antibacterial drug

³ anticonvulsant

⁴ human or veterinary antibiotic

⁵ treatment of hypertension or angina

⁶ antihistamine

⁷ human or veterinary antibiotic

⁸ fibrate drug

⁹ metabolite of Ibuprofen

¹⁰ anti-inflammatory drug

¹¹ anti-inflammatory drug

¹² agricultural fungicide

¹³ human or veterinary antibiotic

¹⁴ antibiotic

¹⁵ psychoactive drug

However, only eleven compounds were detected in WWT samples collected post lagoon discharge when there was a decrease in all human-use pharmaceutical concentrations but an increase in carbadox and thiabendazole concentrations.

3.3.2 Crayfish Lengths and Weights

During the course of the study, 5 crayfish died and 1 escaped before the end of their respective exposure periods. The loss of these samples was spread equally across the recovery and impact treatments.

Total lengths of *O. virilis* captured at the REF site (61.69 ± 5.9 mm) were smaller ($p = 0.012 \times 10^{-6}$) than those captured at the AGR (69.11 ± 7.2 mm) and WWT (68.87 ± 8.1 mm) sites. Initial crayfish mass at the beginning of the experiment differed ($p = 0.0003$) among study catchments (REF = 7.85 ± 1.5 g, AGR = 11.21 ± 3.7 g, WWT = 9.75 ± 3.6 g).

Crayfish growth rates were only significantly different in recovery and impact treatments containing crayfish captured in or exposed to the REF site. Crayfish captured in and exposed to the REF site (RR) had a lower growth rate than the other control crayfish (AA $p = 0.018$, WW $p = 0.016$) and crayfish transferred to the REF site (AR $p = 0.005$, WR $p = 0.016$) after two weeks of exposure (Table 3.6; Fig. 3.2). GLM and Tukey's HSD analyses of the impact treatments found that crayfish captured in and exposed to the AGR site (AA) had a larger growth rate than crayfish transferred to the AGR site from the REF site (RA $p = 0.0003$) and the REF control crayfish (RR $p = 0.007$) after two weeks of exposure (Table 3.6; Fig. 3.3). Crayfish moved from the REF site to the WWT site (RW) had a lower growth rate than the WWT control crayfish (WW $p = 0.033$) after one week of exposure but had a similar growth rate after two weeks

Table 3.6. Significant (\neq) and non-significant ($=$) growth rate differences among samples in recovery and impact treatment groups at study catchments in the Red River Valley, Manitoba, Canada in July 2014.

Scenario	Treatment	Week 1	Week 2
Recovery	AGR to REF	AA=AR; AR=RR; RR=AA	AA=AR; AR \neq RR; RR \neq AA
	WWT to AGR	WW=WA; WA=AA; AA=WW	WW=WA; WA=AA; AA=WW
	WWT to REF	WW=WR; WR=RR; RR=WW	WW=WR; WR \neq RR; RR \neq WW
Impact	REF to AGR	RR=RA; RA=AA; AA=RR	RR=RA; RA \neq AA; AA \neq RR
	AGR to WWT	AA=AW; AW=WW; WW=AA	AA=AW; AW=WW; WW=AA
	REF to WWT	RR=RW; RW \neq WW; WW=RR	RR=RW; RW=WW; WW \neq RR

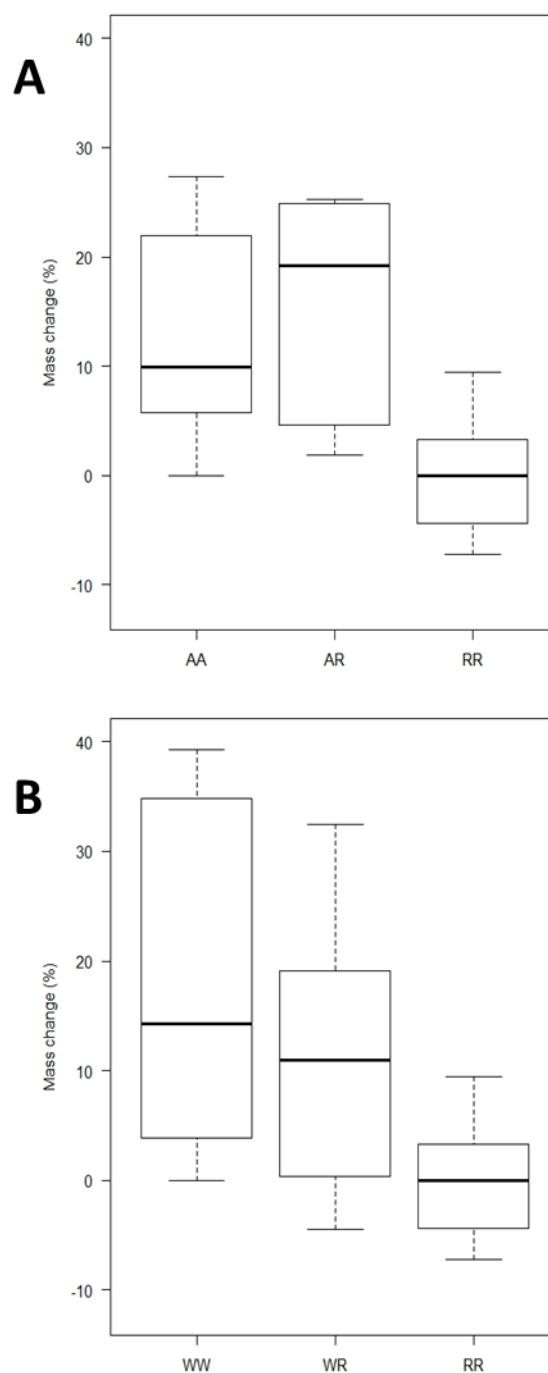


Figure 3.2. Differences in growth rates (mean \pm sd shown) of northern crayfish (*O. virilis*) after two weeks of exposure to the (A) agriculture to reference recovery treatment and (B) wastewater to reference recovery treatment.

(Table 3.6, Fig. 3.3). In contrast, the REF control crayfish (RR) and WWT control crayfish (WW) had similar growth rates after one week of exposure but different growth rates after two weeks of exposure ($p = 0.005$) (Table 3.6; Fig. 3.3).

3.3.3 Metabolomics Analysis of Recovery Treatments

3.3.3.1 AGR to REF

PCA scores of the hepatopancreas metabolome in a multivariate analysis of variance (MANOVA) test showed no difference ($p = 0.49$) by week for the AGR recovery treatment along PC1 or PC2. Metabolome PCA scores were different by week for the tail ($p = 0.001$) and gill ($p = 0.027$) tissues along PC1. Week 1 and week 2 hepatopancreas tissues were combined to create a new AGR recovery treatment group. Tail and gill tissues were separated into week 1 and week 2 AGR recovery treatment groups. These new groups were used in subsequent analyses. PCA scores of the hepatopancreas tissue showed no metabolic separation among metabolomes along PC1 ($p = 0.52$) or PC2 ($p = 0.78$) (Table 3.7). Analyses of tail muscle showed separation between AA/AR and RR metabolomes in the AGR recovery treatment for week 1 ($p = 0.002$) and between AR and RR metabolomes for week 2 ($p = 0.030$) along PC1 (Table 3.7; Appendix B, Fig. B.1). Analyses of gill tissue showed separation between AA and AR/RR metabolomes in the AGR recovery treatment for week 1 ($p = 0.027$) along PC1 and between AA and AR metabolomes for week 2 ($p = 0.034$) along PC2 (Table 3.7). Only the tail and gill AGR recovery treatment groups were used in further metabolic analyses. Leave one out cross validation (LOOCV) found that gill AGR recovery treatments for weeks 1 and 2 had negative Q^2 values and poor model predictability; thus, they were removed from further metabolic analyses. Using VIP scores from PLS-DA

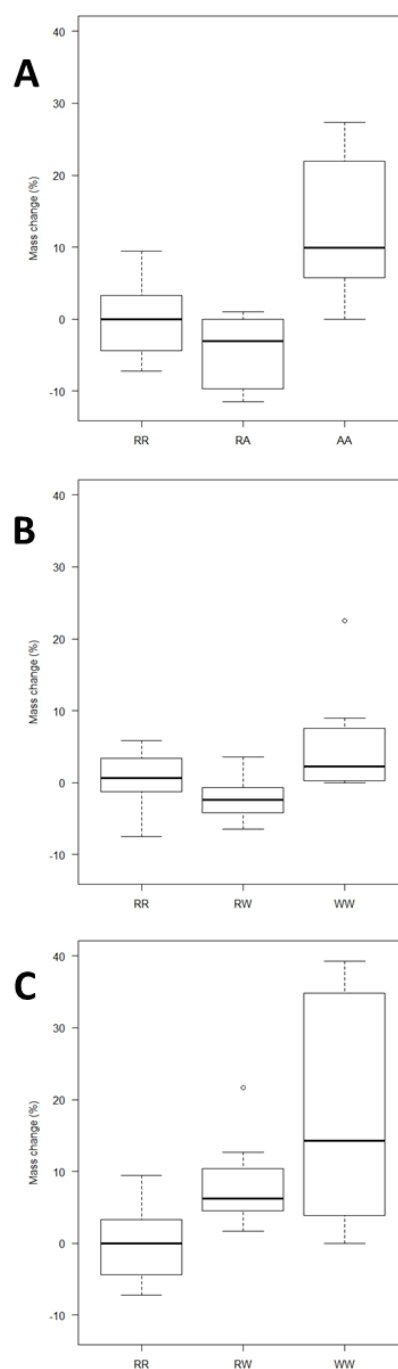


Figure 3.3. Differences in growth rates (mean \pm sd shown) of northern crayfish (*O. virilis*) after (A) two weeks of exposure to the reference to agriculture impact treatment, (B) one week of exposure to the reference to wastewater impact treatment, and (C) two weeks of exposure to the reference to wastewater impact treatment.

Table 3.7. Significant (\neq) and non-significant ($=$) metabolic and weight differences among samples in recovery and impact treatment groups at study catchments in the Red River Valley, Manitoba, Canada in July 2014. Key: *week 1 and week 2*- week significance, *combined*- no significance by week, *red asterisk*- significant differences among samples but poor model predictability (LOOCV).

Scenario	Metric	Treatment	Combined	Week 1	Week 2
Recovery	Hepatopancreas	AGR to REF	AA=AR; AR=RR; RR=AA		
		WWT to AGR	WW=WA; WA=AA; AA=WW		
		WWT to REF	WW \neq WR; WR=RR; RR=WW*		
	Tail	AGR to REF		AA=AR; AR \neq RR; RR \neq AA	AA=AR; AR \neq RR; RR=AA
		WWT to AGR		WW=WA; WA=AA; AA=WW	WW=WA; WA \neq AA; AA=WW
		WWT to REF		WW=WR; WR \neq RR; RR \neq WW	WW=WR; WR=RR; RR=WW
	Gill	AGR to REF		AA \neq AR; AR=RR; RR \neq AA*	AA \neq AR; AR=RR; RR=AA*
		WWT to AGR	WW \neq WA; WA=AA; AA \neq WW		
		WWT to REF	WW \neq WR; WR=RR; RR \neq WW		
Impact	Hepatopancreas	REF to AGR		RR=RA; RA=AA; AA=RR	RR \neq RA; RA \neq AA; AA=RR*
		AGR to WWT	AA=AW; AW=WW; WW=AA		
		REF to WWT		RR=RW; RW=WW; WW=RR	RR \neq RW; RW=WW; WW=RR*
	Tail	REF to AGR		RR=RA; RA=AA; AA \neq RR	RR=RA; RA \neq AA; AA \neq RR
		AGR to WWT		AA=AW; AW=WW; WW=AA	AA=AW; AW=WW; WW=AA
		REF to WWT		RR \neq RW; RW=WW; WW \neq RR	RR \neq RW; RW \neq WW; WW=RR
	Gill	REF to AGR	RR=RA; RA \neq AA; AA=RR		
		AGR to WWT		AA \neq AW; AW=WW; WW \neq AA	AA \neq AW; AW=WW; WW \neq AA*
		REF to WWT	RR \neq RW; RW=WW; WW \neq RR		

models, it was determined that AA/AR tail metabolomes had higher levels of alanine and lower levels of glycine and malonate than RR tail metabolomes in the week 1 AGR recovery treatment, whereas AR tail metabolomes from the week 2 AGR recovery treatment had lower levels of amino acids (i.e., isoleucine, leucine, lysine, threonine, tyrosine), acetoacetate, and malonate than RR tail metabolomes (Table 3.8; Appendix C, Fig C.1).

3.3.3.2 WWT to AGR

MANOVA tests showed no separation by week for hepatopancreas ($p = 0.75$) and gill ($p = 0.50$) metabolome PCA scores along PC1 or PC2 for the WWT to AGR recovery treatment. PCA scores of the tail metabolome showed a difference ($p = 0.049$) by week along PC1. Weeks 1 and 2 hepatopancreas and gill tissues were combined into new WWT to AGR recovery treatment groups. Tail tissues were split by week into separate WWT to AGR recovery treatment groups. Metabolic analyses were conducted using these new groups. There was no difference among hepatopancreas metabolomes in the WWT to AGR recovery treatment along PC1 ($p = 0.53$) or PC2 ($p = 0.90$) (Table 3.7). Tail muscle only showed a metabolic separation ($p = 0.021$) between WA and AA metabolomes during week 2 along PC1 (Table 3.7; Appendix B, Fig. B.1). Analyses of gill tissue showed a separation ($p = 0.002$) between WW and WA/AA metabolomes along PC1 in the WWT to AGR recovery treatment (Table 3.7; Appendix B Fig. B.1). Validated PLS-DA models were only created for week 2 tail and gill WWT to AGR recovery treatments. VIP scores showed that week 2 WA tail metabolomes had higher levels of glutamine, glutamate, and betaine and lower levels of glycine and succinate than week 2 AA tail metabolomes (Table 3.8; Appendix C, Fig. C.2). In the WWT to AGR

Table 3.8. Metabolites in *O. virilis* tail and gill tissues identified by PCA and PLS-DA analyses for **recovery** treatments. Key: --- not analyzed for significant metabolites, - not significantly different among samples, + significantly different among samples. Peak shape key: *s* singlet, *d* doublet, *t* triplet, *dd* double doublet, *m* multiplet.

Metabolites	Chemical Shift [ppm, (multiplicity)]	Tail						Gill			
		AGR to REF		WWT to AGR		WWT to REF		AGR to REF		WWT to AGR	WWT to REF
		1	2	1	2	1	2	1	2	Combined	Combined
<i>Amino Acids</i>											
Alanine	1.48 (d), 3.78 (m)	+	-	---	-	-	---	---	---	-	-
Glutamate	2.05 (m), 2.33 (m), 2.36 (m), 3.75 (t)	-	-	---	+	+	---	---	---	-	-
Glutamine	2.14 (m), 2.44 (m), 3.77 (m)	-	-	---	+	+	---	---	---	-	-
Glycine	3.56 (s)	+	-	---	+	+	---	---	---	-	-
Isoleucine	0.92 (t), 1.00 (d), 1.25 (m)	-	+	---	-	+	---	---	---	-	+
Leucine	0.94 (d), 0.96 (d)	-	+	---	-	+	---	---	---	-	+
Lysine	1.43 (m), 3.01 (t)	-	+	---	-	-	---	---	---	-	+
Serine	3.84 (dd), 3.98 (dd)	-	-	---	-	-	---	---	---	-	-
Threonine	1.32 (d)	-	+	---	-	-	---	---	---	-	+
Tyrosine	3.05 (dd), 6.88 (d), 7.18 (d)	-	+	---	-	+	---	---	---	+	-
Valine	0.98 (d), 1.04 (d)	-	-	---	-	+	---	---	---	-	-
<i>Energy related</i>											
Acetoacetate	2.27 (s) 3.42 (s)	-	+	---	-	-	---	---	---	-	-
Glucose	3.48 (t), 3.52 (dd), 3.70 (t), 3.75 (dd), 3.82 (m), 3.89 (dd), 5.22 (d)	-	-	---	-	-	---	---	---	-	-
Lactate	1.33 (d)	-	-	---	-	-	---	---	---	+	-
Malonate	3.13 (s)	+	+	---	-	-	---	---	---	+	-
NADH	6.94 (s)	-	-	---	-	-	---	---	---	+	-
Succinate	2.39 (s), 3.91 (s)	-	-	---	+	+	---	---	---	-	+
<i>Miscellaneous</i>											
Betaine	3.27 (s), 3.91 (s)	-	-	---	+	-	---	---	---	-	+
Trehalose	3.44 (t), 3.64 (dd), 3.81(m), 3.85 (t), 5.19 (d)	-	-	---	-	-	---	---	---	-	+

recovery treatment, WW gill metabolomes had higher levels of tyrosine, lactate, and NADH but lower levels of malonate than WA/AA gill metabolomes (Table 3.8; Appendix C, Fig. C.2).

3.3.3.3 WWT to REF

Using a MANOVA test, PCA scores showed no difference by week for hepatopancreas ($p = 0.50$) or gill ($p = 0.85$) metabolomes along PC1 or PC2 in the WWT to REF recovery treatments. PCA scores of the tail metabolome were different ($p = 0.0002$) by week along PC1. Hepatopancreas and gill week 1 and 2 tissues were combined into new WWT to REF recovery treatments, while tail week 1 and week 2 tissues were split into separate WWT to REF recovery treatments. These new groups' PCA scores were used in further metabolic analyses. Analysis showed separation between WW and WR hepatopancreas metabolomes approached statistical significance ($p = 0.056$) along PC1 in the WWT to REF recovery treatment (Table 3.7). WW/WR and RR tail metabolomes were only different ($p = 0.002$) during the week 1 WWT to REF recovery treatment (Table 3.7; Appendix B Fig. B.1). A difference ($p = 0.0002$) was seen between WW and WR/RR gill metabolomes along PC2 in the WWT to REF recovery treatment (Table 3.7; Appendix B, Fig. B.1). PLS-DA models were created for all WWT to REF recovery treatments, but LOOCV found that the hepatopancreas treatment had poor model predictability. Hepatopancreas tissues were removed from further metabolic analyses. The week 1 WWT to REF recovery treatment had the most significantly metabolic differences among tail recovery samples (Table 3.8; Appendix C, Fig. C.3). Six amino acids and succinate were found at higher levels, and glycine was found at lower levels in WW /WR tail metabolomes in comparison to RR tail metabolomes. Similarly to the

metabolite changes in the tail muscle, changes in metabolite levels of gill tissues were observed in treatments associated with substantive differences in the stressor environments (i.e., WWT to REF) (Table 3.8; Appendix C, Fig. C.3). In the WWT to REF recovery treatment, WW gill metabolomes had lower levels of amino acids and succinate but higher levels of betaine and trehalose than WR/RR gill metabolomes.

3.3.4 Metabolomics Analysis of Impact Treatments

3.3.4.1 REF to AGR

Multivariate analysis of variance (MANOVA) found metabolome PCA scores were different by week for hepatopancreas tissues ($p = 0.048$) along PC2 and tail tissues ($p = 0.034$) along PC1 in REF to AGR impact treatments. There was no separation ($p = 0.25$) by week for gill metabolome PCA scores along PC1 or PC2. Hepatopancreas and tail week 1 and week 2 tissues were separated into new REF to AGR impact treatment groups. Week 1 and week 2 gill tissues were combined into one REF to AGR impact treatment group. Further metabolomics analyses used the new treatment groups. Metabolic analyses found that RR/AA and RA hepatopancreas metabolomes were only different ($p = 0.009$) along PC1 in the week 2 REF to AGR impact treatment (Table 3.7). PCA scores also showed separation between RR and AA tail metabolomes in the REF to AGR impact treatment for week 1 ($p = 0.052$) and between RR/RA and AA tail metabolomes in the REF to AGR impact treatment for week 2 ($p = 0.005$) along PC1 (Table 3.7; Appendix B, Fig. B.2). PCA scores for the gill metabolome showed separation ($p = 0.046$) between RA and AA gill metabolomes in the REF to AGR impact treatment along PC1 (Table 3.7; Appendix B, Fig. B.2). Only treatment groups with significant metabolic differences among metabolomes were used in subsequent PLS-DA

tests. LOOCV found the week 2 hepatopancreas REF to AGR recovery impact treatment to have poor model predictability, and it was removed from further metabolic analyses. PLS-DA VIP scores determined that RR tail metabolomes had higher levels of glycine and malonate and lower levels of alanine, serine, acetoacetate, glucose and trehalose than AA tail metabolomes in the week 1 REF to AGR impact treatment (Table 3.9; Appendix C, Fig. C.4). In the week 2 REF to AGR impact treatment, RR/RA tail metabolomes had higher levels of leucine and tyrosine but lower levels of alanine, serine, glucose, betaine, and trehalose than AA tail metabolomes (Table 3.9; Appendix C, Fig. C.4). Metabolite identification for gill metabolomes in the REF to AGR impact treatment was not successful with only one significant metabolite identified (Table 3.9; Appendix C, Fig C.4).

3.3.4.2 AGR to WWT

PCA scores of the hepatopancreas metabolomes in a MANOVA test showed no difference ($p = 0.50$) by week along PC1 or PC2 in the AGR impact treatment. However, metabolome PCA scores showed a separation by week for tail tissues along PC1 ($p = 0.003$) and PC2 ($p = 0.025$) and for gill tissues ($p = 0.007$) along PC2. Hepatopancreas tissues were combined to create a new AGR impact treatment group, while week 1 and week 2 tail and gill tissues were separated into separate AGR impact treatment groups. Analyses on these new AGR impact treatment groups showed no separation of hepatopancreas or tail metabolome PCA scores. AA gill metabolome PCA scores were different than AW/WW gill metabolome PCA scores in the AGR impact treatment for week 1 ($p = 0.031$) along PC1 and for week 2 ($p = 0.035$) along PC2 (Table 3.7; Appendix B, Fig. B.2). A PLS-DA model could not be validated for the week 2 gill AGR

Table 3.9. Metabolites in *O. virilis* tail and gill tissues identified by PCA and PLS-DA analyses for **impact** treatments. Key: --- not analyzed for significant metabolites, - not significantly different among samples, + significantly different among samples. Peak shape key: *s* singlet, *d* doublet, *t* triplet, *dd* double doublet, *m* multiplet.

Metabolites	Chemical Shift [ppm, (multiplicity)]	Tail						Gill			
		REF to AGR		AGR to WWT		REF to WWT		REF to AGR	AGR to WWT		REF to WWT
		1	2	1	2	1	2	Combined	1	2	Combined
<i>Amino Acids</i>											
Alanine	1.48 (d), 3.78 (m)	+	+	---	---	+	-	-	-	---	-
Glutamate	2.05 (m), 2.33 (m), 2.36 (m), 3.75 (t)	-	-	---	---	+	-	-	-	---	-
Glutamine	2.14 (m), 2.44 (m), 3.77 (t)	-	-	---	---	+	-	-	-	---	-
Glycine	3.56 (s)	+	-	---	---	+	-	-	-	---	-
Isoleucine	0.92 (t), 1.00 (d), 1.25 (m)	-	-	---	---	-	-	-	-	---	+
Leucine	0.94 (d), 0.96 (d)	-	+	---	---	+	-	-	-	---	+
Lysine	1.43 (m), 3.01 (t)	-	-	---	---	-	-	-	-	---	+
Serine	3.84 (dd), 3.98 (dd)	+	+	---	---	+	+	-	-	---	-
Threonine	1.32 (d), 3.25 (m)	-	-	---	---	-	-	-	-	---	-
Tyrosine	3.05 (dd), 6.88 (d), 7.18 (d)	-	+	---	---	+	-	-	-	---	+
Valine	0.98 (d), 1.04 (d)	-	-	---	---	+	-	-	-	---	-
<i>Energy related</i>											
Acetoacetate	2.27 (s) 3.42 (s)	+	-	---	---	+	-	-	-	---	-
Glucose	3.48 (t), 3.52 (dd), 3.70 (t), 3.75 (dd), 3.82 (m), 3.89 (dd), 5.22 (d)	+	+	---	---	-	-	-	-	---	-
Lactate	1.33 (d)	-	-	----	---	-	-	-	-	---	-
Malonate	3.13 (s)	+	-	---	---	-	-	+	-	---	-
NADH	6.94 (s)	-	-	---	---	-	-	-	-	---	-
Succinate	2.39 (s), 3.91 (s)	-	-	---	---	+	+	-	-	---	+
<i>Miscellaneous</i>											
Betaine	3.27 (s), 3.91 (s)	-	+	---	---	-	+	-	+	---	-
Trehalose	3.44 (t), 3.64 (dd), 3.81(m), 3.85 (t), 5.19 (d)	+	+	---	---	-	+	-	-	---	-

impact treatment, and it was removed from further metabolic analyses. Metabolite identification for the gill metabolomes in the week 1 AGR impact treatment was not successful with only one significant metabolite identified (Table 3.9; Appendix C, Fig. C.5).

3.3.4.3 *REF to WWT*

MANOVA tests found metabolome PCA scores were different by week for hepatopancreas tissues ($p = 0.017$) and tail tissues ($p = 0.014$) along PC2 in REF to WWT impact treatments. However, PCA scores of the gill metabolome showed no separation ($p = 0.13$) by week along PC1 or PC2. Hepatopancreas and tail tissues were organized into week separated REF to WWT impact treatment groups. The gill week 1 and week 2 tissues were combined into one REF to WWT impact treatment. Metabolic analyses were conducted on these new groups. PCA scores showed separation ($p = 0.045$) between RR and RW hepatopancreas metabolomes for the week 2 REF to WWT impact treatment along PC1 (Table 3.7). Analyses also showed separation between RR and RW/WW tail metabolomes in the REF to WWT impact treatment for week 1 ($p = 0.0003$) and between RR/WW and RW tail metabolomes in the REF to WWT impact treatment for week 2 ($p = 0.018$) along PC1 (Table 3.7; Appendix B, Fig. B.2). Significant separation ($p = 0.0003$) was seen between RR and RW/WW gill metabolomes in the REF to WWT impact treatment along PC2 (Table 3.7; Appendix B, Fig. B.2). The hepatopancreas week 2 REF to WWT impact treatment was removed before metabolite identification because the PLS-DA model could not be validated. In the week 1 REF to WWT impact treatment, RR tail metabolomes had higher levels of glycine and lower levels of several amino acids, acetoacetate, and succinate than RW/WW tail metabolomes

(Table 3.9; Appendix C, Fig. C.6). In the week 2 REF to WWT impact treatment, tail metabolite levels were inconsistent and could not be discerned among metabolomes (Table 3.9; Appendix C, Fig. C.6). Isoleucine, leucine, lysine, tyrosine, and succinate were all observed at higher levels in the RR gill metabolomes in comparison to the RW/WW gill metabolomes in the REF to WWT impact treatment (Table 3.9; Appendix C, Fig. C.6).

3.4. Discussion

3.4.1 Evaluation of the Crayfish Metabolome

The present study indicates the potential utility of the crayfish metabolome for assessing aquatic ecosystems as an indicator of stream conditions. One important characteristic of a good indicator is sensitivity to changing environmental conditions (*sensu* Cairns et al., 1993). In the present study, the metabolic sensitivity of the crayfish metabolome to removed (recovery treatments) or added (impact treatments) land-use associated stressors was dependent on tissue. Because the crayfish hepatopancreas plays a significant role in metabolism, immunity, stress response, and detoxification, many studies have detected metabolite changes in the hepatopancreas following exposure to toxicological stress or altered in-stream conditions (Okama and Abe, 1998; Fujimori and Abe, 2002; Alcorlo et al., 2006; Kouba et al., 2010; Shen et al., 2014). Metabolites extracted from hepatopancreas tissue did not detect among site differences for any of the recovery or impact treatments. Thus, the findings of the present study were unexpected. However, a recent study by Watanabe et al. (2015) found that metabolite levels of wet tissue extractions continuously changed during repeated exposure to room temperature, while metabolite levels of dry tissue extractions did not; leading them to conclude that

dry tissue samples have greater stability and may be preferable for NMR analysis. It is possible that metabolites in the present study showed no significant differences among hepatopancreas samples in any recovery or impact treatment because hepatopancreas tissues were extracted wet and degraded during processing. However, other metabolomics studies showed that mussel and clam hepatopancreas tissues extracted wet are sensitive to toxicological pollutants (Liu et al., 2011; Fasulo et al., 2012; Campillo et al., 2015; Ji et al., 2015). Thus, the stressors in this study may not have affected the hepatopancreas. Further studies assessing the impact of land use on dry hepatopancreas tissues are needed to better understand the sensitivity of the hepatopancreas.

Tail tissue was less sensitive than gill tissue to environmental changes associated with recovery treatments. After two weeks of exposure, the tail muscle showed no metabolic recovery in the AGR recovery treatment or in the WWT to AGR recovery treatment. However, tail tissue was more sensitive to impact treatments. Tail tissue exhibited rapid responses to the stressors after one week of exposure, but these responses quickly dissipated before two weeks of exposure. The findings of this study are consistent with other studies that were unable to detect significant metabolic responses after longer exposure periods (Bowron et al., 2009; Davis et al., 2013). Genetic alteration at the population level is a possible explanation for the lack of effect on tail muscle after two weeks of exposure in the recovery and impact treatments. In the present study, crayfish populations captured in sites with more land-use stressors (i.e., AGR, WWT) may have previously (past generations) become tolerant to those stressors through genetic selection. Thus, the stressed metabolic state may have become a tolerable state, reducing or eliminating their ability to “recover” when transferred to a site with fewer land-use

stressors. Additionally, crayfish populations captured in sites with less land-use stressors may have some genetic tolerance to stress, which enabled them to recover after longer exposures to stress. Previous studies have proposed that hypoxia acts as a selective force, leading to genotypic and subsequent phenotypic variations among fish populations (Timmerman and Chapman, 2004; Harniman et al., 2013). Thus, in these studies, populations genetically changed in response to selection pressure for hypoxia tolerant organisms. In a transcriptomics study, Beringer et al. (2014) found gene expression of fathead minnows was significantly different between a site upstream of a WWT effluent source and sites at and below the WWT effluent source. This also suggests that aquatic populations shift in tolerance to aquatic stressors through genetic changes. Overall, the findings of this study suggest that while the tail muscle can rapidly respond to shifts in environmental changes and may be useful as an early detection indicator, it may be ineffective as an indicator of press effects resulting from long term exposure to environmental stress.

The gill tissue most consistently demonstrated sensitivity to recovery and impact treatments. Because gill tissue physically contacts the water and is lipophilic (Alcorlo et al., 2006, Henry et al., 2012), it was expected to show responses to removal (recovery) or addition (impact) of chemical pollutants, such as the veterinary antibiotics and PPCPs in the AGR or WWT sites. The results of this study supported this prediction. Gill tissue response was also consistent regardless of the duration of exposure (i.e., one or two weeks). Thus, the gill may be the more robust tissue for use in long term monitoring programs. The gill metabolome is fast responding (changes after one week) but is still measurable after two weeks of exposure. The findings of this study indicate that

interpretation of metabolic responses may be confounded by tissue sensitivity, so monitoring programs need to consider tissue response before using the crayfish metabolome as an indicator of stream conditions.

Results of this study suggest the crayfish metabolome showed sensitivity to recovery and impact treatments. However, significant metabolic changes among crayfish were most pronounced in treatments associated with substantive differences in the stressor environments (i.e., WWT to REF, REF to WWT). The AGR site had similar DO concentrations as the REF site, but turbidity levels and Erythromycin-H₂O concentrations were more similar to those at the WWT site. Likewise, TN and TP concentrations at the AGR site were higher than at the REF site but lower than at the WWT site. This overlap in stressor conditions between the AGR site and the other two sites may have been the reason few metabolic differences were seen in treatments containing AGR crayfish. Therefore, the crayfish metabolome would be most effective in monitoring sites where the stressor effect is large and substantive differences in concentrations of DO, nutrients, and PPCPs are present.

In the present study, many of the same metabolites were altered in tail and gill tissues when crayfish were transferred between REF and WWT sites. However, the direction of metabolite level changes was dependent on tissue. When crayfish were transferred from the WWT to REF site, WR tail muscle did not recover and remained metabolically similar to the WW tail muscle after one week of exposure. WW/WR metabolomes had higher levels of amino acids and succinate and lower levels of glycine than RR metabolomes. Increased amino acid levels as a response to *in situ* herbicide and heavy metal contaminants have been well documented in metabolomics studies (i.e.,

Tuffnail et al., 2009; Skelton et al., 2014; Fasulo et al., 2012; Campillo et al., 2015; Watanabe et al., 2015) and may be indicative of alterations in cellular energy metabolism (Fasulo et al., 2012; Wagner et al., 2015). Free amino acids would be released from muscle for consumption as an alternative energy source (Fasulo et al., 2012; Wagner et al., 2015). Hypoxic conditions disrupt energy metabolism; thus, a higher level of succinate in WW/WR tail muscles may be indicative of hypoxic stress. Glycine is necessary for the biosynthesis of nitrogenous bases (Fasulo et al., 2012). The high concentrations of TN at the WWT site could have stimulated base biosynthesis, decreasing glycine levels. Alternatively, when crayfish were transferred from the WWT to the REF site, WR gill tissue recovered and became metabolically similar to RR gill samples. WW gill metabolomes had lower levels of amino acids and succinate and higher levels of betaine and trehalose than WR/RR metabolomes. The decrease in amino acid levels of WW gill tissue is in contrast to the increase seen in WW/WR tail muscle. Toxicological contaminants (e.g., herbicides) have been shown to reduce the permeability of gill cell membranes, causing leakage of amino acids from the cells (Cheney et al., 2008). Thus, high concentrations of PPCPs at the WWT site may have altered gill membrane structure and amino acid levels in WW crayfish. Lower gill succinate levels in WW crayfish were unexpected because hypoxic conditions are known to disrupt cellular energy metabolism, decreasing succinate dehydrogenase and increasing succinate levels (Fujimori and Abe, 2002; Nystrom, 2002; Bonvillain et al., 2012). There is one hypothesis as to why lower levels of succinate were measured in WW crayfish. Generations of the crayfish population at the WWT site have been exposed to low DO concentrations. Over time, the fittest crayfish at the WWT site were those who could

tolerate lower DO concentrations, and they were the ones to survive and pass on their genes. Hypoxia thus acted as a selective force, and the crayfish population at the WWT site may have become more genetically tolerant to low DO concentrations. Thus, aerobic respiration continued uninterrupted in gill cells. Additionally, high nitrogen concentrations have been shown to alter acid-neutralizing capabilities of freshwater systems (Camargo and Alonso, 2006); thus, higher levels of betaine suggest WW crayfish experienced oxidative stress in the presence of high TN concentrations. Lastly, higher concentrations of trehalose could be expected in WW crayfish because energy consuming activities would decrease with hypoxia. A similar pattern in metabolite levels as seen in the recovery treatments was observed in tail and gill tissues in the REF to WWT impact treatments. RW/WW tail muscles were metabolically similar and once again had higher amino acid and succinate levels (plus higher acetoacetate levels) and lower glycine levels than RR metabolomes after one week of exposure. RW/WW gill metabolomes were metabolically similar and had lower amino acid and succinate levels than RR metabolomes. In conclusion, recovery and impact treatments alter crayfish metabolites associated with cellular energy metabolism and osmoregulation, but the direction of metabolite changes is dependent on tissue.

It is apparent that metabolic changes in this study could be associated with land-use stressors, but there was no diagnostic power between metabolite changes and specific stressors. Because many site characteristics (e.g., DO, nutrient concentrations) were different among sites, metabolite differences could not be associated to one specific site characteristic. This is in contrast to the controlled laboratory study, which established potential diagnostic relationships between specific stressors (i.e., DO, food stress) and

specific metabolic changes (Izral, Chapter 2). Results were also inconsistent between the lab and field studies. Changes in tail metabolite levels diagnostic of DO stress in the lab experiment were not observable in the tail muscle of crayfish in the WWT site. This suggests that more crayfish laboratory studies evaluating the individual and combined metabolic effects of other natural and toxicological stressors are needed. Such studies would help disentangle cumulative effects *in situ*. Therefore, the field study determined that the crayfish metabolome is sensitive to sites with substantive differences in the stressor environments, but the metabolome was not diagnostic or able to disentangle all the land-use stressors associated with land activities.

One of the most, if not the most, important characteristics of a good indicator is ecological relevance (*sensu* Cairns et al., 1993). The current study did not attempt to relate metabolite changes to individual fitness (e.g. fecundity, survival). However, mass measurements were taken on the first day of the study and after one and two weeks of exposure. These changes in mass measurements are not indicative of individual fitness, but they do provide indirect relationships between weight groups and metabolite groups (Table 6). In the present study, RR crayfish in the AGR to REF recovery treatment had a lower growth rate than AA and AR crayfish after two weeks of exposure. AA and AR crayfish had lower levels of amino acids, suggesting their use in protein synthesis and subsequent mass gain. RR crayfish in the WWT to REF recovery treatment had a lower growth rate than WW and WR crayfish after two weeks of exposure. Levels of amino acids varied in WW and WR gill and tail tissues, and a relationship between amino acid use and mass gain could not be determined. Control crayfish at the two sites with greater land-use associated stressors (AGR (AA) and WWT (WW)) experienced the same

growth rate as their respective experimental crayfish that went into the recovery REF site (AR, WR). Therefore, crayfish that live in sites with greater land-use associated stressors may be genetically tolerant to those stressors and experience no negative effects on individual fitness. Thus, they have no need for recovery. In the impact treatments, crayfish transferred from the REF site to either impact site (AGR, WWT) had a lower growth rate than the control crayfish in those exposure sites (AA or WW) after one or two weeks of exposure. REF crayfish were more stressed in their new exposure environments. Thus, crayfish transferred from a site with less land-use associated stressors to a site with more could experience stress that alters growth. In order to evaluate the effect of altered metabolic pathways on higher levels of biological organization, more studies that combine direct measurements of growth and reproduction with analysis of metabolic changes are needed (Hines et al., 2010).

3.4.2 Summary and Conclusions

Results from the present study demonstrate the potential effectiveness of using the crayfish metabolome as a sensitive bioindicator of added or removed land-use associated stressors. More importantly, the present study shows that tail and gill tissues acclimate to exposure sites. This implies that future monitoring experiments should be conducted as bioassays using crayfish captured or harvested at the same location or site. The results also demonstrate the complexity of metabolomics. Before using the crayfish metabolome in monitoring studies, careful consideration of the monitoring design needs to be taken. This includes tissue choice, monitoring duration (i.e., short vs. long term), site and land-use selection, and deciding to use lab raised or captured crayfish. The results of the present study provide a foundation for using the crayfish metabolome as an indicator, but

further metabolomics studies are needed to disentangle cumulative metabolic effects and relate metabolic responses of land-use associated stressors to crayfish fitness.

3.5 References

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Chapter 4: General Discussion

4.1 Summary

The overall goal of this thesis was to assess the suitability of the crayfish metabolome as an indicator of stream conditions. While aquatic metabolomics studies are becoming more common, both components of this thesis were novel. The laboratory study assessed metabolite sensitivity and diagnostic power of natural in-stream conditions (i.e., DO, food availability). The field study assessed metabolite response to changes in land-use associated stressors. Both studies were the first in the field of metabolomics to test the suitability of the crayfish metabolome as a bioindicator. As stated in the introduction chapter, criteria of good aquatic bioindicators have been well documented and include the following: 1) measurability; 2) sensitivity to stressors; 3) diagnostic of the stressor causing the problem 4) predictive or anticipatory of future conditions; and 5) biologically and socially relevant (*sensu* Hammons, 1981; Suter, 1989; Hunsaker and Carpenter, 1990; Kerr, 1990; Cairns et al., 1993). The main purpose of this summary chapter is to assess how (or if) the crayfish metabolome meets each criterion in the laboratory and/or field studies.

4.1.1 Measurable

Levels of crayfish metabolites were measurable in both the laboratory and field studies. The collection of metabolite samples was easy and quick. Crayfish were sacrificed, and tissues were dissected and flash frozen in liquid nitrogen. However, the identification of significant metabolite peaks posed a greater challenge. Because aquatic metabolomics analysis is relatively new, there is no established protocol to identify significant spectral bins. In this thesis, PCA scores and PLS-DA VIP scores were used

conjointly to detect significant spectral bins. In some other metabolomics studies, spectral bins are identified through other multivariate analyses. Once the significant spectral bins were identified in the lab and field studies, the significant metabolite at each bin had to be identified. Dozens of metabolites were present at some of the spectral peaks, making the identification of the significant metabolite difficult. There was ultimately some level of subjectivity in the identification. Thus, although the crayfish metabolome is measurable, additional metabolomics studies are needed to establish standard NMR analysis and metabolite identification protocols.

4.1.2 Sensitive to Stressors

In both the laboratory and field studies, the crayfish metabolome was sensitive to stressors. In the laboratory study, after fourteen days of exposure, amino acids and osmolytes in tail muscle were sensitive to changes between fed and starved treatments. Amino acids, energy metabolites, and trehalose in tail muscle were sensitive to changes between high and low DO concentration treatments during a fourteen day exposure period. In the field study, tail muscle was sensitive enough to measure metabolic responses to removed (recovery) or added (impact) land-use associated stressors. Gill tissue was more metabolically sensitive to the removal and addition of land-use stressors over a longer exposure period. Both the lab and field studies showed the following: 1) metabolic responses to a specific stressor are dependent on tissue; 2) the crayfish metabolome is sensitive enough to change during seven or fourteen day exposure periods (fast-responding); and 3) significant metabolic responses are most identifiable between treatments or sites with more substantive differences among stressors. However, although the metabolome is quick to respond to changing environmental conditions, the

metabolome may recover quickly as well. Therefore, the crayfish metabolome is sensitive enough to quickly respond to a variety of stressors, such as changes in food availability and DO concentrations and varying land-use activities (i.e., REF, AGR, WWT), but sensitivity varies across tissues and exposure periods.

4.1.3 Diagnostic

The crayfish metabolome showed diagnostic potential in the laboratory study. Glutamine, isoleucine, leucine, lysine, valine, and betaine were identified as metabolites that may be sensitive enough to diagnose food stress in *P. clarkii* tail muscle. Similarly, alanine, glutamate, acetoacetate, succinate, and trehalose were identified as metabolites that may be sensitive enough to diagnose DO stress in *P. clarkii* tail muscle. It is easy to establish cause and effect in controlled laboratory experiments because they lack the ecological complexity of *in situ* field experiments (Munkittrick and McCarty, 1995). In a natural stream ecosystem, anthropogenic inputs (e.g., non-point agricultural runoff, wastewater effluent) and natural in-stream conditions interact to create complex mixtures (Munkittrick and McCarty, 1995; Adams and Greeley, 2000; Malmqvist and Rundle, 2002; Allan, 2004). Thus, it is hard to establish specific cause and effect relationships in field experiments. This was evident in the field study described in chapter 3 of this thesis. *O. virilis* metabolic differences were associated with land-use (REF, AGR, WWT), but many different site-associated stressors were present. Sites had different concentrations of DO, turbidity, total nitrogen, total phosphorous, and PPCPs. The metabolic differences observed could not be definitively linked to any one of these stressors. One of the reasons the lab study was conducted was to establish crayfish biomarkers of food or DO stress that may be identified in the field study. Although some metabolite biomarkers of DO

stress were identified in crayfish exposed to the WWT site (Tables 3.8, 3.9), those metabolite changes lacked true diagnostic power. The lab and field studies provided a foundation for disentangling cumulative effects and identifying crayfish metabolite biomarkers of specific stressors *in situ*, but future metabolomics studies need to further evaluate the crayfish metabolome response to complex mixtures.

4.1.4 Predictive, Biologically and Socially Relevant

The importance of predictability, biological relevance, and societal relevance in biomonitoring is well known. Good bioindicators have greater implications at higher levels of biological organization and are important in maintaining ecosystem integrity (Munkittrick and McCarty, 1995; Adams and Greeley, 2000). However, many ecologically relevant bioindicators are at the population or community levels where ecosystem changes have already occurred (Munkittrick and McCarty, 1995; Adams and Greeley, 2000). The crayfish metabolome was studied as a potential bioindicator in this thesis to see if it could quickly and definitively predict the effect of stressors on metabolic pathways, which may affect organism fitness. Although the lab and field studies assessed the sensitivity and diagnostic power of the crayfish metabolome to a variety of stressors, they did not measure the ecological implication of the observed metabolite changes. Metabolites associated with cellular energy metabolism and osmoregulation were altered in crayfish exposed to adverse natural in-stream conditions (i.e., starvation, low DO concentrations) and sites with greater non-point and point source pollutants (i.e., AGR and WWT sites). However, the disruptions in crayfish cellular energy metabolism and osmoregulation may not ultimately affect individual fitness (e.g., fecundity, survival). These responses may be trivial and of no importance to the organism

or aquatic ecosystem as a whole. Although this thesis did not measure the predictability or biological relevance of crayfish metabolic responses, a study by Hines et al. (2010) discovered that metabolic biomarkers of copper and pentachlorophenol were relevant to organismal fitness in mussels (i.e., scope for growth). Moreover, when indicators have ecological relevance, they have greater societal relevance. Stakeholders are most concerned with alterations at the aquatic ecosystem level, so bioindicators that do not predict ecological changes are less likely to be used in aquatic monitoring programs. Future studies need to measure the ability of the crayfish metabolome to predict more ecologically relevant changes in the environment.

4.1.5 Conclusions and Recommendations

The lab and field studies showed that the crayfish metabolome is measurable, fast responding, sensitive, and potentially diagnostic of specific environmental stressors. However, metabolomics multivariate analyses, differences in tissue and exposure time period sensitivity, and changes in diagnostic power between lab and field studies need to be taken into consideration before using the crayfish metabolome as a bioindicator. Additionally, the crayfish metabolome was not shown to be predictive or biologically and socially relevant. This knowledge gap needs to be assessed further before the crayfish metabolome is widely used as a bioindicator in aquatic biomonitoring programs. Although a large knowledge gap on the utility of the crayfish metabolome as a bioindicator exists, information from this thesis provided the following recommendations for applying the metabolome to biomonitoring programs in the short term: 1) monitored sites need to have substantive differences in site characteristics (e.g., DO concentrations, types of non-point or point source pollutants); 2) experiments should conduct bioassays

with crayfish captured from the same site or lab raised; and 3) tail muscle should be assessed in short term assays, while gill tissue could be applied in longer term assays. Before more research can be conducted on the crayfish metabolome, aquatic biomonitoring programs may be able to use the metabolome as an indicator by following these recommendations.

4.2 Future research

The results of this thesis provided valuable knowledge on the suitability of the crayfish metabolome as a bioindicator of stream conditions; however, it also generated a lot of questions regarding the use of the crayfish metabolome in aquatic monitoring. First of all, the laboratory study only assessed the separate effects of two natural in-stream stressors. This raises the question as to whether the crayfish metabolome can distinguish between food availability and DO stress when exposed to them concurrently. Evaluating the combined effect of multiple natural in-stream stressors and/or anthropogenic stressors (e.g., heavy metal contamination, pharmaceutical and personal care products) on the crayfish metabolome in controlled laboratory studies may help disentangle cumulative effects in future field studies. As a result, the crayfish metabolome would be even more powerful as a bioindicator in aquatic biomonitoring. Moreover, the field study showed that significant metabolic differences were most pronounced in crayfish exposed to sites associated with substantive differences in the stressor environments (e.g., REF to WWT treatment). This suggested that WWT effluent greatly altered the aquatic environment. A gradient metabolomics study measuring metabolic changes across a wide range of exposure levels would holistically assess the impacts and attenuation of wastewater effluent down a stream reach. Additionally, a shorter time-sensitive field study (e.g.,

daily measurements over a two week time frame) evaluating crayfish metabolic responses to land-use associated stressors can better establish the time needed for the metabolome to respond to and recover from such stressors. Findings from such a study would help aquatic biomonitoring programs decide whether to use the crayfish metabolome as an indicator of short duration environmental changes or long term environmental changes. Most importantly, as previously stated, future research needs to assess the ability of the crayfish metabolome to predict ecologically relevant changes. The findings from these future studies in combination with the findings of this thesis would provide a much better understanding of the crayfish metabolome as a bioindicator.

4.3 References

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Appendix A

Table A.5. Pharmaceutical and personal care products (PPCPs) measured at study catchments in the Red River Valley, Manitoba, Canada in July 2014.

Compound	
1	Acetaminophen
2	Azithromycin
3	Bisphenol A
4	Caffeine
5	Carbadox
6	Carbamazepine
7	Cefotaxime
8	Ciprofloxacin
9	Clarithromycin
10	Clinafloxacin
11	Cloxacillin
12	Dehydronifedipine
13	Diphenhydramine
14	Diltiazem
15	Digoxin
16	Digoxigenin
17	Enrofloxacin
18	Erythromycin-H ₂ O
19	Furosemide
20	Flumequine
21	Fluoxetine
22	Gemfibrozil
23	Glipizide
24	Glyburide
25	Hydrochlorothiazide
26	2-Hydroxy-Ibuprofen
27	Ibuprofen
28	Lincomycin
29	Lomefloxacin
30	Miconazole
31	Naproxen
32	Norfloxacin
33	Norgestimate
34	Ofloxacin
35	Ormetoprim
36	Oxacillin
37	Oxolinic Acid
38	Penicillin G
39	Penicillin
40	Roxithromycin
41	Sarafloxacin
42	Sulfachloropyridazine
43	Sulfadiazine
44	Sulfadimethoxine
45	Sulfamerazine
46	Sulfamethazine
47	Sulfamethizole
48	Sulfamethoxazole
49	Sulfanilamide
50	Sulfathiazole
51	Thiabendazole
52	Triclocarban
53	Triclosan
54	Trimethoprim
55	Tylosin
56	Virginiamycin M1
57	Warfarin
58	1,7-Dimethylxanthine

Appendix B

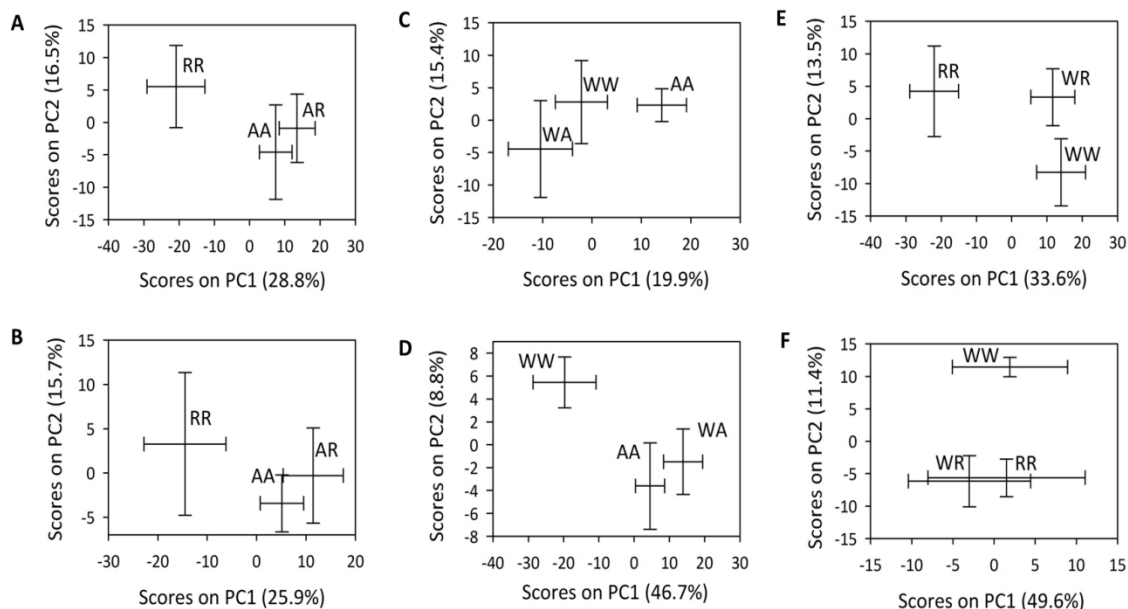


Figure B.1. PCA score plots of northern crayfish (*O. virilis*) metabolomes used in recovery treatments. (A) week 1 tail AGR to REF recovery treatment, (B) week 2 tail AGR to REF recovery treatment, (C) week 2 tail WWT to AGR recovery treatment, (D) gill (weeks 1 + 2) WWT to AGR recovery treatment, (E) week 1 tail WWT to REF recovery treatment, and (F) gill (weeks 1 + 2) WWT to REF recovery treatment. The bars represent the mean of the score for each tissue and its corresponding standard error.

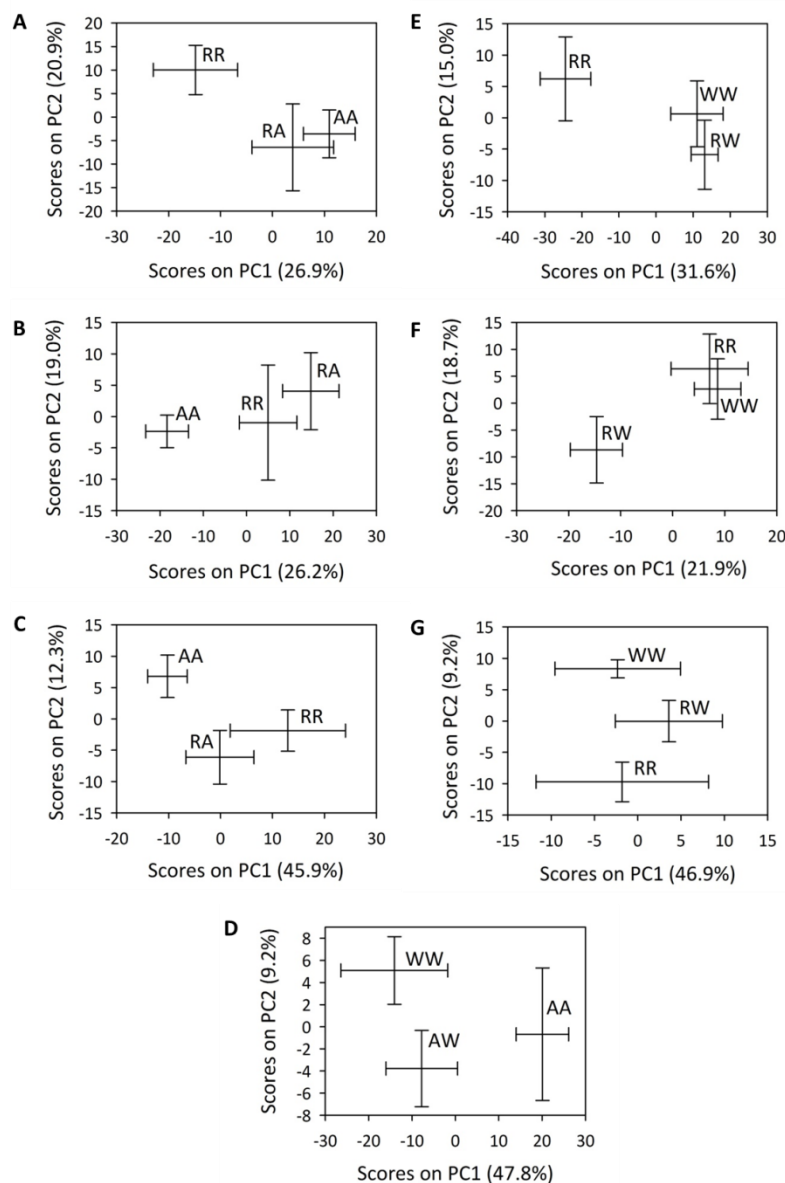


Figure B.2. PCA score plots of northern crayfish (*O. virilis*) metabolomes used in impact treatments. (A) week 1 tail REF to AGR impact treatment, (B) week 2 tail REF to AGR impact treatment, (C) gill (weeks 1 + 2) REF to AGR impact treatment, (D) week 1 gill AGR to WWT impact treatment, (E) week 1 tail REF to WWT impact treatment, (F) week 2 tail REF to WWT impact treatment, and (G) gill (weeks 1 + 2) REF to WWT treatment. The bars represent the mean of the score for each tissue and its corresponding standard error.

Appendix C

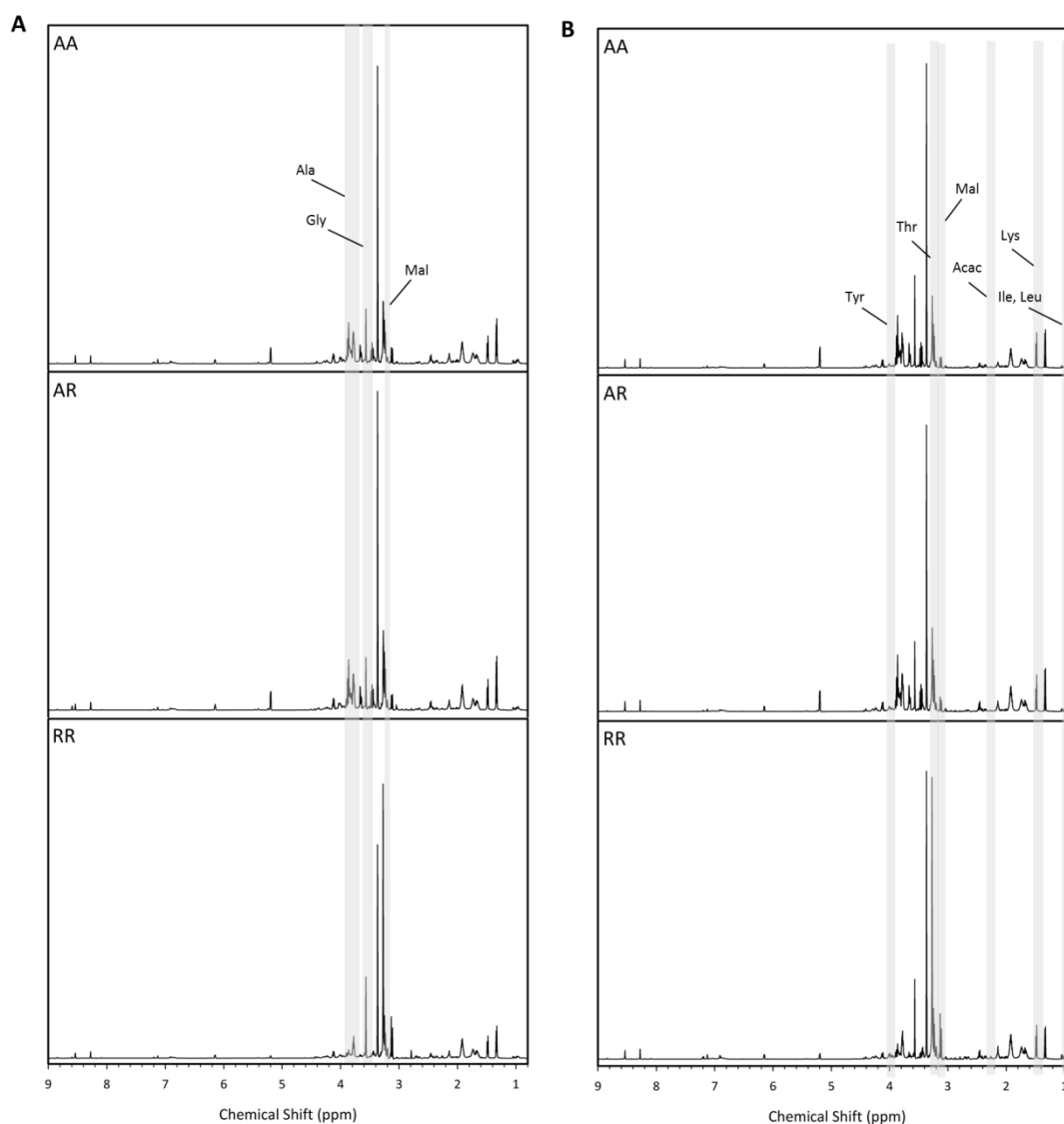


Figure C.4. Average 1-D 600 MHz ^1H NMR spectra from the tail metabolomes of northern crayfish (*O. virilis*) exposed to the AGR to REF recovery treatment for (A) 1 week and (B) 2 weeks. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Acac*- acetoacetate, *Ala*- alanine, *Gly*- glycine, *Ile*- isoleucine, *Leu*- leucine, *Lys*- lysine, *Mal*- malonate, *Thr*- threonine, *Tyr*- tyrosine.

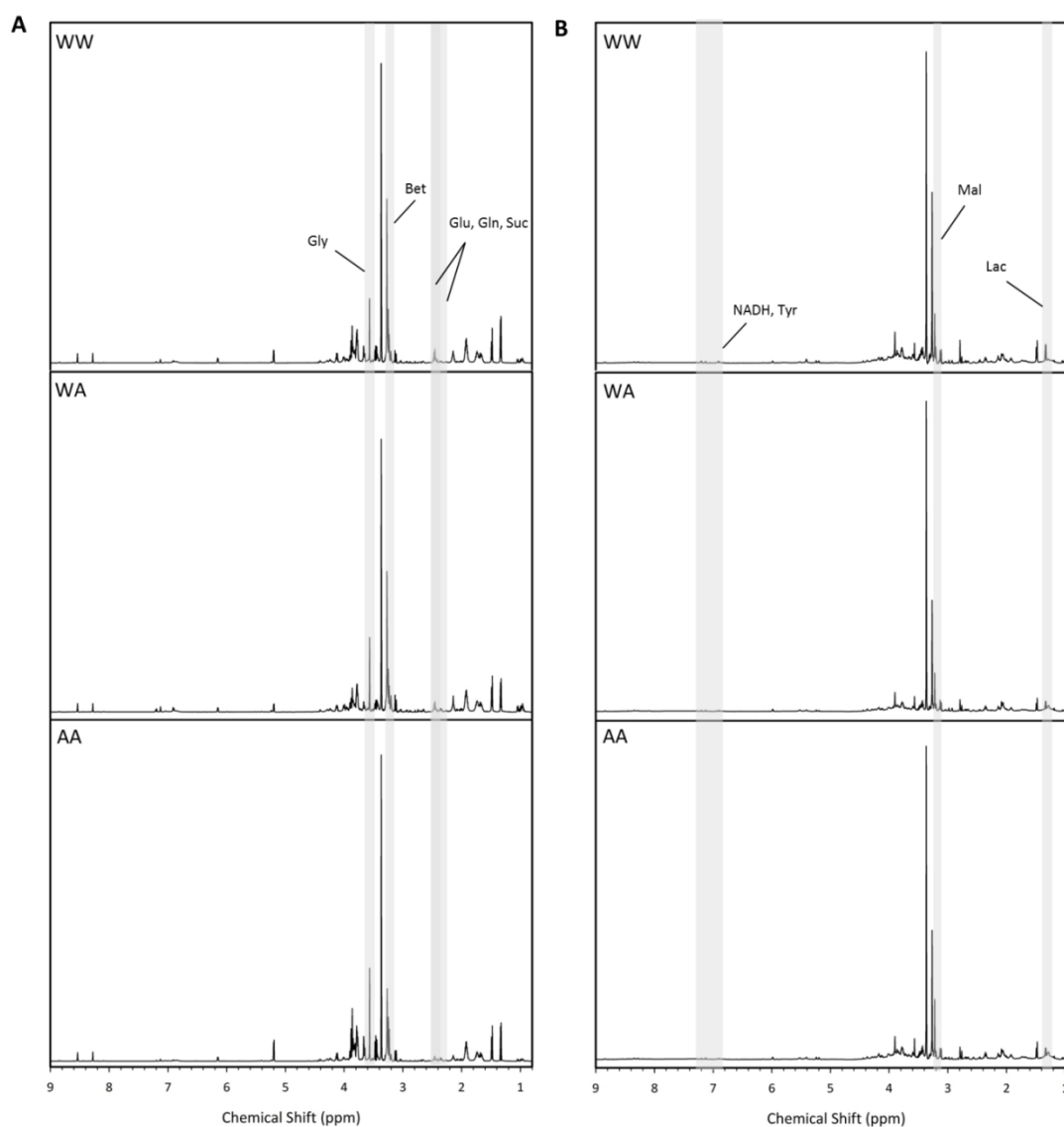


Figure C.2. Average 1-D 600 MHz ^1H NMR spectra of northern crayfish (*O. virilis*) metabolomes exposed to the WWT to AGR recovery treatment for (A) week 2 tail muscle and (B) gill tissue. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Bet*- betaine, *Gln*- glutamine, *Glu*- glutamate, *Gly*- glycine, *Lac*- lactate, *Mal*- malonate, *NADH*- nicotinamide adenine dinucleotide, *Suc*- succinate, *Tyr*- tyrosine.

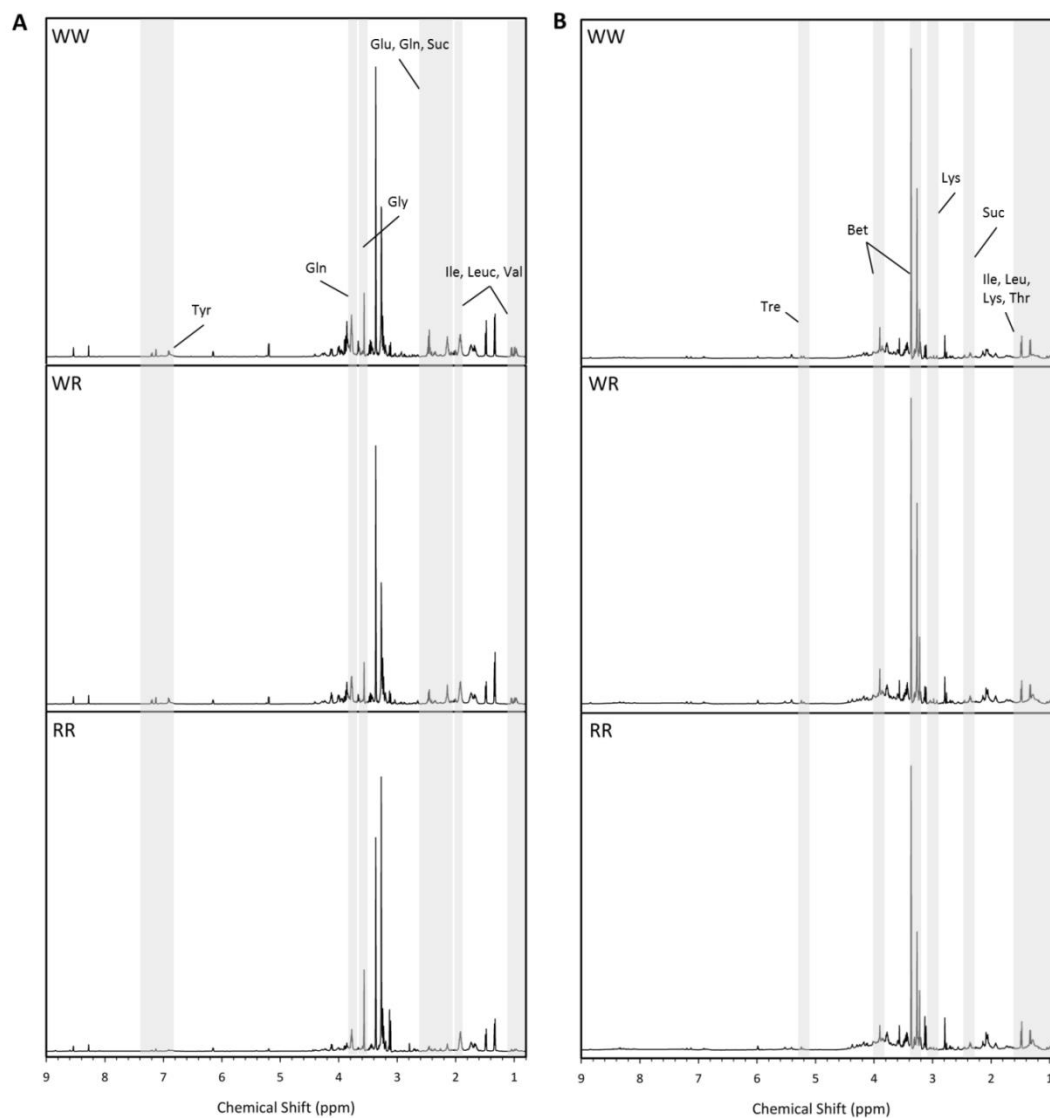


Figure C.3. Average 1-D 600 MHz ^1H NMR spectra of northern crayfish (*O. virilis*) metabolomes exposed to the WWT to REF recovery treatment for (A) week 1 tail muscle and (B) gill tissue. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Bet*- betaine, *Gln*- glutamine, *Glu*- glutamate, *Gly*- glycine, *Ile*- isoleucine, *Leu*- leucine, *Lys*- lysine, *Suc*- succinate, *Thr*- threonine, *Tre*- trehalose, *Tyr*- tyrosine, *Val*- valine.

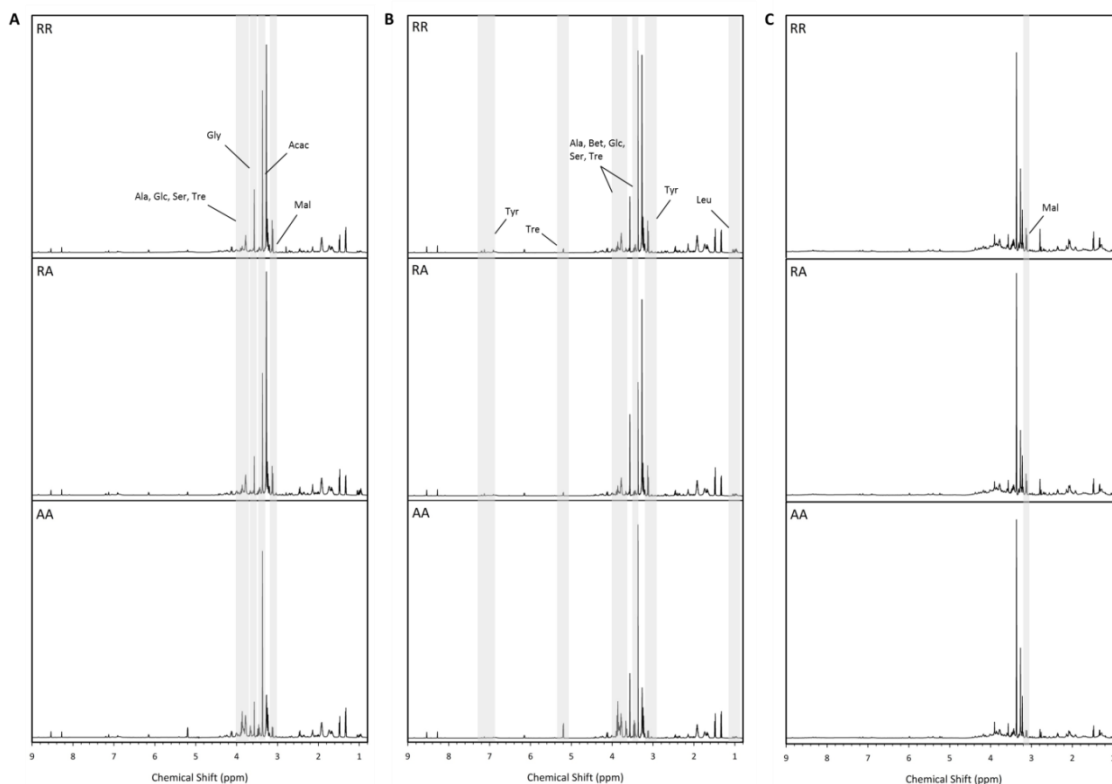


Figure C.4. Average 1-D 600 MHz ^1H NMR spectra of northern crayfish (*O. virilis*) metabolomes exposed to the REF to AGR impact treatment for (A) week 1 tail muscle, (B) week 2 tail muscle, and (C) gill tissue. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Acac*- acetoacetate, *Ala*- alanine, *Bet*- betaine, *Glc*- glucose, *Gly*- glycine, *Leu*- leucine, *Mal*- malonate, *Ser*- serine, *Tre*- trehalose, *Tyr*- tyrosine.

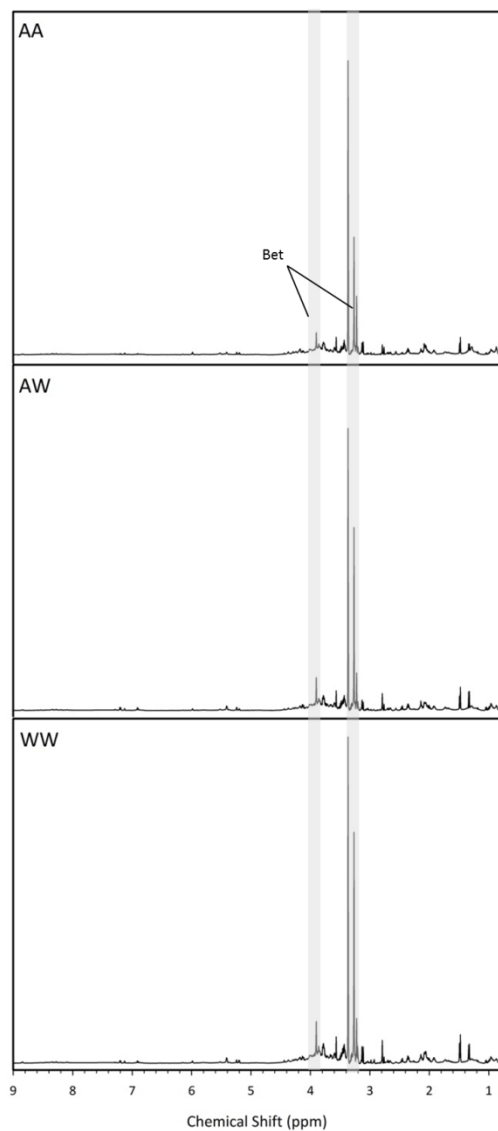


Figure C.5. Average 1-D 600 MHz ¹H NMR spectra of northern crayfish (*O. virilis*) gill tissue exposed to the AGR to WWT impact treatment for one week. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Bet*- betaine.

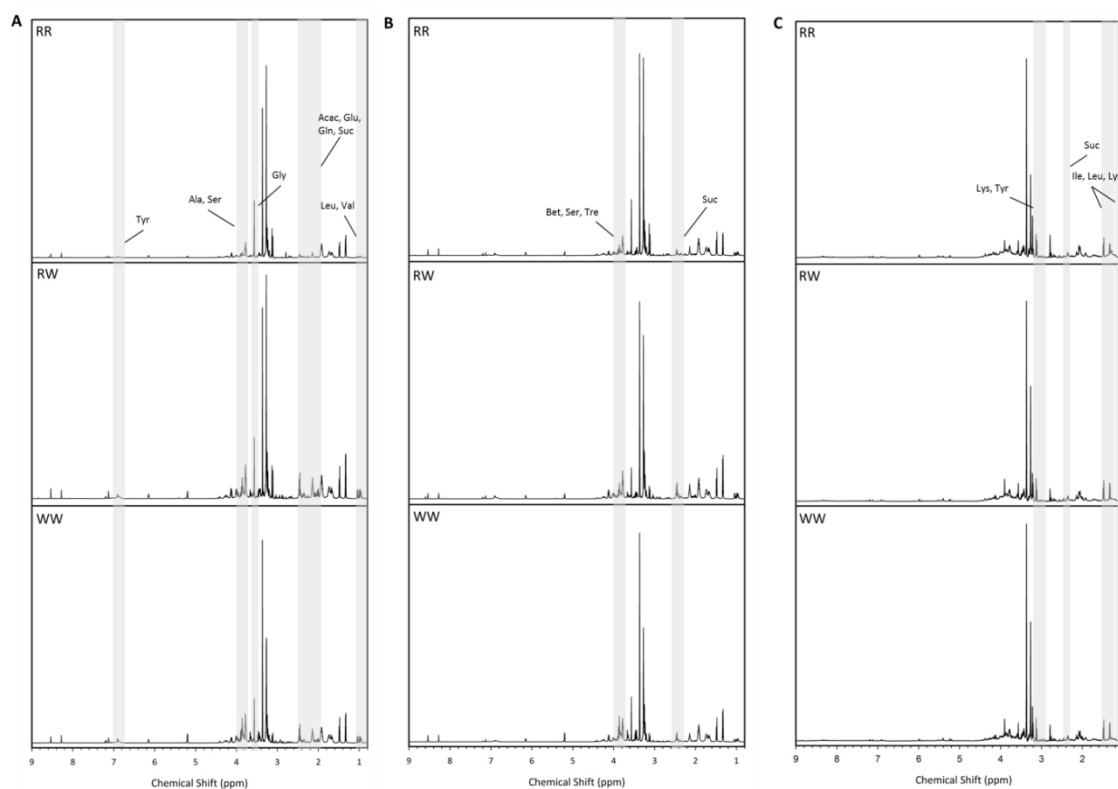


Figure C.6. Average 1-D 600 MHz ^1H NMR spectra of northern crayfish (*O. virilis*) metabolomes exposed to the REF to WWT impact treatment for (A) week 1 tail muscle, (B) week 2 tail muscle, and (C) gill tissue. The spectra are displayed using the same Y-scale. Significant metabolite peak changes addressed in the text are emphasized with shaded bars. Key: *Acac*- acetoacetate, *Ala*- alanine, *Bet*- betaine, *Gln*- glutamine, *Glu*- glutamate, *Gly*- glycine, *Ile*- isoleucine, *Leu*- leucine, *Lys*- lysine, *Ser*- serine, *Suc*- succinate, *Tre*- trehalose, *Tyr*- tyrosine, *Val*- valine.

Curriculum Vitae

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EDUCATION

University of Western Ontario; London, ON, Canada; *2013-2016*

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- Canadian Rivers Institute Days (PEI), Poster presenter, *Oct 2014*
- MRS (CO) Summer Undergraduate Research Symposium I, Oral Presenter, *Aug 2011*

RESEARCH AND RELEVANT EXPERIENCE

Graduate Student Teaching Assistant **Fall 2013, Spring 2014, Spring 2015**

University of Western Ontario, Dept. of Geography, London ON

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Dr. Adam Yates and Dr. Bob Brua, National Hydrology Research Centre, Saskatoon SK

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- Identified benthic invertebrate samples from streams in the Grand River Watershed, ON, Canada. Investigated hydrological and vegetation sampling techniques appropriate for assessing water quality/quantity impacts associated with agricultural and wastewater treatment activities and BMPs in the Lake Winnipeg Basin, MB, Canada.

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Field Assistant**May 2012 – Aug 2012**Dr. Sean Cahoon, Penn State University, *in situ* Greenland

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- Spanish language comprehension
- Aquatic Wild Certification and youth and community outreach experience
- Experience living and working in remote areas