Biomolecular Responses of Freshwater Organisms to Environmental Stressors

Submitted by

Manisha Shakya

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Department of Ecology, Environment and Evolution

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Table of Contents

List of Figures

List of Tables

Abstract

Environmental stressors such as changes in water quality and presence of contaminants in freshwaters can cause negative impacts on aquatic organisms by altering their molecular and physiological processes. Changes to the biomolecular makeup of aquatic organisms such as changes to their proteome and amino acid (AA) profile have been suggested as a potential sensitive indicator of environmental stress. However, information regarding the influence of environmental stressors on the overall AA profile (i.e. protein + peptide + free AAs) and proteome of aquatic organisms is currently limited. This project aimed to explore if these biomolecules are significantly affected by environmental stressors, using both field and laboratory experiments. The major objectives of this project were to determine: (i) if changes in water quality along a river continuum influence the AA profile of freshwater macroinvertebrates using two case studies: an uncontaminated river system (Murray-Darling Basin) and a contaminated river (Dee River), and (ii) the effect of waterborne copper (Cu) on the biomolecular makeup (AA profile (algae and fish), proteome (algae and fish); element distribution (fish) and chemical composition of tissues (fish)) of freshwater organisms at environmentally realistic concentrations using algae and fish as model organisms.

The field-based studies showed that the AA profile of macroinvertebrates is directly related to taxonomy with individuals from different taxa containing different AA profiles. Within the uncontaminated river system, season and sites (geographic distribution) were shown to influence the AA profile of decapods, however, no effect was shown for the caddisfly (*Triplectides* sp.) and the snail (*Physa* sp.). Contamination of a river system with acid mine drainage significantly influenced the AA profile of all macroinvertebrate taxa studied and also altered the overall macroinvertebrate community composition at contaminated sites. This study highlights the potential of using AA profiling to study the response of aquatic organisms to environmental stressors.

In controlled laboratory studies, Cu was shown to be highly toxic to *Chlorella* sp. (growth inhibition; EC_{10}: 1 µg L^{-1} and EC_{50}: 2 µg L^{-1}: effective concentration (EC_x) at which x% effect observed) and moderately toxic to Purple-spotted Gudgeons (Loss of equilibrium: EC_{10}: 12 µg L^{-1} and EC_{50}: 22 µg L^{-1}). Analysis of the green alga (*Chlorella* sp.) exposed to Cu concentrations representative of EC_{50} (2 µg L^{-1}) revealed a significant change in the AA profile and proteome of the algae. Proteomic analysis showed that exposure to Cu was associated with the up-regulation of proteins involved in cellular processing and signalling

and down-regulation of proteins involved in information storage and processing. Copper also affected proteins involved in the regulation of AAs as well as carbohydrate and energy metabolism. Similarly, the exposure of Purple-spotted Gudgeon sacfry to Cu > 5 µg L^{-1} over 96 h significantly changed the AA profile of the organism. Using proton-induced x-ray emission (PIXE) microscopy, Cu was found to be accumulated in the retinal tissues of sacfry and was associated with an increase in zinc (Zn) concentrations. The absence of Zn in the growth media suggests that copper exposure causes a redistribution of Zn in the organism and this is related to the role of Zn in rhodopsin. Fourier transform infrared analysis also showed a shift in protein structure of the retinal tissues in response to Cu exposure.

Overall, exposure to natural and anthropogenic environmental stressors caused significant changes to the whole body AA profile of aquatic organisms. Changes to the biomolecular makeup (AA profile, proteome, elemental distribution, chemical composition of tissues) in response to Cu exposure were shown at concentrations lower than those required to cause significant effects to chronic toxicity indicators such as growth rate and loss of equilibrium. The use of biomolecular markers to determine responses to environmental stressors such as Cu may prove a more sensitive endpoint then traditional chronic toxicity endpoints.

Statement of Authorship

“Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.”

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MS performed experimental design, sample analysis, data analysis and wrote the manuscript. AH, GR and ES provided supervision and contributed to experimental design, data analysis and editing. JL and JCM contributed to Proton induced X-ray emission (PIXE) analysis of samples and data acquisition.

Chapter One

General Introduction

1.1 Background to the research

Environmental stressors in waterways, both natural (rising temperatures; salinity) and anthropogenic (metals, contaminants, etc.) negatively impact aquatic biota and are of great concern globally (Gonçalves et al., 2017). Changes in water quality and the presence of contaminants in waterbodies can have negative effects on the metabolic and physiological processes in aquatic organisms (Filimonova et al., 2016a; Mahboob et al., 2019). Upon exposure to stressors, organisms are generally able to adapt to a small range of fluctuations. Beyond a certain limit, detrimental changes to organisms occur, triggering responses within different levels of biological organisation within freshwater ecosystems (Figure 1.1) (Arambourou et al., 2020; Gambi et al., 2020).

At the organism level, responses to stressors can be observed through changes in growth rate, reproduction, behaviour and even death of the organism. The impact of stressors may then magnify up to influence populations, communities and eventually affect the entire ecosystem (Figure 1.1) (Taylor & Maher, 2010). For instance, the impact of environmental stressors on organism will change population dynamics like age/sex ratio, genetic structure and abundance (Zvereva & Kozlov, 2006; Howse, 2007; Fischer et al., 2012). Furthermore, serious adverse effects such as decreased species richness, loss of species, changes in community composition, loss of biodiversity, changes in the food web and biogeochemical cycles have been noticed at the community and ecosystem levels (Mackey, 1988; Chi et al., 2017; Gambi et al., 2020). Studies into the responses of environmental stressors at the population, community and ecosystem levels have high ecological relevance, however, they generally do not show a direct link between the organism and the stressor (Taylor & Maher, 2010). Concentrations of contaminants in water might not reflect potential effects to organisms either as this will depend on their bioavailability and accumulation potential, and the organism's ability to respond and regulate defence mechanisms (Taylor & Maher, 2010).

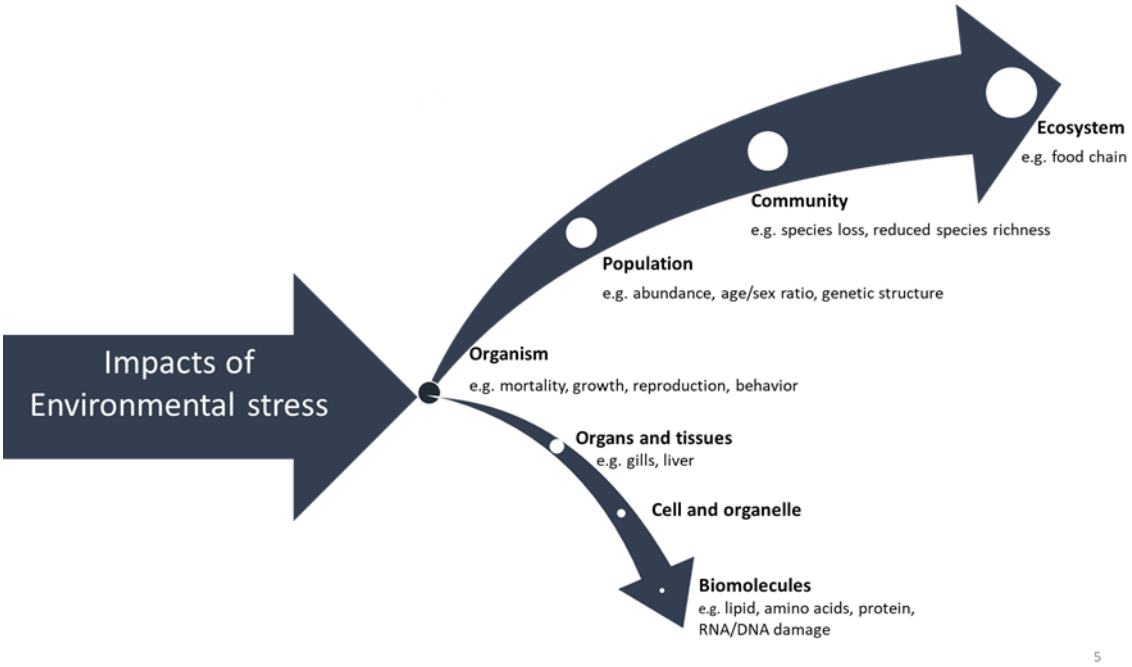

Figure 1.1. Impacts of environmental stressors on different levels of biological organisation.

At the organism level, response mechanisms are the result of a process in which organisms try to adapt to the stressor. Studies of the biomolecular changes in organisms due to exposure to contaminants or natural environmental stressors help to establish a connection between the external stressor and adverse impacts at the cellular level and provides insight into the mechanisms underlying the effects observed at the organism level (Anjum et al., 2014). Recent studies have shown that abiotic stressors can damage biomolecules such as proteins, deoxyribonucleic acid (DNA), fatty acids, and amino acids (AAs) of aquatic organisms (Hussain et al., 2019; Shekh et al., 2020; Previšić et al., 2020; León-Vaz et al., 2020). Biomolecular measures of toxicant-induced stress are suggested to be specific and sensitive indicators of exposure to a stressor (Rajeshkumar et al., 2017). Given that changes occur initially at the biomolecular level before impacts are observed at the organism level and higher levels of biological organisation, such measures may be useful to predict the potential impacts of stressors within freshwater ecosystems (Gonçalves et al., 2017). This thesis explores the effects of abiotic environmental stressors on the biomolecular composition of aquatic organisms with a particular focus on changes to whole body AA profiles.

1.2 Fundamentals of amino acids

Amino acids are organic molecules that contain an amino ($-NH_2$) and a carboxylic acid (-COOH) functional group attached to a carbon atom, hence the name “amino acids”. The properties and function of each AA are defined by a unique side chain group known as the

R-group attached to the carbon atom (Table 1.1) (Wu, 2009). These organic molecules are generally stable in aqueous solutions at the physiological pH range except for glutamine (Gln) and cysteine (Cys). Gln is cyclized to pyroglutamate and Cys undergoes rapid oxidation to form cystine (Cys-Cys) which is the dimer of Cys (Wu, 2009). Amino acids in a biological system are either metabolized or incorporated into three 'domains' that include free amino acids (FAAs), peptide AAs, and proteome AAs (Gu et al., 2015). The AA profile of an organism consists of AAs from all three domains. Amino acids assemble via amide bonds (also known as peptide linkages) to form peptides and proteins (Kelly & Pearce, 2020). Each protein is unique and has a definite function based on which AAs it contains and how they are arranged (Betts & Russell, 2003). Among different AAs found in an organism, the twenty standard AAs are listed in Table 1.1 and are the common building blocks of proteins (Wu, 2009).

Not all organisms are capable of synthesizing all AAs and instead rely on their food sources to obtain AAs they cannot produce. For instance, only autotrophs such as plants, algae and bacteria can synthesize all 20 primary AAs by assimilating inorganic forms of nitrogen like ammonium (NH_4^+) and nitrate (NO_3^-). Animals on the other hand are not capable of synthesizing all AAs and must obtain certain AAs from their diet (Kelly & Pearce, 2020). This requirement leads to the division of AAs into two categories: essential and non-essential. Amino acids that cannot be produced in sufficient amounts by animals (eg: mammals, fish, macroinvertebrates) are classified as nutritionally essential AAs (EAA) and consist of arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val), whereas those that can be produced are categorised as non-essential amino acids (NEAA). Non-essential AAs consist of alanine (Ala), asparagine (Asn), aspartate (Asp), glutamate (Glu), Gln, glycine (Gly), serine (Ser), tyrosine (Tyr), Cys and proline (Pro) (Wu, 2009).

Table 1.1. Properties of twenty proteogenic amino acids.

Name	Short-form	Properties	Essential/Non-essential
Alanine	Ala	Non-polar, Aliphatic	Non-essential
Arginine	Arg	Positively charged, Basic, Polar	Essential
Asparagine	Asn	Polar, Non-charged	Non-essential
Aspartic acid	Asp	Negatively charged, Polar	Non-essential
Cysteine	Cys	Polar, Non-charged	Non-essential
Glutamic acid	Glu	Negatively charged, Acidic, Polar,	Non-essential
Glutamine	Gln	Polar, Non-charged	Non-essential
Glycine	Gly	Non-polar, Aliphatic residue	Non-essential
Histidine	His	Positively charged, Basic, Polar	Essential
Isoleucine	Ile	Non-polar, Aliphatic residue, BCAA*	Essential
Leucine	Leu	Non-polar, Aliphatic residue, BCAA*	Essential
Lysine	Lys	Positively charged, Basic, Polar	Essential
Methionine	Met	Polar, Non-charged	Essential
Phenylalanine	Phe	Aromatic	Essential
Proline	Pro	Non-polar, Aliphatic residue	Non-essential
Serine	Ser	Polar, Non-charged	Non-essential
Threonine	Thr	Polar, Non-charged	Essential
Tryptophan	Trp	Aromatic	Essential
Tyrosine	Tyr	Aromatic	Non-essential
Valine	Val	Non-polar, Aliphatic residue, BCAA*	Essential

*BCAA (Branched-chain amino acid)

1.3 Role of amino acids in organisms

Amino acids are the fundamental units of life with a crucial role in the maintenance of physiological functions within organisms (Bröer & Bröer, 2017). The role of AAs in protein synthesis is well documented (Betts & Russell, 2003). As a component of proteins, AAs are necessary for the production of enzymes, hormones, structural materials, and also play a role in nutrient regulation for the growth of organisms (Hayat et al., 2012). Every AA has a unique function in protein synthesis (Betts & Russell, 2003). For instance, the sulfur (S)-containing AA, Cys, helps in protein folding and stabilization of proteins by forming disulphide bonds (Betts & Russell, 2003). Glycine with its single hydrogen makes it the only AA that can bind with adenosine triphosphate (ATP) (Betts & Russell, 2003).

Apart from protein synthesis, AAs take part in many metabolic processes that help in the growth and development of organisms (Figure 1.2). Some of the AAs are important regulators of key cellular metabolic pathways for energy production like glycolysis and the tricarboxylic acid (TCA) cycle (Nie et al., 2018; Kelly & Pearce, 2020). For instance,

BCAAs: Ile, Leu and Val help in translocation of glucose transporters to promote glycolysis and provide coenzyme A (CoA) intermediates to the TCA cycle (Nie et al., 2018). Amino acids are converted to acetyl-CoA and oxidized to CO_2 and H_2O via the TCA cycle, however, the use of AAs is less efficient for ATP production compared to fat and glucose (Wu, 2009). Sulfur-containing AAs (Cys and Met) help in sulfur and redox metabolism by taking part in the synthesis of glutathione that helps to detoxify reactive oxygen species (ROS) produced and maintains the intracellular redox balance in cells (Kelly & Pearce, 2020). During stressed conditions, cells require a high amount of energy in the form of ATP (Bonora et al., 2012). Animals obtain their energy through glycolysis, the TCA cycle and oxidative phosphorylation, with AAs regulating these interconnected processes (Kelly & Pearce, 2020). Besides, energy production and protein synthesis, AAs have different regulatory functions in organisms. For example, AAs also help with the synthesis of important metabolites like glutathione, glycine betaine, ammonia, nitric oxide, urea, polyamines, fatty acids, purines, and other nitrogenous substances that are of biological importance (Conceição et al., 1998; Wu, 2009). They also play major roles in signalling pathways, regulating gene expression and immunity systems during stressed conditions (Yang et al., 2001; Kilberg et al., 2005; Abdel-Salam, 2014; Xie et al., 2015). Amino acids also play significant roles in the stress response and environmental stressors can change the AA profile of living organisms (Batista-Silva et al., 2019).

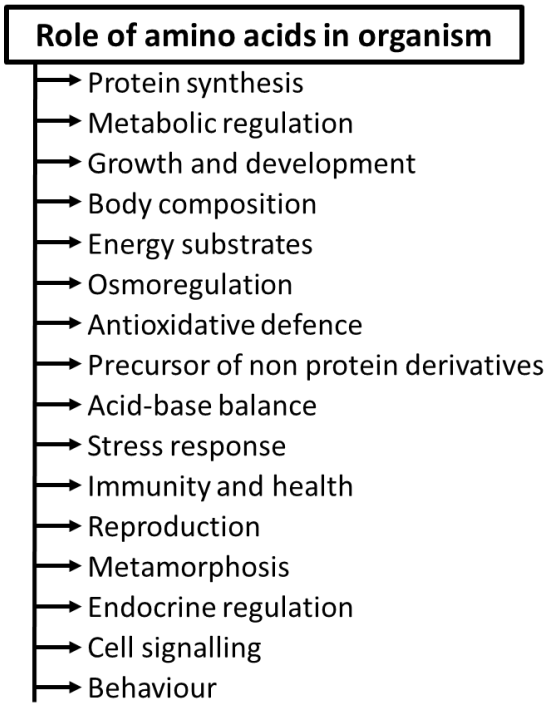

Figure 1.2. The metabolic role of amino acids.

All twenty AAs are required for the proper functioning of cells (Wu, 2009; Kelly & Pearce, 2020). A constant turnover of proteins helps in recycling of most of the AAs, with the loss of AAs mainly due to oxidation for energy production. Homeostasis is maintained through a highly regulated process involving the exchange of EAA with NEAA and transfer of amino groups from oxidised AAs to biosynthesis pathways of AAs (Bröer & Bröer, 2017). Whole body AA homeostasis can be disturbed due to deficiency of EAAs in the diet as well as abnormal metabolism of AAs, impacting growth and development of organisms and even causing death.

1.4 Interspecific variation in amino acid profiles

Energy transfer efficiency in foodwebs is largely regulated by the nutritional quality of food at each trophic level (Vesterinen et al., 2020). Amino acids are one of the main biomolecules transferred across the food web (Dwyer et al., 2018). The nutritional value of diets depend on many factors; the AA profile being one of them (Ruess & Müller-Navarra, 2019). Producers such as algae and other green plants are the major source of EAAs in a food web (Kolmakova & Kolmakov, 2019). Consumers have to depend on dietary sources to acquire EAAs. This dependency has developed AAs as an informative biomolecular tracker for assessing the prey-predator relationship in aquatic food webs (Ventura & Catalan, 2010). Food must contain the right proportion of EAAs, as EAAs determines the growth and development, fitness, and reproductive success of an organism in each trophic level within a food web (Guisande, 2006). However, the AA profile of organisms is one of the least investigated areas in the field of ecology (Ruess & Müller-Navarra, 2019; Kolmakova & Kolmakov, 2019).

Amino acid profiles of living organisms have distinct patterns controlled by their genetic information encoded by DNA (Sorimachi, 1999; Pe'er et al., 2004; Bogatyreva et al., 2006; Moura et al., 2013) and suggest that even small variations in AA profiles may be biologically significant in distinguishing different species (Gurure et al., 2007). Each taxon therefore should have a unique AA profile (Guisande, 2006). This has been demonstrated for a few aquatic organisms present in lakes (James et al., 1989; Conceição et al., 1998; Thera et al., 2020; Vesterinen et al., 2020). For example, phytoplankton have a higher protein content and hence AA content compared to aquatic macrophytes, due to the presence of other structural components like lignin, cellulose and hemicellulose in the aquatic macrophytes (Ruess & Müller-Navarra, 2019). A recent study conducted by Thera et al. (2020) on the AA profiles of benthic macroinvertebrates from different functional

feeding groups, zooplankton, biofilm and fish from temperate lakes showed each taxon had a significantly distinct AA profile, controlled by both phylogeny and functional feeding groups. They further reported seasonal variations in the AA profile of biofilms, due to change in the community composition of biofilms between seasons (Thera et al., 2020). Vesterinen et al. (2020) also analysed the EAA content of benthic macroinvertebrate taxa along with cladocerans and copepods from the pelagic and littoral habitat of subarctic lakes and found taxon-specific relationships in the AA profile of studied taxa. Moreover, they found that based on the EAA content, zooplankton were higher quality food for fish compared to macroinvertebrates. They further suggested that climate induced changes in the community composition of zooplankton and littoral macroinvertebrates will affect the availability of EAAs to top consumers like fish (Vesterinen et al., 2020).

Compared to studies on aquatic organisms collected from lake and marine environments (Guisande et al., 1999; Guisande, 2006; Aranguren-Riaño et al., 2018; Thera et al., 2020), limited studies have been conducted on understanding the taxa specific variation in the AA profile of freshwater organisms collected from riverine ecosystems, despite AAs playing a major role in the quality of diet ('nutritional landscape') of river ecosystems (Sharma & Dietz, 2006). The study conducted by Kolmakova et al. (2013) is the only study conducted to date that compared the AA profile among biofilms, zoobenthos and fish collected from a river system. Dwyer et al. (2018) studied the AA profile of different insect taxa collected from one site along a river and found taxa specific AA profiles, which was correlated with phylogeny but not with functional feeding groups. Hence, more studies are necessary to understand how freshwater organisms vary in their biomolecular composition especially in regard to their AA profiles.

1.5 Influence of environmental stressors on intraspecific variation in taxa amino acid profiles

Environmental stressors may impact the biomolecular makeup of aquatic organisms, influencing aquatic food webs via the alteration of essential biomolecules such as AAs and their trophic transfer (Guisande et al., 1998; Ruess & Müller-Navarra, 2019). Studies of the biomolecular impacts of environmental stressors on aquatic organisms have focused mainly on fatty acids, especially in regards to the essential polyunsaturated fatty acids (PUFA), due to their major role in the growth and survival of consumers (Ruess & Müller-Navarra, 2019; Sushchik et al., 2020). Stressors such as temperature, salinity, and toxic chemicals have been demonstrated to cause changes to the fatty acid composition of aquatic organisms (Gonçalves et al., 2012; Filimonova et al., 2016b; Mesquita et al., 2018; Mahboob et al.,

2019). Beside PUFAs, EAAs also play a major role in the growth and development of organisms as explained in section 1.3, with lower protein content and mismatch between AA ratios between predator and prey likely to also impact the consumer's fitness (Lin et al., 2015).

Amino acid metabolism plays a central role during the stress response (Figure 1.3) (Hildebrandt, 2018; Batista-Silva et al., 2019). During stressed conditions, FAAs can be produced due to protein degradation. For example, Batista-Silva et al. (2019) reported an accumulation of FAAs due to degradation of highly abundant proteins such as the subunits of photosystems and ribosomes in *Arabidopsis thaliana* during osmotic stress caused by exposure to high salinity. Furthermore, cells are also known to increase biosynthesis of certain FAAs that provide protection during stressed conditions. For instance, Pro has been reported to be accumulated during a variety of stressed conditions in higher and lower plants (Hayat et al., 2012; Hildebrandt, 2018; Batista-Silva et al., 2019). Pro acts as an osmolyte, a chelator, antioxidative defence molecule and a signalling molecule (Hayat et al., 2012). Accumulation of free Pro in algae has been reported after Cu exposure in *Chlorella* sp. (Wu et al., 1998), *Anacystis nidulans* (Wu et al., 1995) and *Chlorella vulgaris* (Mehta & Gaur, 1999) and Cd exposure in *Chlamydomonas reinhardtii* (Siripornadulsil et al., 2002). Proline (free) has also been reported to increase during salinity stress in algae (Carillo et al., 2020) and in response to exposure to low temperatures in wheat seedlings (Naidu et al., 1991). Accumulation of free Cys and Glu has been shown for the green microalga *Scenedesmus* sp. when exposed to arsenic (Arora et al., 2018).

During exposure to environmental stressors, the FAA pool can be used to form other biomolecules such as proteins, peptides and secondary metabolites (Figure 1.3) (Wu, 2009; Wu et al., 2014; Batista-Silva et al., 2019). To illustrate, AAs like Cys, Met, Gly help in the synthesis of different stress-related proteins like metallothionine, phytochelatin and glutathione that help to chelate metal ions and aid in an organism's tolerance to metal stress (Grusak et al., 1999; Arora et al., 2018; Gauthier et al., 2020). Secondary metabolites which use FAAs as precursors have also been reported to provide protective roles during stressful conditions (Gauthier et al., 2020). Along with this, oxidation of AAs helps in the generation of energy to fulfil the high demand for ATP during the stressed condition (Wu, 2009; Jiang et al., 2020). For example, Xupeng et al. (2017) demonstrated the role of non-protein AAs in the regulation of energy metabolism in the microalga *Isochrysis zhanjiangensis* during nitrogen depletion. Jiang et al. (2020) showed that the oxidation of Glu and Asp help to maintain energy balance during heat stress in the marine mollusc *Scapharca subcrenata*.

During such interactions (Figure 1.3), the whole body AA composition of organisms might change causing the intraspecific variation in the overall AA profile of aquatic organisms, depending on the environmental conditions the organism is exposed to (Conceição et al., 1998; Thera et al., 2020). Studies conducted on understanding the variability in whole body AA profiles within freshwater organisms and the role external environmental factors play in changing the AA profiles of aquatic organisms are mostly limited to those species which have, or have potential, to be used as food resources for humans (Boyd, 1973; Adeyeye, 2005; Yanar & Çelik, 2006; Barroso et al., 2014; Liu et al., 2018). Some studies have demonstrated the impact of habitat conditions on AA profile of marine organisms. For example, Riveiro et al. (2003) showed that whole body AA profiles (FAA + protein bound) of larval pelagic fish and their eggs collected along the west Atlantic coast of Spain and the Cantabarian sea were species specific and also differed among areas suggesting an influence of environmental variables such as temperature, salinity, food quality and/or quantity on the AA profile of fishes. Cuttitta et al. (2006) also demonstrated that the whole body AA profile of anchovy larva collected from five different coastal nurseries with different habitat conditions such as water turbulence, primary productivity, temperature etc differed significantly. Increasing temperatures have also been shown to change the AA profile of the marine alga *Cylindrotheca fusiformis* (Bermudez et al., 2015).

Compared to marine organisms, limited studies have been conducted on the role environmental factors or stressors have on defining the AA profile of freshwater organisms under ecological settings. Such studies are mostly related to the lentic environment. For example, Vesterinen et al. (2020) found that the AA profile of benthic macroinvertebrates along with cladocerans and copepods from the pelagic and littoral habitats of subarctic lakes were not influenced by habitat conditions. In contrast, Thera et al. (2020) reported within taxon variation in AA profiles in organisms collected from different lakes with varying water quality. Seasonal variations in the AA profile of biofilms collected from the lakes were also reported (Thera et al., 2020). Temperature has also been reported to affect the AA profile of larval African catfish, with higher temperatures leading to increased absorption and depletion of AAs from the yolk sac (Conceição et al., 1998). Temperature effects on the AA profile of algae have also been reported (James et al., 1989). For instance, a change in temperature from 15 to 25 °C increased the content of EAAs in *Chlorella* sp. by 5% and similar results were reported for another alga *Nannochloropsis* sp. (James et al., 1989). Similarly, Ala, Gly, Arg, Pro and Lys were accumulated in the freshwater prawn

Macrobrachium rosenbergii when exposed to high salinity concentrations compared to controls (Yang et al., 2001).

So far, most AA profiling studies have focused on looking at variations between taxa and within taxa collected from different habitats with varying water quality, with only a few investigating the effects of contaminants. Man et al. (2019) found a positive correlation between the content of different AAs (FAA + protein bound) and methyl mercury accumulated in different fish tissues. Variations in AA profiles of the fish species *Cirrhinus mrigala* and *Labeo rohita* upon exposure to different levels of metal pollution were found, with high levels of Cys reported in fish from the polluted river site (28 ± 2 mg g^{-1} dry weight in unpolluted site and 94 ± 3 mg g^{-1} dry weight in most polluted site) (Hussain et al., 2016). Cadmium (Cd) exposure altered the AA profile of *Chlorella vulgaris*, with metal-induced accumulation of Pro, His and Glu reported in Cd stressed cells (Chia et al., 2015). Further studies are needed to investigate the potential of AA profiling in determining potential effects of contaminants with initial studies suggesting that AA profiling may be a sensitive and efficient tool to monitor toxicological effects on aquatic biota (Guisande et al., 1998).

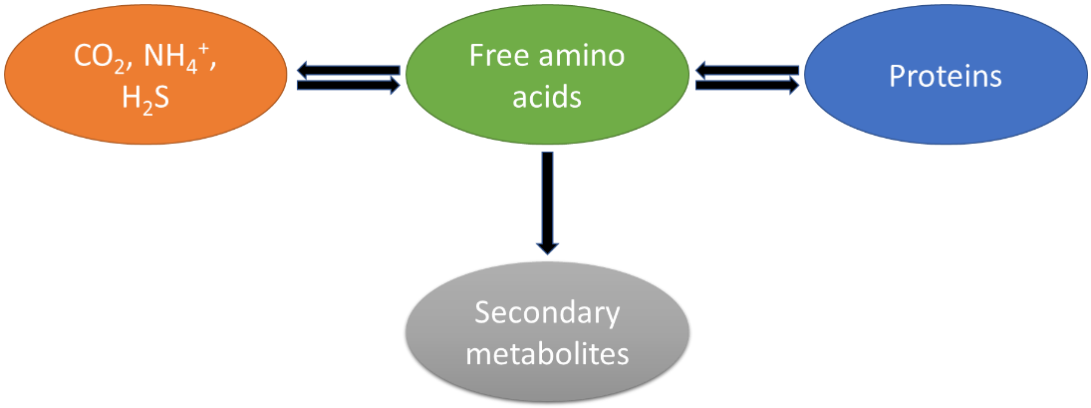

Figure 1.3. Role of amino acid metabolism in the abiotic stress response (Modified from Hildebrandt, 2018).

Understanding the AA composition of freshwater organisms and the inter and intraspecies variations under various ecological settings is essential to determining the influence of AAs on an organism's growth, development and survival, along with understanding potential impacts on the nutritional landscape of ecosystems (Dwyer et al., 2018). As outlined above, environmental stressors may induce alterations in the biomolecular composition of organisms which may alter the nutritional value of organisms to higher trophic levels that depend upon them as source of essential biomolecules (Bermudez et al., 2015). Stressor driven changes in the AA profile of organisms are hence ecologically relevant, as the quality of food (i.e. amount and composition of EAAs) is equally important as quantity

(number of organisms) to ensure the efficient trophic transfer of energy across the food web. Therefore, more studies are necessary to understand taxa specific AA profiles and how external factors such as natural and anthropogenic stressors influence the whole body AA profile of freshwater organisms under ecological settings. Further laboratory studies are also necessary to understand the stressor specific responses as it is often difficult to identify the stressor responsible for specific AA profile changes under ecological settings due to the complex nature and interaction of multiple variables within natural ecosystems.

1.6 Copper as a case study

Anthropogenic activities such as mining and industrial activities have increased global fluxes of trace elements like copper (Cu) to water bodies, negatively impacting aquatic organisms (Kamunde & Wood, 2004). Being an essential micronutrient, a certain concentration of Cu is needed to maintain physiological processes, however, Cu becomes harmful to organisms at higher concentrations (Kamunde & Wood, 2004). Copper poses a serious threat to aquatic ecosystem because of its toxicity and persistence nature (Wood et al., 2011). Once Cu is released into freshwater systems, it is accumulated in aquatic organisms like algae and fish and can get biomagnified in the ecosystem through the food web (Mann et al., 2011). Algae has been shown to be sensitive to Cu, with Cu inhibiting algal growth which in turn affects the amount of food available for consumers such as zooplankton, macroinvertebrates and fish (Burns & Walker, 2000). Copper has also been reported to damage the olfactory function in salmon populations, causing disorientation and leading to difficulties in salmon migration (Sandahl et al., 2004). Moreover, sensitive species may disappear and other species that can tolerate Cu pollution may become more abundant, changing the entire community composition of a freshwater ecosystem (Johnson et al., 1993).

Copper toxicity is usually assessed by conducting acute and chronic laboratory bioassays (Giesy & Graney, 1989; Franklin et al., 2005). For example, the acute and chronic Cu toxicity to model freshwater organisms like fish and algae have been well-studied over the years (Riethmuller et al., 2000; Furuta et al., 2005; Hernandez et al., 2011; Adams et al., 2016; Guo et al., 2016; Yong et al., 2020; Shekh et al., 2020). Several biological and behavioural endpoints like survival, growth, hatching success, deformities, swimming activities and molecular endpoints have been identified to access Cu toxicity (Santos et al., 2019). Algae and fish have shown marked variation in their susceptibility to Cu. Some green algae are very sensitive to Cu (Franklin et al., 2000; Franklin et al., 2001; Franklin et

al., 2002; Wilde et al., 2006). For instance, Cu as low as 2 µg L^{-1} has been found to inhibit growth of *Chlorella* by 50% (Macoustra et al., 2019; Macoustra et al., 2020). Copper toxicity has also been shown to impact cell division, photosynthesis, respiration and different cellular metabolic pathways in algae (Pistocchi et al., 1997; Wei et al., 2014; Hamed et al., 2017). Larval stages of fish have also been reported to be sensitive to Cu. Vardy et al. (2013) determined the lethal concentration (LC_{50}) at 96 h of Cu exposure for larva of white sturgen and rainbow trout to be 10 and 21 µg L^{-1}. Riethmuller et al. (2000) reported the LC_{50} of Cu was between 13 µg L^{-1} – 26 µg L^{-1} for sacfry of fish- *Mogurnda mogurnda* and dependent on water hardness. It has been shown that Cu enters the gills cells of fish via the epithelial sodium (Na^+) channel despite the difference in the chemistry between Cu and Na^+ (Kamunde & Wood, 2004). Environmentally realistic concentrations of Cu have been reported to result in reduced growth and deformities as well as affect reproduction in fish (McKim et al., 1978; Dave & Xiu, 1991; Furuta et al., 2005; Sfakianakis et al., 2015; Santos et al., 2019). Copper induced inhibition of development of embryos and larval stages of fish has been reported in many studies (Buckley et al., 1982; Stouthart et al., 1996; Wu et al., 2003; Jezierska et al., 2009), but the mechanism of inhibition is not entirely clear. It is believed that Cu affects the yolk resorption rate from the sac after hatching of larvae and delays exogenous feeding of sacfry, which has been shown for larvae of the Common Carp (Jezierska et al., 2009). Buckley et al. (1982) suggested that the inhibition of growth of fish larva is related to the inhibition of metabolic processes, however, this has yet to be explored. Copper has also been reported to impact the development of the swim bladder and its inflation in sacfry of several fish species, impairing their ability to float and swim (Stouthart et al., 1996; Wu et al., 2003; Jezierska et al., 2009).

In multicellular organisms, contaminant metals tend to accumulate in particular organs and tissues. Accumulation of Cu has been reported in several parts of adult fish such as gills, brain, liver, kidney, heart, bones, muscles and the digestive track (Kamunde & Wood, 2004; Padrilah et al., 2018; Liu et al., 2020) causing histological alterations in associated tissues (Hansen et al., 1999; Rajeshkumar et al., 2017; Padrilah et al., 2018). According to Jezierska and Witeska (2006), accumulation of metals is higher in smaller and younger fish compared to adults despite them containing underdeveloped organs (Jezierska et al., 2009; Mohammed, 2013). Guo et al. (2016) reported higher amounts of Cu accumulation in larval stages of fish compared to the adult stage, with Cu shown to damage tissues and their chemical composition. Other research suggests Cu affects eyes of larval and adult fish

(Bodammer, 1985; Korbas et al., 2013; Wang et al., 2020; Liu et al., 2020). According to Erie et al. (2005), the pigmented tissues of the eye such as the retinal pigment epithelium, choroid and iris have a high affinity for metal ions, however, further study is needed to clarify this phenomenon. Limited studies have been conducted to study the mode and mechanism of metal accumulation in larval stages of fish.

Recent studies have shown the applicability of technologies such as Proton induced x-ray emission and infrared spectroscopy in studying metal accumulation and chemical alterations in tissues and organs which have occurred due to metal exposure (D'Souza et al., 2008; Penner-Hahn, 2013; Korbas et al., 2013). The application of such technologies would help to better understand the location of metal accumulation and biochemical changes in larval fish exposed to metals.

Generally, Cu shows toxicity when it enters the cell, binding to proteins and nucleic acids and disturbing the normal function of the cell (Padrilah et al., 2018). Excessive amounts of Cu in cells and tissues can cause oxidative stress through the production of reactive oxygen species (ROS), like hydroxyl radicals and hydrogen peroxide that have the potential to damage proteins through the oxidation of Cys and Met (Kamunde & Wood, 2004; Kültz, 2005). Copper also interferes with the homeostasis of Na, chlorine (Cl) and zinc (Zn), and can also bind to Cys, His and Met residues of proteins inactivating them (Kamunde & Wood, 2004). Proteome profiling has led to the identification of several pathways such as glutathione metabolism, sulfur metabolism, taurine and hypotaurine metabolism that are involved in algal response to Cu stress, aiding in explaining the cellular and molecular responses of green algae to Cu exposure (Khatiwada et al., 2020; Yong et al., 2020). Numerous studies have characterised the role of Cys rich protein metallothioneins in the regulation of the cytosolic concentrations of free metal ions in green algae (Linder & Hazegh-Azam, 1996; Morris et al., 1999; Gaur & Rai, 2001; Hasan et al., 2017) and fish (Linder & Hazegh-Azam, 1996; Pedersen et al., 1997; Wu et al., 1998; Krasnići et al., 2019; Yong et al., 2020). Algae can also produce peptides named phytochelatins that help them to sequester Cu (Pawlik-Skowrońska et al., 2007; Ritter et al., 2010). Copper is also complexed by other thiol-containing molecules such as glutathione and S-containing AAs (Pawlik-Skowrońska et al., 2007). A recent study by Yong et al. (2020) showed that AAs (Phe), sulfur-containing AAs (Cys and Met) and amines were significantly upregulated in response to Cu in green algae suggesting a role in metal chelation. Accumulation of free Pro, when exposed to Cu, has also been reported in algae (Mehta & Gaur, 1999; Chia et al., 2015). As discussed above the accumulation of Pro may be related to a tolerance

mechanism (Wu et al., 1998) or it may act as an antioxidant (Singh et al., 2016). Zhang et al. (2008) reported the accumulation of Pro in *Chlamydomonas reinhardtii* after exposure to 2.5 and 5.0 µM Cu(II) and investigated Cu-induced nitric oxide (NO) generation and its relationship to Pro synthesis.

Several studies have shown the impact of Cu contamination on the biomolecular composition of fish and the role of different AAs in stress responses. Hussain et al. (2016) reported elevated levels of Cys, Asp, Glu and Ala in muscles of fish collected from a river polluted with various metals including Cu. Changes in the activity of enzymes important in the metabolism of certain AAs such as aspartate transaminase and alanine transaminase have also been reported in Cu exposed fish (Öner et al., 2009). Many studies have shown changes in the content of the peptide glutathione within larval fish in response to exposure to Cu, possibly linked to its antioxidant activity (Wood et al., 2011; Shekh et al., 2020; Naeemi et al., 2020). These findings suggest that AAs play a major role in freshwater organism responses to metal stress and may aid in attenuating Cu toxicity. However, the direct impact of Cu on the AA profile of freshwater organisms has yet to be explored.

Overall, contamination of water bodies with Cu can adversely impact aquatic organisms with changes seen at the ecosystem, community, organism, organs, tissue/cell and biomolecular levels. Detailed understanding of the sensitivity of various toxicity endpoints at the organism, biomolecular and cellular level are needed for monitoring potential Cu induced changes at the various levels of biological organisation within an aquatic ecosystem (Giesy & Graney, 1989; Wei et al., 2014). Very few studies have combined different toxicity endpoints to understand the relationship between biomolecular responses and the various observed effects at the organism level (Wei et al., 2014; Santos et al., 2019). Currently, there is a gap in understanding the relationship between the macroscopic observations of toxicological response (i.e. chronic toxicity endpoints) used to derive water quality guidelines, with the underlying molecular mechanisms behind toxicity. Such investigations would lead to a better understanding of the mechanisms behind Cu toxicity and may lead to the development of relevant toxicity biomarkers. This would lead to improvements in risk assessment and environmental regulatory approaches for Cu.

1.7 Current scientific knowledge gaps

The available literature suggests that homeostasis of body protein and therefore AA profiles may be a highly conserved pattern within a taxon (Moura et al., 2013). On the other hand, however, there is some evidence suggesting that proteome and AA profiles of aquatic

organisms may also change in response to the external environment (Pe'er et al., 2004; Chen et al., 2013; Moura et al., 2013). Proteins and AAs are major biomolecules that not only define the growth, development and fitness of freshwater organisms but also the fitness and stability of aquatic ecosystems through trophic transfer (Ruess & Müller-Navarra, 2019). Stress-induced alterations or deficiencies in these biomolecules will impact different levels of biological organization as shown in Figure 1.1. However, studies exploring the effect of various environmental stressors on these biomolecules has been limited. The major questions that still need to be addressed are:

i. How do freshwater organisms vary in their biomolecular composition based on AA profile?
ii. What are the major factors that determine the biomolecular composition (AA profile) of freshwater organisms?
iii. Are stress-induced biomolecular changes detectable in the field i.e. within river systems?
iv. At what level/concentrations of a stressor(s) can we detect an organism's response at the biomolecular level?
v. How sensitive are biomolecular changes to stressors compared to traditional endpoints?
vi. Can biomolecular changes explain the macroscopic effects observed at higher levels of biological organisation?

1.8 This study

This thesis explores the impact of environmental stressors on the biomolecular makeup of aquatic organisms with a focus on whole body AA profiles using both field and laboratory experiments. The major objectives of this project were to determine: (i) if changes in water quality along a river continuum influence the AA profile of freshwater macroinvertebrates using two case studies: an uncontaminated river system (Murray-Darling Basin) and a contaminated river (Dee River), and (ii) the effect of waterborne Cu on the biomolecular makeup (AA profile (algae and fish), proteome (algae and fish); element distribution (fish) and chemical composition of tissues (fish)) of freshwater organisms at environmentally realistic concentrations, using algae and fish as model organisms. This study improves our understanding of the effects of environmental stressors on freshwater organisms at the biomolecular level and reveals the role of AA metabolism in the stress response of freshwater organisms. These objectives will be discussed in the following five chapters.

Chapter Two: This chapter was designed to answer the first and second knowledge gaps outlined above in section 1.7, by exploring the variation in the AA profile of macroinvertebrates collected from seven sites along the Murray River, Australia. The target species of this study were *Macrobrachium australiense, Paratya australiensis, Physa* sp. and *Triplectides* sp. This work further focused on understanding seasonal and spatial variations in the AA profile of each taxon to better understand how these factors shape the AA profile of selected macroinvertebrates within the Murray River.

Chapter Three: This chapter addresses the first three knowledge gaps in section 1.7, by investigating the impact of a contamination gradient (acid mine drainage (AMD)) within the Dee River, Queensland on macroinvertebrate community composition and the AA profiles of tolerant taxa: Chironomidae, Ceratopogonidae, Dytiscidae and Gomphidae. This chapter improves our understanding of how contamination within freshwater systems may affect the AA profile of aquatic organisms.

Chapter Four: This chapter was designed to address the final three knowledge gaps (section 1.7) by determining the toxicity of Cu to a unicellular test organism (*Chlorella* sp.) under controlled laboratory conditions. Furthermore, changes in the AA profile and proteome of *Chlorella* sp. in response to exposure to sublethal Cu concentrations were determined to understand the impact Cu has on AA metabolism and other cellular processes. Biomolecular changes were used to explain macroscopic toxicity responses in the alga such as growth inhibition, changes in cell size and integrity.

Chapter Five: This chapter also addresses the final three knowledge gaps (section 1.7) by determining the toxicity of Cu to a multi-cellular organism and the impact of exposure on the AA profile of Purple-spotted Gudgeon sacfry (*Mogurnda adspersa*). The sacfry stage was used because it is often the most sensitive life stage to stress (Barron & Adelman, 1984). This work further explored changes in elemental distribution and the biomolecular and chemical composition of areas known to accumulate Cu such as the retinal tissues.

Each of the research chapters is written as discrete papers, with one accepted for publication in Aquatic Sciences (chapter 2), one under review in Environmental Pollution (chapter 3), one submitted to Environmental Science and Technology (chapter 4) and one (chapter 5) in preparation for submission to peer-reviewed journals. As such, each chapter contains its own discrete reference list and supplementary material.

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Chapter Two

Taxonomic, Seasonal and Spatial Variation in the Amino Acid Profile of Freshwater Macroinvertebrates

2.1 Abstract

Macroinvertebrates play a key role in aquatic food webs, with amino acids (AA) playing an important role in determining their nutritional value to higher consumers. This study aimed to determine whether the AA profile varies among four macroinvertebrate taxa, spatially and seasonally (summer/winter). The freshwater prawn, *Macrobrachium australiense*; freshwater shrimp, *Paratya australiensis*; freshwater snail, *Physa* sp. and caddisfly, *Triplectides* sp. were collected from seven sites along the Murray-Darling Basin, Australia. Sampling was conducted during summer 2015 (October-December), winter 2016 (May-June) and summer 2016 (October-December). Amino acid profiles were found to be significantly different among the four taxa, with the highest total amino acid content found in decapods (*M. australiense* and *P. australiensis*). Based on the total essential AA content from our study, decapods had higher nutritional value compared to *Physa*. Seasonal variations in AA profiles of decapods were observed, with an increased proportion of the non-essential amino acid glycine and decreased proportion of different essential AAs found in individuals collected during winter compared to summer. No seasonal variation in the AA profiles of *Physa* sp. or *Triplectides* sp. was shown. Spatial variation in the AA profile of macroinvertebrates was only recorded in *P. australiense* during winter 2016. These findings expand the current understanding of the AA profile of freshwater macroinvertebrates showing that AA profiles are taxa specific and vary seasonally and spatially depending on taxa.[1]

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2.2 Introduction

The nutritional value of prey items within a natural ecosystem can be assessed using a range of techniques, with amino acid (AAs) content being one of them (Vesterinen et al., 2020). Amino acids are one of the main biomolecules transferred across aquatic food webs (Dwyer et al., 2018). Apart from their role as building blocks of protein, they play a key role in cellular metabolism and function as energy metabolites (Vesterinen et al., 2020). The AA profile of an organism consists of 20 proteinogenic AAs from three domains: free amino acids (FAAs), peptide AAs, and proteome AAs (Gu et al., 2015). Not all organisms can synthesize all AAs. Fish, for example, must obtain ten of twenty proteinogenic AAs from their diet (Li et al., 2009). Depending on organism's ability to synthesise AAs de-novo AAs are categorised into two groups -essential amino acids (EAA) and non-essential amino acids (NEAA) (Supplementary Table S2.1). A diet with a high proportion of EAAs is considered to have a higher nutritional value (Peres & Oliva-Teles, 2006). For instance, fish must obtain lysine (Lys) (EAA) from their diet and as such Lys is considered as one of the major AAs that determine the nutritional quality of fish feeds (Kolmakova et al., 2013). More broadly, distributions of AAs in the diet of predatory fish have been reported to affect their metabolism, immunity, growth rate and fecundity (Rosa et al., 2005). Therefore a better understanding of the distribution and availability of AAs within a riverine ecosystem is important for assessing potential effects of resource depletion on different trophic levels.

The nutritional quality of different aquatic organisms to higher order consumers based on their AA profile has been studied previously (Yang et al., 2001; Chikaraishi et al., 2007; Kolmakova et al., 2013; Pereira et al., 2013; Man et al., 2019; Vesterinen et al., 2020). Nevertheless, studies on macroinvertebrates are still limited. Macroinvertebrates play a key role in the food web of aquatic ecosystems, forming a link between primary producers and higher order consumers (Bian et al., 2016; Paul et al., 2018) and have been suggested to contain a variety of different EAAs, making them a good quality food source for top predators such as fish (Singh et al., 2017; Bowman et al., 2019; Vesterinen et al., 2020). By comparison with marine invertebrates, studies on riverine macroinvertebrates are comparatively sparse (Dwyer et al., 2018; Thera et al., 2020). Understanding the AA profile (protein bound + FAA) of macroinvertebrates as prey items are, therefore, an important step towards understanding the availability of macronutrients to higher order consumers in riverine food webs. Such studies may also help to predict the ecological consequences of changes to the community composition of macroinvertebrates on the nutritional landscape (Dwyer et al., 2018).

The AA profiles of living organisms including macroinvertebrates have been known to have a conserved pattern defined by their taxonomy (Dwyer et al., 2018). Dwyer et al. (2018) showed that the AA profile of twenty aquatic insect taxa from six orders was taxa specific and correlated with phylogeny. Thera et al. (2020) and Vesterinen et al. (2020) also reported the taxonomic control on the AA profile of macroinvertebrates collected from different lakes. However, more studies are needed to improve present knowledge of species-specific AA variability in freshwater macroinvertebrates.

Intraspecies variation in AA profile has also been reported in various aquatic organisms (Aranguren-Riaño et al., 2018; Thera et al., 2020). Seasonal and spatial variations in environmental factors such as temperature, discharge, riparian shading, and water quality can alter the potential food sources for macroinvertebrates and cause biochemical changes within the organism such as changes in AA profile (Boëchat & Adrian, 2005; Leiwakabessy & Lewerissa, 2017; Aranguren-Riaño et al., 2018). In addition to this, unfavourable changes in the water quality itself can cause stress to macroinvertebrates leading to the up/down-regulation of certain proteins and FAAs (Tomanek, 2011; Lane et al., 2019). For instance, Binoy et al. (2012) reported spatial and seasonal differences in the AA profile of the fish species *Labeo gonius* collected from lentic and lotic water bodies. Intraspecific variability in AA profile has also been reported in different zooplankton taxa (Ventura & Catalan, 2010; Aranguren-Riaño et al., 2018). However, seasonal and spatial variations in the AA profiles of macroinvertebrates in riverine ecosystems are not well understood (Thera et al., 2019; Thera et al., 2020). Moreover, the mechanisms that might drive these variations are largely unknown.

This study aimed to characterise the AA profile of four macroinvertebrate taxa to determine whether there is: (1) taxa specific variation in the AA profile of four taxa studied; *Macrobrachium australiense*, *Paratya australiensis*, *Physa* sp. and *Triplectides* sp. 2) seasonal variation in the AA profile of each taxon and 3) spatial variation in AA profile within each taxon. Addressing these knowledge gaps will allow us to better understand how season and location shape the potential nutritional value of aquatic macroinvertebrates as a food resource for consumers.

2.3 Methods

2.3.1 Study sites

Macroinvertebrates were collected from seven sites within the Murray-Darling Basin, Australia, from Jingellic in the upper Murray to Woods Point near where the Murray River

enters Lake Alexandrina in South Australia, covering a vast geographic scale that is ideal for the study of spatial variations in the AA profiles of macroinvertebrates (Figure 2.1). These sites are listed in Table 2.1 with details provided elsewhere (Paul et al., 2013; Paul et al., 2018). The sites vary widely in annual rainfall with the uppermost site (Jingellic) recording the highest annual rainfall (810 mm), the five intermediate sites (Yarrawonga, Euston, Lock 9, Burtundy, Murtho) are situated in the semiarid zone with an annual rainfall of approximately 260 mm and the lowest site (Woods Point) having a more temperate climate with a mean annual rainfall of approximately 400 mm.

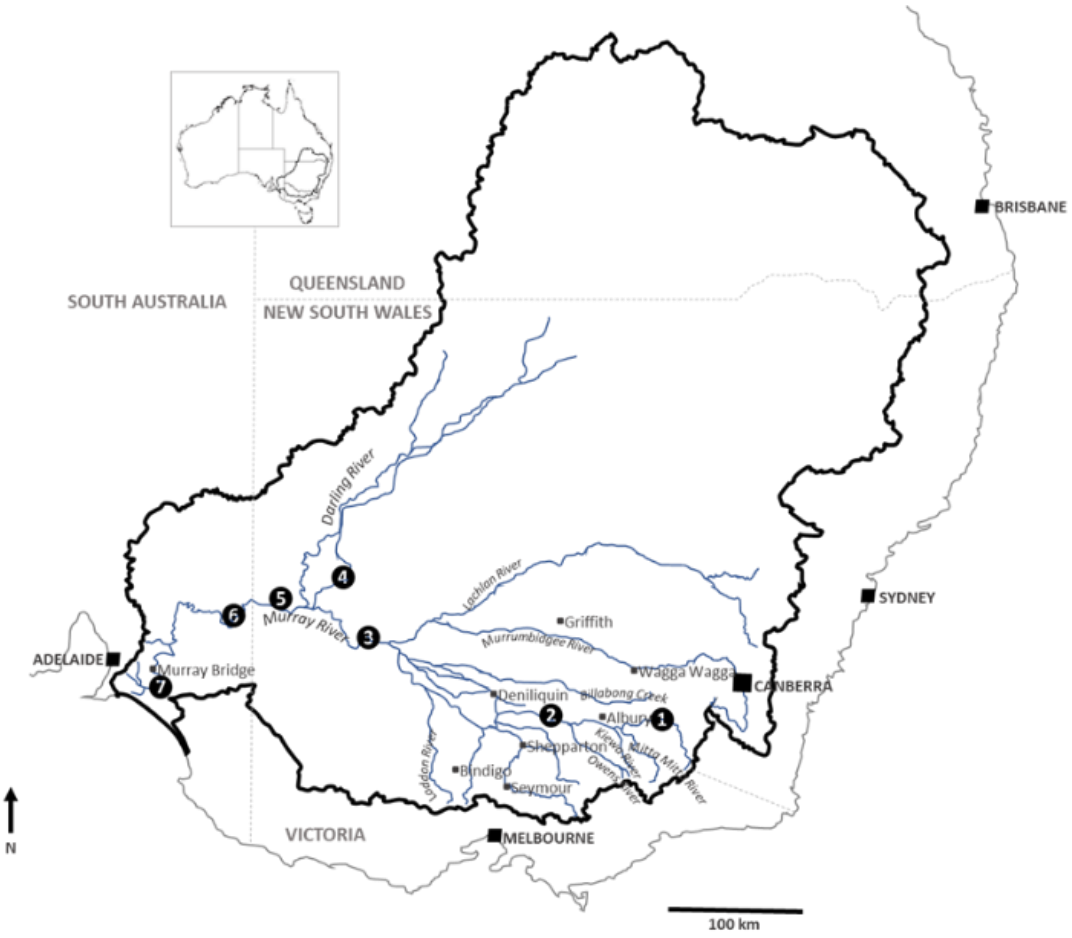

Figure 2.1. Murray-Darling Basin in south-eastern Australia, showing the location of the seven sites used in this study: 1 = Jingellic, 2 = Yarrawonga, 3 = Euston, 4 = Burtundy, 5 = Lock 9, 6 = Murtho, 7 = Woods Point.

Table 2.1. Details of River Murray Biological Monitoring program monitoring sites, modified from Paul et al. (2018).

Site no.	Name	Latitude-Longitude	Distance from the source (km)	Site description
1	Jingellic	S 35°57.748′ E 147°30.517′	258	Below the junction of the Swampy Plains and Indi Rivers but upstream of the maximum extent of Lake Hume
2	Yarrawonga	S 36°00.524′ E 145°57.571′	527	4 km below Yarrawonga Weir
3	Euston	S 34°35.403′ E 142°45.190′	1389	3 km below of Euston Weir
4	Burtundy	S 33°45.010′ E 142°15.580′	2607	On the Darling River, downstream of a small weir
5	Lock 9	S 34°11.081′ E 141°36.204′	1737	1 km upstream of Lock 9 weir, lentic environment being within weir pool
6	Murtho	S 34°06.8396′ E 140°81.1894′	1910	In the South Australian section of the River Murray, about 12 km north of Paringa
7	Woods Point	S 35°13.966′ E 139°24.895′	2416	South Australian River section, about 16 km south of Murray Bridge at the end of Craton Lane

2.3.2 Sample collection and study species

Macroinvertebrates were collected in summer 2015 (October-December), winter 2016 (May-June) and summer 2016 (October-December) as part of a long-term (but since discontinued) monitoring program for the Murray River (Paul et al., 2018). A combination of artificial substrate samplers (ASS) deployed for six weeks and sweep net sampling was used to collect the macroinvertebrates that colonized the substrate as well as those found in the major stream habitats such as: macrophytes, leaf packs and the water surface (Paul et al., 2013). Environmental variables including: water temperature (°C), pH, conductivity (µS cm^{-1}) and turbidity (NTU) were also recorded using a Yellow Springs Instruments (YSI) Pro DSS water quality meter (YSI Environmental, Yellow Spring, OH). Macroinvertebrates were identified to species level where possible and enumerated (Paul et al 2013). Four taxa that represent key components of the food web were chosen for use in this study. These were: 1) a freshwater prawn, *Macrobrachium australiense* (Decapoda: Palaemonidae), 2) a freshwater shrimp, *Paratya australiensis* (Decapoda: Atyidae), 3) a freshwater snail, *Physa* sp. (Basommatophora: Physidae) and 4) a caddisfly, *Triplectides* sp. (Trichoptera: Leptoceridae). One to five specimens of each taxon from each site and season were analysed (Supplementary Table S2.2). *Physa* sp. and *Triplectides* sp. were collected only in 2016 summer and 2016 winter, whereas, the decapods (*M. australiense* and *P. australiensis*) were collected over all sampling periods.

In a preliminary study to test if gut contents contribute significantly to the overall AA profile of macroinvertebrates, 20 individuals of *P. australiensis* were sampled from the edge and benthic zones of a site at Wodonga creek. Ten individuals were frozen after transporting the samples to the laboratory, while the remaining 10 individuals were held overnight in river water to void their gut content prior to freezing (all specimens were frozen at -80°C). The AA profile of whole body tissue of *P. australiensis* with and without gut contents was found to not significantly differ (PERMANOVA, pseudo-F = 1.57, p = 0.2) indicating that gut content does not significantly affect the AA profile of an organism (Supplementary Figure S2.1); all subsequent AA analyses were conducted on whole organisms containing their gut contents.

2.3.3 Amino acid analysis

Sample processing and AA analysis broadly followed that described previously by Dwyer et al. (2018). Samples for AA analysis (whole animal) were first homogenized with Mini beadbeater-16 (Biospec) using approximately 0.2 g of 0.5 mm diameter glass beads (Biospec) in 1 mL of Milli-Q and then stored at -80 °C. 100 µL of the thawed sample (10% of total dry biomass of individual sample) was freeze-dried in pyrolysed tubes (550 °C) and hydrolysed with 6 N HCl containing 0.02% phenol at 110 °C for 24 h under an argon atmosphere (Fountoulakis & Lahm, 1998). After removing the acid in a rotary vacuum concentrator (RVC 2-18 CDplus; Martin Christ, Germany) at 40 °C for 4.5 h, the AA mixture was reconstituted with 0.1% formic acid (using volume in µL = 1,000,000×original weight of sample (g) in 1000 µL; this step should give approximately 100 pmol μL^{-1} of each AA). The AA mixture was then filtered through a 0.45 µm cellulose acetate membrane filter. 20 µL of the filtrate was buffered with 60 µL borate buffer (pH = 9, Merck Centripur, Germany) and derivatized with 20 µL of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; Synchem UG & Co. KG) at room temperature. The tagged solution was then heated at 55 °C (10 mins) and diluted (10×) with 0.1% formic acid prior to analysis.

Tagged AA samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of a Shimadzu Nexera X2 UPLC coupled to a Shimadzu 8030 triple quadrupole mass spectrometer, operated in positive ion electrospray ionisation (ESI) mode (Shimadzu corporation Kyoto, Japan). Individually tagged AAs were detected using multiple reaction monitoring (MRM), with collision parameters optimized individually. The separation was achieved using gradient elution (0.55 mL min^{-1}) through a Waters Aquity UPLC BEH C18 column (2.1×150 mm; pore size

1.7 µm) maintained at 50 °C (5 µL injection volume; 10 min run time). For the mass spectrometer the gas temperature, neutralizer gas flow, drying gas flow and interface voltage were set at 275 °C, 3.0 L min^{-1}, 17.0 L min^{-1}, 2.5 kV respectively. System control and data analysis were performed using LabSolutions software (Shimadzu, Tokyo, Japan).

Nineteen AAs quantified were: arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), valine (Val), alanine (Ala), asparagine (Asn) + aspartic acid (Aps) as Asx, cystine (Cys-Cys), glutamine (Gln) + glutamic acid (Glu) as Glx, glycine (Gly), proline (Pro), serine (Ser) and tyrosine (Tyr). In order to identify and quantify these AAs, calibration standards were prepared from an amino acid standard H (Waters Corporation) spiked with Gln, Asn and Tryptophan (Trp), prepared at final concentrations in the range 0.01 – 2 pmol μL^{-1}. Reagent blank (20 µL of 0.1% formic acid, 80 µL borate buffer and 20 µL AQC), sample blanks (Milli-Q treated as samples that went through the entire hydrolysis procedure) and 0.1% formic acid were also prepared for quality assurance (QA) along with quality control (QC) samples with selected AAs (His, Arg, Glu, Lys and Ile) to check peak positions and drift. Bovine Insulin (Sigma-Aldrich) was used as QC for the hydrolysis procedure with recoveries of each AA reported in Supplementary Table S2.3.

The AA profile for each sample is expressed as the relative abundance (mol%) of the AA pool. The total amino acid (TAA) content (mg per g dry biomass) in each sample was calculated by summing up the 17 AA masses (expressed as the polymerised molecular weights: Supplementary Table S2.1) obtained after acid hydrolysis (Lourenço et al., 2002). Similarly, the total essential amino acid (TEAA) content and total non-essential amino acid (TNEAA) content (mg per g dry biomass) were calculated from the sum of the 9 EAAs (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val and 8 NEAAs (Ala, Asx, Cys-Cys, Glx, Gly, Pro, Ser and Tyr) masses. During acid hydrolysis, Asn and Gln are deaminated to Asp and Glu, although they were specifically measured in this work to check for partial deamination. As is common practice, Asx (= Asn+Asp) and Glx (= Gln+Glu) are used to denote the combination of these AA pairs (Harris et al., 2016). Recoveries of Cys-Cys were very poor (< 30% in Bovine Insulin; BVI) but similar to those previously reported in the literature (Dwyer et al 2018; Harris et al. 2016). Trp cannot be measured in this procedure as it is destroyed during hydrolysis with hydrochloric acid.

2.3.4 Data analysis

Changes in the physical and chemical water quality parameters among sites during the three sampling periods were analysed by bar plots and principal components analysis (PCA). The relative abundance of each AA (expressed as mol%) were used in all statistical analyses. After testing for normality and homogeneity of variables using Shapiro-Wilk test, significant differences among species in regards to TAA content, seasonal difference in TAA content within each taxon were tested using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's tests ($p < 0.05$). If the conditions for ANOVA were not fulfilled, a non-parametric Kruskal-Wallis's H test (Kruskal & Wallis, 1952) was used ($p < 0.05$) in R (version 3.5.1). Bar plots and box plots were also made in R using the package ggplot2 (Wickham, 2009).

All other statistical analyses were conducted using the PERMANOVA+ V7.0.11 add-on to the Primer 7 statistical package (Anderson et al., 2008). Permutational multivariate ANOVA (PERMANOVA) was conducted to assess variation in AA profile between the four taxa and three seasons, using a two-factor design based on Euclidean distances. Permutation of residuals under a reduced model and Type III (partial) sums of squares type were used for PERMANOVA. When the main test showed significant differences between taxa and season, pairwise comparisons were performed within the PERMANOVA routine to determine taxa and seasonal differences (summer 2015, summer 2016 and winter 2016) in AA profile for each taxon. No significant seasonal difference was observed in the AA profile between two summers (summer 2015 and summer 2016) for *M. australiense* and *P. australiensis.* Hence, data from two summers (summer 2015 and summer 2016) were combined (as summer) for all the statistical analysis of TAA content, taxonomic variation and site variation in AA profile of these two taxa. Site variation in the AA profile of each taxon was also studied using PERMANOVA and pairwise analysis. Due to lack of adequate samples per site for *Triplectides* sp., variation in AA profile by site was not analysed for this taxon. The Mann Whitney U test was used to check the significant seasonal variation in relative abundance of each AA per taxa.

Environmental variables were used as predictors in distance-based linear models' (distLM) and were fitted individually (marginal test) or together in AA (relative abundance) matrices data sets (sequential test) for each taxonomic profile (*M. australiense* and *P. australiensis* only). The Akaike Information Criterion (AIC) was used to establish the selection criteria, based on the specified selection procedure, to evaluate the 'best' model (for each taxonomic group) that explains AA profile patterns and their responses to water quality. For visual

interpretation of the models in multidimensional space, we used distance-based redundancy analysis (dbRDA) to generate ordination plots to illustrate associations between environmental variables and biological data (AA profile).

2.4 Results

2.4.1 Water quality of the sampling sites

Average water quality parameters of the seven sites at the time of macroinvertebrate collection during the three sampling periods are shown in Figure 2.2a-d. Electrical conductivity (EC) in the Murray River gradually increased from upstream to downstream sites (site 1 to 7) and were all less than 500 µS cm^{-1}. Salinity at the Burtundy site on the Darling River was noticeably higher with EC > 500 µS cm^{-1}.

Principal component analysis of the water quality data (Figure 2.2e) shows a clear separation in water quality between summer and winter sampling. The first principal axis (PC1) explained 42.2% of the variation and was strongly correlated with temperature and pH. The second PC axis explained 32.5% of the variation and was correlated with EC and turbidity. The PCA also shows that seasonal variation in water quality within a site is higher than the spatial variation among sites. Within the sampling sites, Burtundy (site 4), which is the only sampling site from the Darling River is quite separated from all other sites along the Murray River due to higher EC and turbidity.

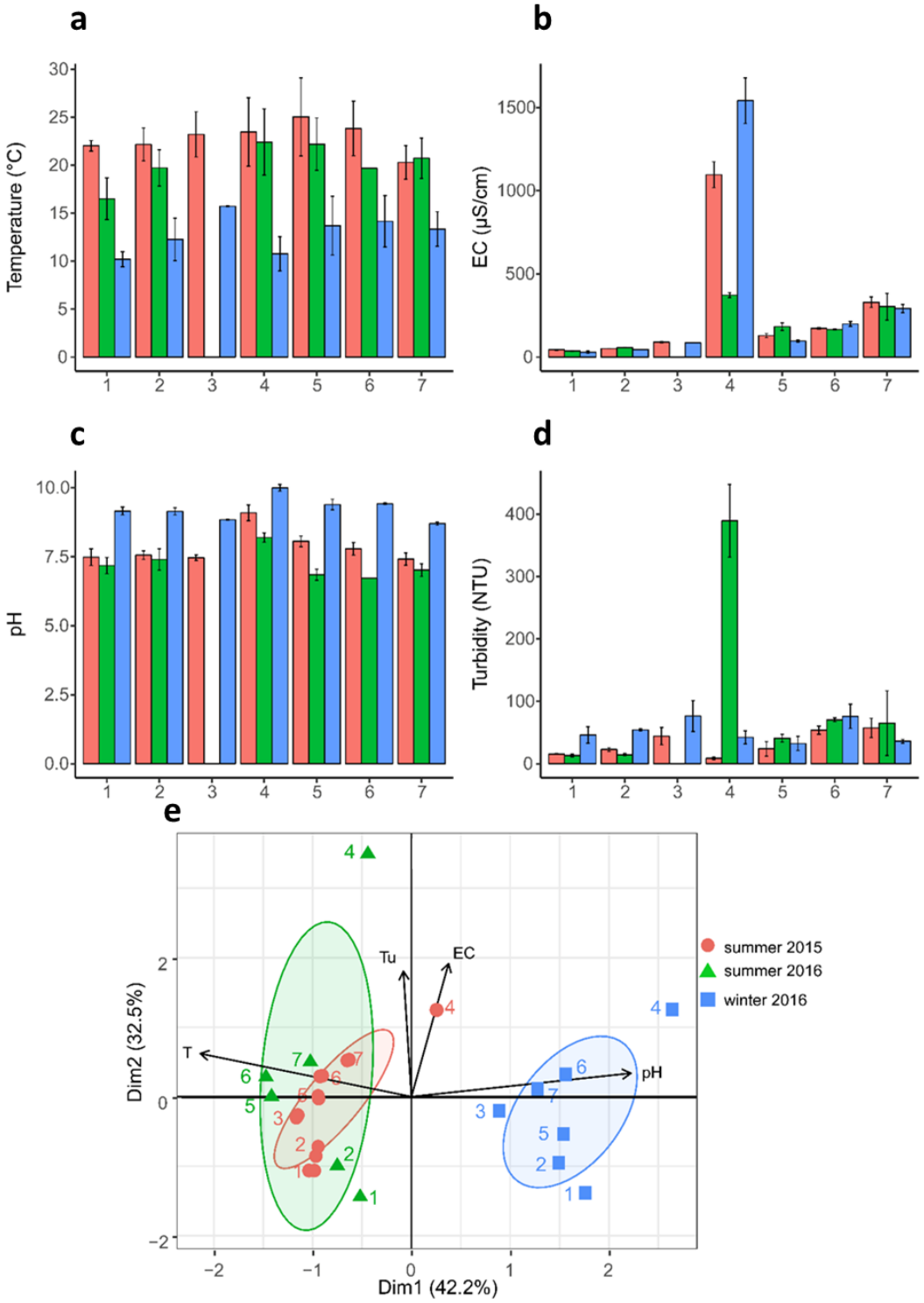

Figure 2.2. Water quality parameters for each site measured during summer 2015, winter 2016 and summer 2016. Values represent the average (± SD) calculated from four measurements taken during each sampling period; two measurements were at the beginning of deployment of artificial substrate and two measurements were at the end of deployment (**a-d**). **e**. Biplot of principal component analysis of water quality parameters at the seven samplings whereby the numbers represent sites: 1 = Jingellic, 2 = Yarrawonga, 3 = Euston, 4 = Burtundy, 5 = Lock 9, 6 = Murtho, 7 = Woods Point and colours represent seasons: summer 2015 (●), summer 2016 (▲), winter 2016 (■). No data were recorded for Euston in summer 2016 and data was not available for the end of deployment period for Murtho in summer 2016. The measured variables are T: temperature (°C), EC: conductivity (µS cm^{-1}), pH, and Tu: turbidity (NTU).

2.4.2 Total amino acid content

Macroinvertebrate taxa significantly varied in their TAA content (Kruskal-Wallis test, H = 30.1, df = 2, p < 0001 for summer; Kruskal-Wallis test, H = 19.6, df = 2, p < 0001 for winter; (Figure 2.3)). Among the three studied taxa, the TAA and TEAA content in *Physa* sp. was significantly lower than that of the two other taxa: *M. australiense* and *P. australiensis* (Table 2.2). The two decapods did not differ in TAA content. Data are not presented for *Triplectides* sp. due to the insufficient sample material for this taxon. The AA profile (mg per g of dry weight) is shown in Supplementary Table S2.5. No significant seasonal variation (summer and winter) were observed in the TAA concentration *of M. australiense* (Kruskal-Wallis test, H = 3.02, df = 1, p = 0.08), *P. australiensis* (Kruskal-Wallis test, H = 0.80, df = 1, p = 0.37) and *Physa* sp. (Kruskal-Wallis test, H = 3.48, df = 1, p = 0.06).

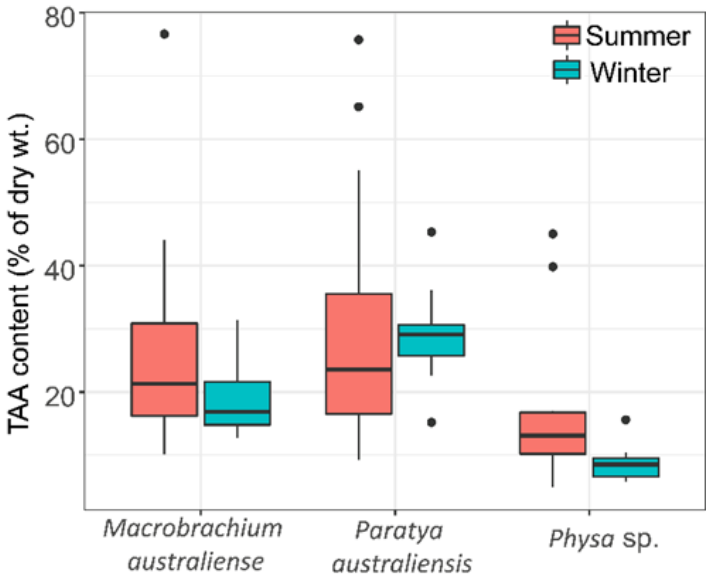

Figure 2.3. Total amino acid content (TAA) in *Macrobrachium australiense*, *Paratya australiensis*, and *Physa* sp. during summer (2015 and 2016 combined) and winter seasons.

Table 2.2. Total essential amino acids (TEAA) and Total non-essential amino acids (TNEAA) in three macroinvertebrate taxa expressed as mg per g dry weight, across the two seasons.

Taxa	Summer			Winter		
	TEAA	TNEAA	TAA	TEAA	TNEAA	TAA
Macrobrachium australiense	117.3 ± 5.7	138.0 ± 7.4	255.3 ± 12.9	95.9± 2.7	98.3 ± 3.7	194.2 ± 6.2
Paratya australiensis	129.4 ± 6.2	148.1 ± 8.0	277.5 ± 12.9	144.4 ± 3.4	143.5 ± 3.7	287.9 ± 6.9
Physa sp.	74.4 ± 5.6	104.5 ± 8.1	178.9 ± 13.7	39.0 ± 1.3	51.0 ± 1.8	90.0 ± 3.17

2.4.3 Taxa specific amino acid profile

We quantified 17 AAs of which four NEAAs: Glx, Gly, Ala and Asx, were dominant in the four taxa studied (Figure 2.4a-b). Met and His were the least abundant EAAs in all taxa. Comparing the relative abundance of TEAAs and TNEAAs, TNEAAs were relatively higher in all the studied taxa (Supplementary Table S2.4). A clear separation among the four studied taxa based on their AA profile was observed during summer (Figure 2.4c) and winter (Figure 2.4d), with AA profiles significantly different among the four taxa (summer: PERMANOVA, pseudo – F = 13.94, p = 0.001; winter: PERMANOVA, pseudo – F = 45.02, p = 0.001). The AAs Gly, Glx, Asx, Phe and Tyr were the major AAs that drove the differences among taxa.

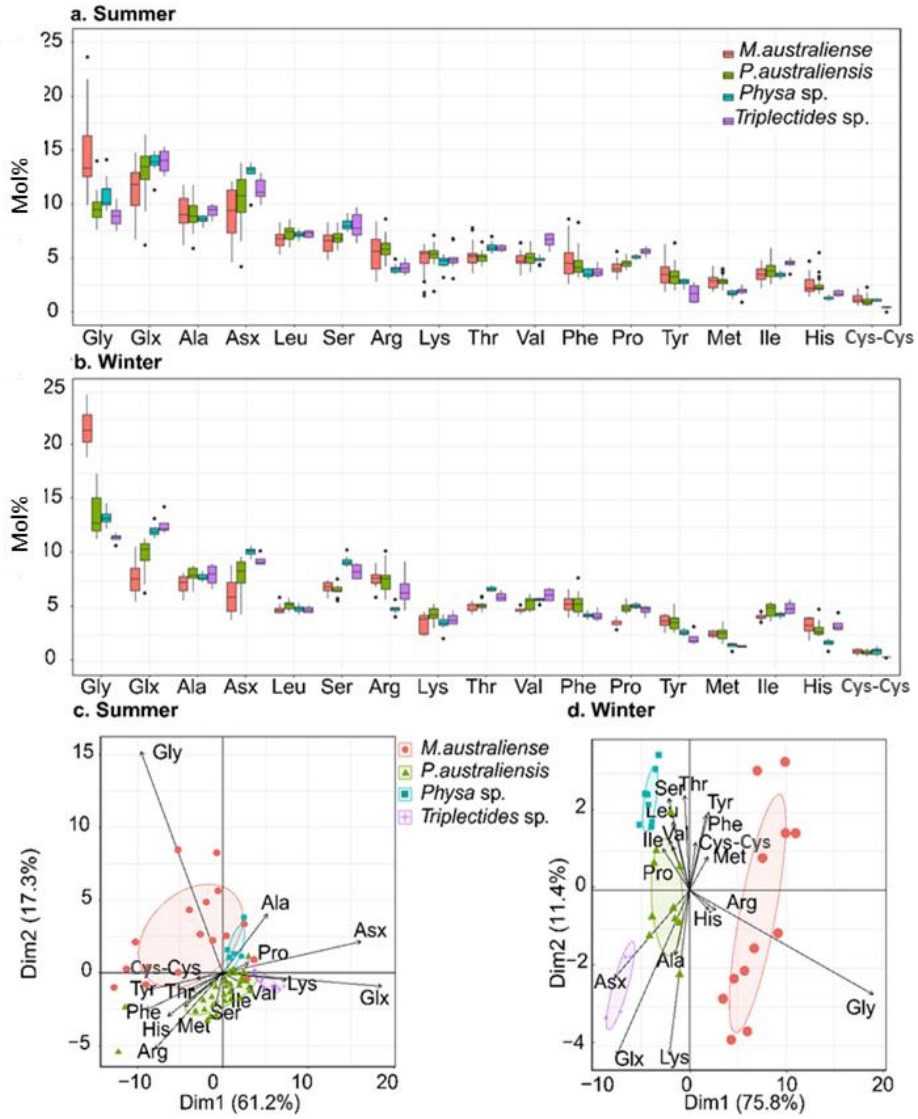

Figure 2.4. The amino acid profiles (relative abundance expressed as mol%) for *Macrobrachium australiense*, *Paratya australiensis*, *Physa* sp. and *Triplectides* sp. during: **a**. summer (2015 and 2016 combined) and **b**. winter (2016). Also shown are principal component analysis (PCA) of the amino acid profiles of the four macroinvertebrate taxa over two seasons: **c**. summer *(*2015 and 2016 combined) and **d**. winter (2016) with vectors shown for amino acids.

2.4.4 Seasonal patterns of amino acid profile in macroinvertebrates

Intra-species variation in AA profile was also observed between seasons in some taxa (Figure 2.5a) (PERMANOVA, pseudo-F = 8.7369, p = 0.001) with a significant interaction between taxa and sampling period (PERMANOVA, pseudo-F = 2.7972, p = 0.004). Significant intra-specific seasonal variations in AA profile of *M. australiense and P. australiensis* were observed, with winter 2016 samples significantly different from both summers (2015 and 2016) sampling periods. However, no seasonal variation in AA profile was observed for *Physa* and *Triplectides* sp. (Table 2.3). Furthermore, Gly, Leu and Val were shown to be the major AAs that changed in their proportions between seasons (summer and winter) in *M. australiense* (Figure 2.5b, Supplementary Table S2.4) whereas, in *P. australiense*, seasonal variation was observed in Gly, Ser, Thr, Met, Lys, Ile and Pro (Figure 2.5c, Supplementary Table S2.4). Both the decapods had a higher content of Gly in winter compared to summer (7% higher in *M. australiense* and 3% higher in *P. australiensis)*. The TEAA and TNEAA content for each taxon over different seasons are shown in Table 2.2. Substantial seasonal variations were observed in TEAA content in all studied taxa with less TEAA content during winter compared to summer.

Table 2.3. Permutational multivariate ANOVA (PERMANOVA) pairwise comparisons for macroinvertebrate amino acid profile using 'taxa' and 'season' as fixed factors. *p*-values in bold correspond to significantly different amino acid profiles ($p < 0.05$).

Taxa	Groups	*t*	*p* (perm)	Unique permutations
Macrobrachium australianse	Winter 2016-Summer 2015	4.16	**0.001**	999
	Winter 2016-Summer 2016	2.16	**0.018**	819
	Summer 2015-Summer 2016	0.97	0.335	772
Paratya australiensis	Winter 2016-Summer 2015	2.52	**0.001**	998
	Winter 2016-Summer 2016	2.15	**0.003**	999
	Summer 2015-Summer 2016	0.60	0.696	999
Physa sp.	Winter 2016-Summer 2016	1.30	0.161	708
Triplectides sp.	Winter 2016-Summer 2016	0.96	0.451	773

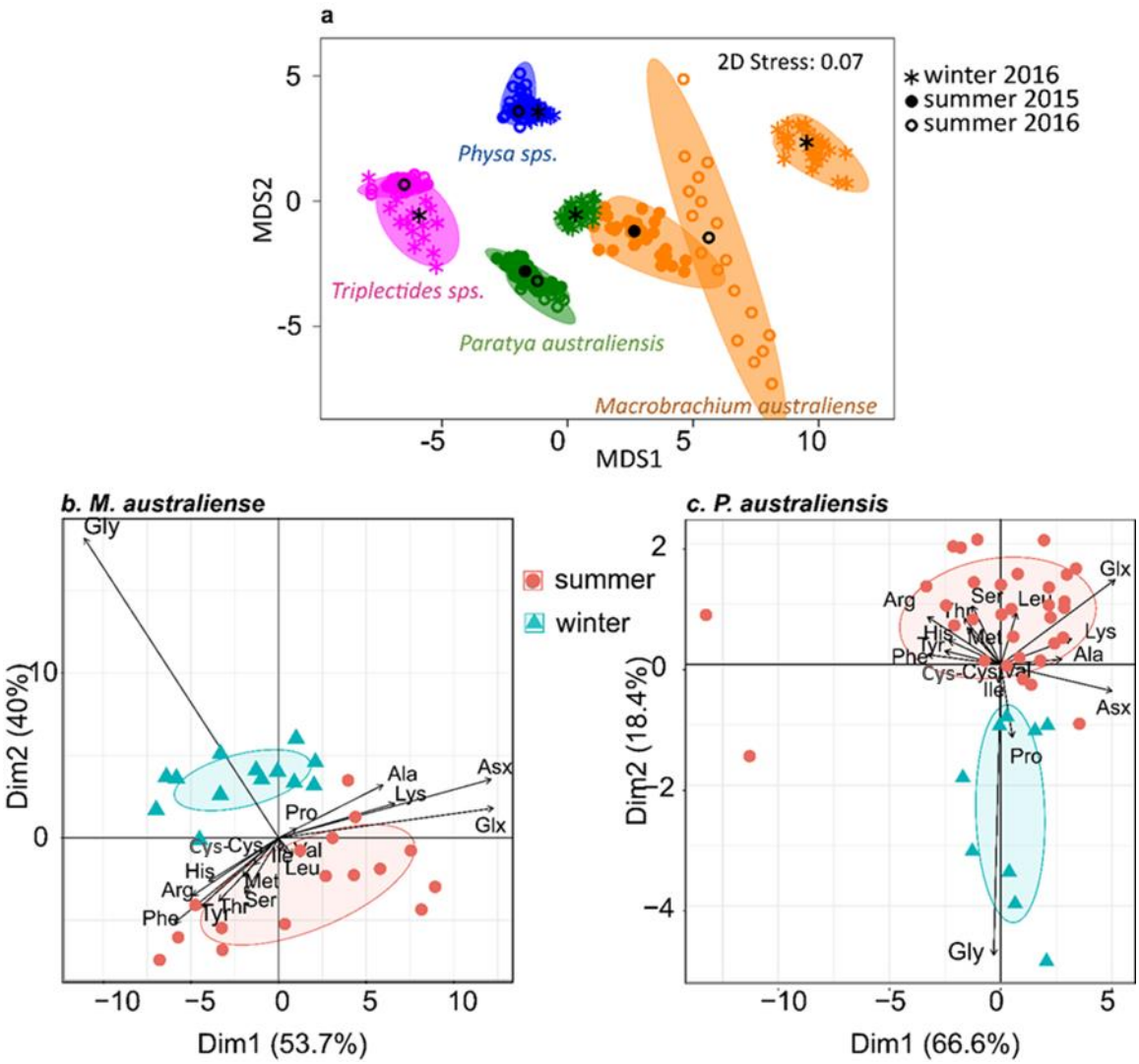

Figure 2.5. **a**. Multidimensional scaling (MDS) ordination based on 95% confidence interval bootstrap average showing seasonal differences in the amino acid profile of *Macrobrachium australiense*, *Paratya australiensis*, but not *Physa* sp. or *Triplectides* sp. collected over three sampling periods (summer 2015, summer 2016 and winter 2016). Note *Physa* sp. and *Triplectides* sp. were not sampled during summer 2015. Black symbols represent average location for particular groups. Principal component analysis plots showing seasonal differences in amino acid profile for **b**. *Macrobrachium australiense* and **c**. *Paratya australiensis* (summer 2015 and summer 2016 combined).

2.4.5 Spatial variability of amino acid profiles

Amino acid profile of *M. australianse* showed no significant variation between sites over all seasons (summer 2015: PERMANOVA, pseudo-F = 0.86, p = 0.61; summer 2016: PERMANOVA, pseudo-F = 0.75, p = 0.64; and winter 2016: PERMANOVA, pseudo-F = 0.20, p = 0.95). Likewise, no spatial variation was observed in the AA profile of *Physa* sp. collected during summer and winter 2016, despite specimens being collected from four widely separated sites: Woods Point (site 7), Murtho (site 6), Lock 9 (site 5) and Jingellic (site 1) (PERMANOVA, pseudo-F = 1.74, p = 0.17).

P. australiensis was collected from three locations (Woods Point (site 7), Murtho (site 5), and Yarrawonga (site 2)) during summer 2016 with no significant difference in the AA profile between sites (PERMANOVA, pseudo-F = 1.6, p = 0.14). Similarly, no site effect was detected in the AA profile of *P. australiensis* collected from all six sites along the Murray River during summer 2015 (PERMANOVA, pseudo-F = 1.01, p = 0.31). During winter 2016, *P. australiensis* was found only at three sites: Woods Point (site 7), Yarrawonga (site 2) and Jingellic (site 1) with significant spatial variation in the AA profile (PERMANOVA, pseudo-F = 14.46, p = 0.001) found. Pair-wise comparisons revealed that *P. australianse* from Woods Point (site 7) contained significantly different AA profile than specimens from Yarrawonga (site 2) (t = 4.4, p < 0.05) and Jingellic (site 1) (t = 3.4, p < 0.05); *P. australiensis* from Yarrawonga (site 2) and Jingellic (site 1) were not significantly different in their AA profile (t = 2.06, p = 0.052). Leu, Thr, Val, Phe, Ile, Tyr, Met and Cys-Cys were all found to be significantly lower in the Woods Point samples whereas Gly, Asx and Glx were significantly higher compared to the other two sites (Figure 2.6).

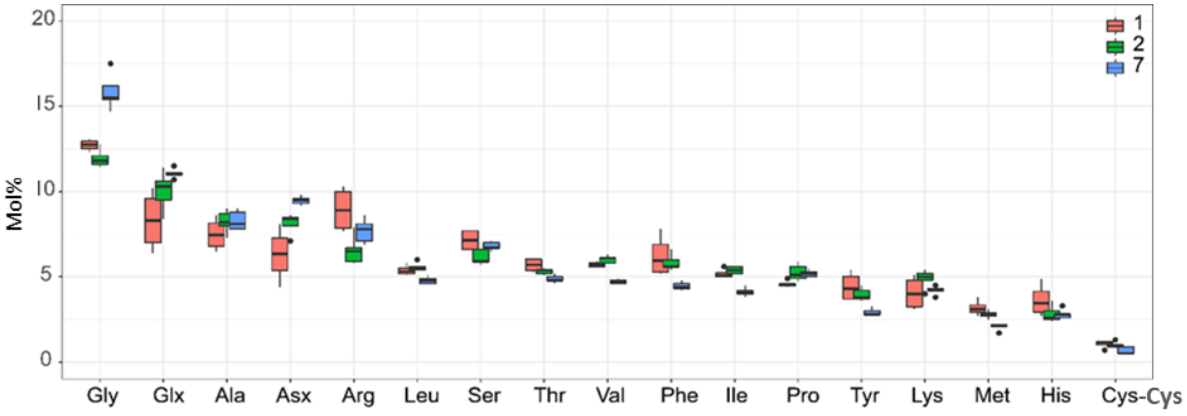

Figure 2.6. Amino acid profile (relative abundance expressed as mol%) in *Paratya australiensis* collected from three sites along the Murray River during winter 2016 (site 1: Jingellic, site 2: Yarrawonga, site 7: Woods Point).

2.4.6 Environmental predictors of amino acid profile

Amino acid profile of decapods (*M. australiense* and *P. australiensis*) differed between the seasons (summer and winter), whereas, spatial variation was observed only for *P. australiensis* during winter 2016. The DistLM analysis with specified selection sequential tests of four environmental variables indicated that temperature and pH explained 51.3% of the variation in AA profile of *M. australiense* based on the AIC values (Table 2.4 and Figure 2.7a). In the case of *P. australiensis*, temperature and turbidity were the only two significant environmental parameters (Table 2.4), explaining around 23.6 % of the variation in the AA profile (Figure 2.7b). Temperature was the most important single environmental predictor of decapod AA profiles (34.7% and 17.8% of the variation in the AA profile in *M. australiense* and *P. australiensis*, respectively).

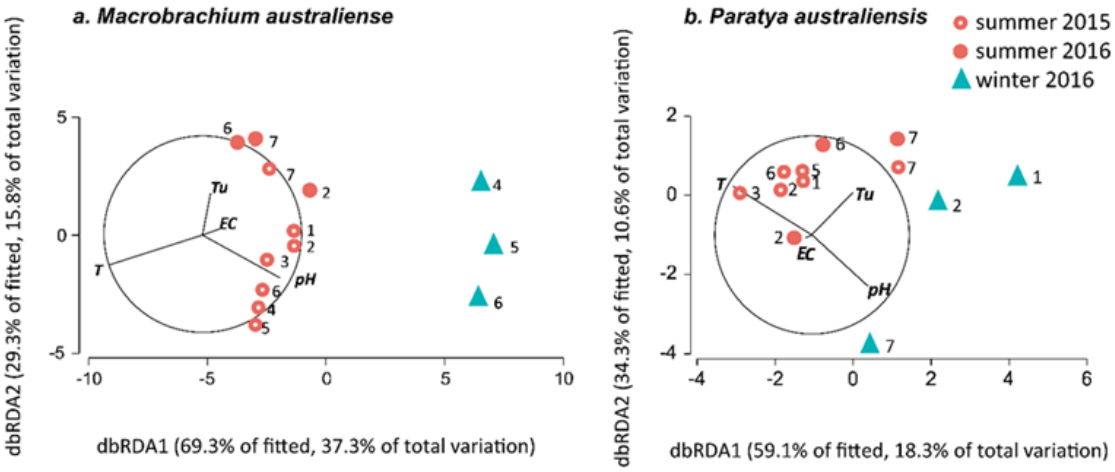

Figure 2.7. Distance-based redundancy analysis (dbRDA) of Euclidean distance matrix calculated from standardised amino acid data for: **a**. *Macrobrachium australiense* and **b**. *Paratya australiensis* showing water quality parameters (T: temperature, Tu: turbidity, EC: conductivity and pH) as predictor variables. Numbers represent sites: 1 = Jingellic, 2 = Yarrawonga, 3 = Euston, 4 = Burtundy, 5 = Lock 9, 6 = Murtho, 7 = Woods Point.

Table 2.4. DistLM results of amino acid profile for *Macrobrachium australiense* and *Paratya australiensis* against 4 predictor water quality variables (999 permutations). Bold indicates variables significantly correlated with amino acid profile at $p < 0.05$.

Predictor variable	Taxa	Marginal test			Specified selection sequential tests			
		Pseudo-*F*	*p*	PVX *	Pseudo-*F*	*p*	PVX*	CVX**
Temperature (°C)	*M. australiense*	24.45	**0.001**	34.7	24.45	**0.001**	34.7	34.7
	P. australiensis	8.67	**0.001**	14.8	8.67	**0.003**	14.8	14.8
pH	*M. australiense*	16.07	**0.001**	25.9	15.22	**0.001**	16.6	51.3
	P. australiensis	4.98	**0.014**	9.1	0.64	0.454	1.1	15.9
Turbidity (NTU)	*M. australiense*	1.63	0.181	3.4	1.25	0.266	1.3	52.6
	P. australiensis	3.06	**0.046**	5.7	4.58	**0.025**	7.3	23.2
Conductivity (µs cm^{-1})	*M. australiense*	0.57	0.579	1.2	0.43	0.667	0.6	53.2
	P. australiensis	0.36	0.766	0.7	0.81	0.390	1.3	24.5

* Proportion variation explained ** Cumulative variation explained

2.5 Discussion

2.5.1 Variation in total amino acid content among taxa

Overall, in our study decapods were found to contain a higher TAA content compared to the snail indicating their high nutritional value. Higher protein concentration in decapods compared to *Physa* could be due to their omnivorous feeding habit and also may be due to decapods containing more muscle tissue than exoskeleton (Abdel-Salam, 2014). Zukowski and Walker (2009) reported that *Physa acuta* is the most abundant freshwater snail in the lower River Murray. *Physa acuta* is an introduced species and their ascendancy has been linked to the decline of native species (Zukowski & Walker, 2009). Given the differences in nutritional quality between the snail *Physa* sp. and the other taxa in this study, the increasing population of this species could affect the availability of TAA/protein to higher order consumers in the Murray River.

2.5.2 Amino acid profiles in macroinvertebrates are taxa specific

In line with findings from previous studies (Dwyer et al., 2018; Aranguren-Riaño et al., 2018), AA profiles successfully discriminated between the four taxa (*M. australiense, P. australiensis, Physa* sp. and *Triplectides* sp.) regardless of having been collected from a wide geographic range and across various seasons. This supports the assumptions made by

Bogatyreva et al. (2006) and Dwyer et al. (2018) that AA profile in living organisms is a highly conserved pattern and controlled by genetic information.

Apart from Ala, the proportion of all other (16) AAs differed among taxa. Differences in AA profile among macroinvertebrate taxa also suggest that streams and rivers with a diverse macroinvertebrate community structure may provide a wider variety of AAs for top consumers than rivers with lower diversity. In addition to this, changes in community composition of macroinvertebrates will alter the nutritional landscape available to top predators such as fish (Dwyer et al., 2018). Previous studies have reported that diets higher in EAAs compared to NEAAs lead to better development and protein synthesis in fish (Peres & Oliva-Teles, 2006). Based on the TEAA content from our study, decapods had higher nutritional value compared to *Physa* sp. A higher amount of TEAA in decapods has also been reported in previous studies (Yanar & Çelik, 2006; Bhavan et al., 2010). The EAA- Val was found in the highest proportion in *Triplectides* but this taxon contained a lower proportion of other EAA such as Leu, Phe and Met. Among the nine EAAs, the abundance of Met and His are lowest in all the studied taxa. Many studies have found that Met and His are the limiting EAA for fish and other higher consumers feeding on macroinvertebrates (Reed & D'Abramo, 1989; Yanar & Çelik, 2006). Glx, Ala, Asx and Gly were found to be the dominant NEAAs in the studied macroinvertebrates which were in accordance with the findings from previous studies (Reed & D'Abramo, 1989; Çagiltay et al., 2011; Leiwakabessy & Lewerissa, 2017).

2.5.3 Amino acid profile in macroinvertebrates with respect to seasons

Many studies have emphasized season as one of the major factors that shape the AA profile of organisms (Çagiltay et al., 2011; Binoy et al., 2012; Çaglak & Karsli, 2017; Ghribi et al., 2018). However, the study of food webs in rivers and streams are often limited to a single point of time, which makes our understanding about riverine nutritional ecology incomplete. Given rivers are dynamic systems, interactions between different trophic levels and energy flow could be affected by seasonal changes in food sources or food nutritional quality. Our results show that even though there is no significant seasonal change in the TAA content of macroinvertebrates, the AA profile of the two decapod species *M. australiense* and *P. australiensis* show significant seasonal variation; *Physa* sp. and *Triplectides* sp. on the other hand, showed no such variation.

The changes in the AA profile of decapods with season is consistent with a previous study on the mussel *Mytilus edulis* (Li et al., 2015). The nutritional value of decapods is higher

during summer, with respect to their TEAA content. For *M. australiense* the EAAs Ser, Val, Ile decreased in winter compared to summer while Gly increased. Similarly, for *P. australiensis* the EAAs Lys, Ser, Thr, Met decreased during winter while Ile and Gly increased. Lys and Met are considered to be important indicators of the nutritional value of fish diets (Li et al., 2009). Our results showed a significant decrease in the Lys and Met levels in winter compared to summer in *P. australiensis.* A decrease in Lys content was also observed in *Physa* sp. in winter even though the overall AA profile did not significantly differ. A decrease in the availability of different EAAs in macroinvertebrates during winter may result in a poorer quality diet with decreased nutritional value for higher consumers like fish. Top predators therefore may need to adopt different feeding strategies to meet their AA requirements across seasons. This highlights a need to assess the transfer of EAAs between macroinvertebrates and their predators to understand the effects of fluctuating EAA contents on growth and metabolism of higher order consumers in a riverine environments.

Seasonal changes in water temperature can change the basal food resources in rivers. One reason for the changes in the AA profile of decapods during summer and winter could be the result of varied food resources available to the decapods during the two different seasons. The similarity of AA profile within two summer sampling periods (summer 2015 and summer 2016) in *M. australiense* and *P. australiensis* provide some support for this idea. Decapod diets generally consist of a wide variety of foods such as biofilms, littoral plants and fine particulate organic matter (Burns & Walker, 2000). Seasonal variation in discharge, temperature and shading of the riparian region in rivers can alter food resources for decapods through changes in basal community composition, productivity or changes in food web (Torres-Ruiz et al., 2007). Such variation in diet might be a reason for seasonal intraspecies variation in the AA profile of decapods. However, previous studies suggest that diet may not be an influencing factor defining the AA profile of organisms. Brückner et al. (2017) showed diet did not play a role in defining the AA profile of oribatid mites (microarthropods). These soil microarthropod's feeding habits are diverse, ranging from decomposers to scavengers and predators, but their AA profile was similar despite significant differences in the AA profile of their food. Moreover, none of the measured AAs correlated with the AA profile of these resources. Srivastava et al. (2006) reported that, despite feeding the rotifer *Brachionus plicatilis* on five varieties of food, this caused no changes to their AA profiles even though the food differed in AA profile. Similarly, Boëchat and Adrian (2005) showed that two species of freshwater ciliate have significantly

different AA profiles despite feeding on the same algae as a food resource. Hence, further evidence would be required to support the idea that changing diet is a major factor behind seasonal and temporal variations in the AA profiles of the decapods studied here.

We also showed that the AA profile of purged and unpurged shrimp was not found to be different, suggesting that gut content does not significantly affect the AA profile of decapods. Therefore, the seasonal variation in AA profile of decapods observed in our study is not due to differences in the gut content of the decapods at time of sampling. The lack of effect of gut contents on AA profile is likely due to the relatively small contribution of the gut compared to the whole body mass.

Another possible reason for the seasonal variation in the nutritional quality of decapods could be due to changes in the biochemical response (AA or protein) of organisms triggered by changes in water quality parameters driven by season. Water quality parameters, especially temperature and pH, differed substantially between seasons (Figure 2.2). DistLM results showed around 53% of AA profile variation of *M. australiense* and 24% of AA profile variation in *P. australiensis* are explained by the water quality parameters, with temperature alone explaining the most seasonal and spatial variation (34% and 15%, respectively). Water temperature is the main abiotic driver that influences feeding, growth, behaviour, life cycle and metabolism in macroinvertebrates (Dallas & Rivers-Moore, 2012; Li et al., 2013). Previous studies have demonstrated that temperature changes alter the FAA content in different organisms like adult barnacle *Balanus balanoides* (Cook et al., 1972); white shrimp, *Litopenaeus vannamei* (Zhou et al., 2011); and the beetles *Sitophilus granarius* and *Cryptolestes ferrugineus* (Fields et al., 1998). Further investigation is required to determine the influence temperature has on determining the AA profile of freshwater organisms and thus potential for altering the nutritional landscape of aquatic ecosystems. This is greatly needed given the threat of climate change and anthropogenic changes to temperature regimes within aquatic ecosystems through cold water pollution associated with river regulation.

The greatest seasonal variation in AA profile was seen in the Gly content with a difference of around 7% in *M. australiense* and 3% in *P. australiensis*, with a higher content during winter. Gly is synthesized from Ser and Thr (Wang et al., 2013; Xie et al., 2014). Decreases in Ser and Thr were also shown between summer and winter suggesting that the up-regulation of Gly in winter might have decreased the amount of Ser and Thr (Supplementary Table S2.4) in decapods during this season. In contrast, previous studies have shown that Gly content (protein bound + free) decreased in winter compared to

summer (Yanar & Çelik, 2006). This has also been shown for other decapods such as the green tiger shrimp and speckled shrimps from the eastern Mediterranean coast (Yanar & Çelik, 2006), red shrimp, pink shrimp and Norway lobster (Rosa and Nunes (2004). According to Zhou et al. (2011), response of individual AA to temperature depends upon the species. Even though Gly is one of the NEAAs for macroinvertebrates and fish, it is known to participate in gene expression and regulation and has a role in the immune response of fish. Gly has also been reported as an important AA in the osmoregulatory responses of aquatic animals to environmental stress (Xie et al., 2014) by improving oxidation resistance capacity. They reported that increases in Gly in the diet of shrimps significantly increased the survival of shrimps exposed to low salinity with increases in growth rate, protein production as well as whole body concentrations of Mg, Ca and Fe (Xie et al., 2014). The change in Gly content therefore might also reflect a protective mechanism in response to changing temperatures in the decapods in this study.

2.5.4 Spatial pattern of amino acid within taxa

No variation in AA profile of *M. australiense*, *P. australiensis* (winter is an exception) *and Physa* sp. collected from different sites along the Murray River was found in this study despite the EC and turbidity changes along the Murray River. Conductivity varied from 24 to 373 µS cm^{-1} at sites along the Murray and was substantially higher at Burtundy (Darling River) ranging between 359 and 1660 µS cm^{-1}. Such variation in water quality among sites, however, seems to have less impact on the AA profile of macroinvertebrates compared to the seasonal factors and shows macroinvertebrates may be able to maintain AA homeostasis in a wide range of habitats. In contrast, various studies have shown that salinity impacts on the FAA profile of decapods due to role of FAA (Glu, Ala, Gly, Arg, Pro and Lys) in osmoregulation (Yang et al., 2001; Koyama et al., 2018). Yang et al. (2001) reported a relationship between salinity and changes in AAs of freshwater prawns however the effects on FAAs were seen at much higher salinity concentrations then what were present at sites in this study.

Even though site variation was not observed in the AA profile of *P. australiensis* collected from six sampling sites (Murtho, Woods point, Yarrawonga, Lock 9, Jingellic, Euston) in summer 2015 and three sampling sites (Murtho, Woods point and Yarrawonga) in summer 2016, there was a significant difference in the AA profile of *P. australiensis* collected from three sites (Woods point, Yarrawonga and Jingellic) in winter 2016. All the EAAs except Arg, His and Lys were found to be lower in the shrimps from Woods Point compared to

that of the upper two sites (Yarrawonga and Jingellic). DistLM analysis showed that temperature and EC explained around 24% of the variation in the AA profile of *P. australiensis* between sites during winter. Marie et al. (2017) also reported changes to the transcriptome in the shrimp *Palaemon carideans* due to the combined stress of temperature and salinity which is likely to have altered the AA profile of this taxa. Another possible explanation for the observed spatial variation could be due to possible genetic differences in the population of *P. australiensis* between upstream and downstream of the Murray River. However, this is less likely given no site difference was shown during summer. The observed site variation in AA profile of *P. australiensis* during winter may be attributed to other factors like ontogeny (Roustaian et al., 2000) or sex (Bhavan et al., 2010). Further research is thus needed to tease out the factors contributing to spatial variations in AA profiles of *P. australiensis* during winter.

2.6 Conclusion

This study shows that the AA profile of freshwater macroinvertebrates is not only taxon specific but can vary temporally (between seasons) and spatially between sites depending on taxa. A change in the community composition of macroinvertebrates can therefore potentially alter the nutritional landscape available to higher order consumers within riverine environments. Further research is required to determine the relative influence of seasonal and spatial variation in AAs due to inter and intraspecies differences, on the growth and metabolism of top predators and to understand the cascading effects across the food web.

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2.9 Supplementary materials

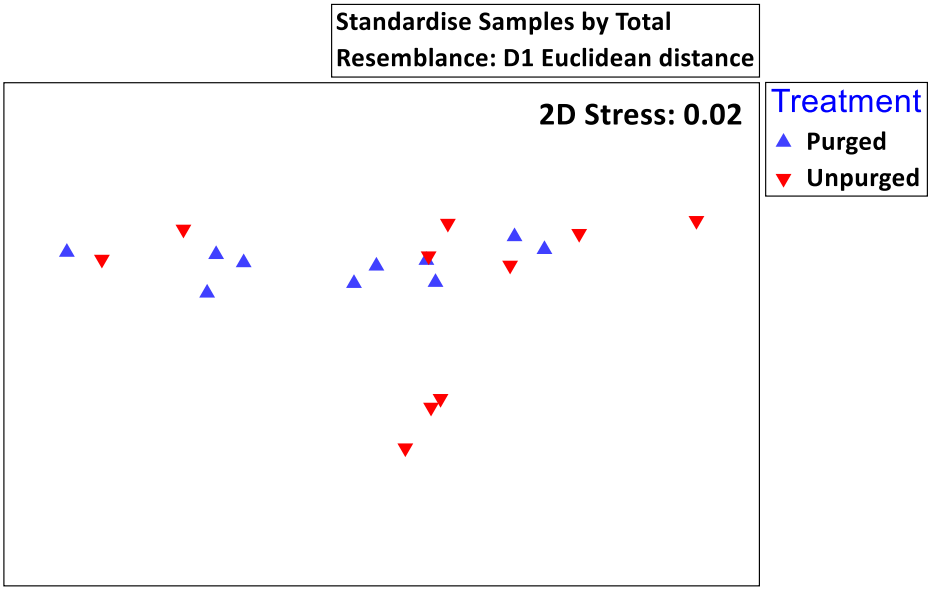

Figure S2.1. NMDS plot of amino acid profiles of purged and unpurged *Paratya australiensis* collected from Wodonga Creek.

Table S2.1. Amino acid properties, including: polymerised molecular weight (MW.poly), abbreviated amino acid identifier (AA.ID.short) and essential and non-essential (EAA/NEAA) based on (Li et al., 2009).

AA.ID	MW.poly	AA.ID.short	EAA/NEAA
Histidine	137.14	His	Essential
Asparagine	114.11	Asn	Non-Essential
Arginine	156.19	Arg	Essential
Serine	87.08	Ser	Non-Essential
Glutamine	128.14	Gln	Non-Essential
Glycine	57.05	Gly	Non-Essential
Aspartate	115.09	Asp	Non-Essential
Glutamate	129.12	Glu	Non-Essential
Threonine	101.11	Thr	Essential
Cystine	204.3	Cys-Cys	Non-Essential
Alanine	71.09	Ala	Non-Essential
Proline	97.12	Pro	Non-Essential
Cysteine	103.15	Cys	Non-Essential
Lysine	128.17	Lys	Essential
Tyrosine	163.18	Tyr	Non-Essential
Methionine	131.19	Met	Essential
Valine	99.14	Val	Essential
Isoleucine	113.16	Ile	Essential
Leucine	113.16	Leu	Essential
Phenylalanine	147.18	Phe	Essential
Tryptophan	186.21	Trp	Essential
ASX	115.09	Asx	Non-Essential
GLX	129.12	Glx	Non-Essential

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Table S2.2. Number of specimens collected from each site and season.

		Site	1. Jingellic			2. Yarrawonga			3. Euston			4. Burtundy			5. Lock 9			6. Murtho			7. Woods Point		
Taxa	Order	Family	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W	2015 S	2016 S	2016 W
Macrobrachium australiense	Decapoda	Palaemonidae	1			2	5		5			5		5	5		5	3	4	3	1	5	
Paratya australiensis	Decapoda	Atyidae	5		4	5	5	5	1						2			5	5		5	5	5
Physa sp.	Basommatophora	Physidae		3												4	3		1	2		5	3
Triplectides sp.	Trichoptera	Leptoceridae		2												1	1		4			5	3

2015 S = 2015 Summer; 2016 S = 2016 Summer; 2016 W =2016 Winter

Table S2.3. Percent recovery of 16 amino acids with 6 N HCl containing 0.02% phenol hydrolysis. Note: Bovine Insulin does not include methionine (Met).

Amino acid	His	Arg	Ser	Gly	Asx	Glx	Thr	Cys-Cys	Ala	Pro	Lys	Tyr	Val	Ile	Leu	Phe
% Recovery	37	86	90	96	80	76	91	28	74	77	76	58	46	26	57	57

Table S2.4. Amino acid profile expressed as the relative abundance of each amino acid (mol%) (mean ± SD) of 17 amino acids in macroinvertebrates during summer and winter. *p*-values are from Mann-Whitney U test to determine differences between seasons. TEAA = sum of essential amino acids, TNEAA = sum of non-essential amino acids.

AAs	*Macrobrachium australiense*			*Paratya australiensis*			*Physa* sp.			*Triplectides* sp.		
	Summer	Winter	*p*	Summer	Winter	*p*	Summer	Winter	*p*	Summer	Winter	*p*
Arg*	6.0 ± 1.5	5.8 ± 0.8	0.93	6.4 ± 1.2	5.7 ± 0.5	0.05	3.1 ± 0.4	3.4 ± 0.3	0.24	4.7 ± 0.2	4.8 ± 0.5	0.95
His*	2.8 ± 1.3	2.6 ± 0.5	0.91	2.9 ± 0.9	2.5 ± 0.2	0.43	1.6 ± 0.3	0.8 ± 0.2	0.14	1.6 ± 0.4	1.8 ± 0.3	0.27
Ile*	3.3 ± 0.3	3.0 ± 0.3	0.06	3.8 ± 0.4	4.1 ± 0.2	**0.003**	4.1 ± 0.2	4.2 ± 0.3	0.62	4.1 ± 0.5	3.8 ± 0.5	0.17
Leu*	6.6 ± 0.6	6.3 ± 0.2	**0.04**	7.3 ± 0.7	7.2 ± 0.5	0.35	8.5 ± 0.6	8.0 ± 0.2	0.09	5.8 ± 1.5	6.6 ± 1.3	0.50
Lys*	4.6 ± 1.6	4.3 ± 1.0	0.33	5.9 ± 1.2	5.7 ± 0.4	**0.04**	3.6 ± 0.4	3.1 ± 0.4	**0.03**	5.8 ± 0.7	7.0 ± 1.3	0.10
Met*	2.9 ± 0.7	2.6 ± 0.5	0.27	2.9 ± 0.6	2.4 ± 0.1	**0.02**	1.5 ± 0.5	1.8 ± 0.1	0.14	1.6 ± 0.4	1.9 ± 0.4	0.07
Phe*	5.3 ± 1.9	4.6 ± 0.9	0.72	4.5 ± 1.2	4.2 ± 0.3	0.46	4.1 ± 0.4	4.3 ± 0.2	0.34	3.5 ± 0.3	3.3 ± 0.5	0.43
Thr*	6.0 ± 1.0	5.3 ± 0.8	0.09	5.4 ± 0.6	4.9 ± 0.3	**0.02**	6.1 ± 0.4	6.5 ± 0.3	**0.03**	5.6 ± 0.3	5.6 ± 0.4	0.50
Val*	4.9 ± 0.5	4.5 ± 0.3	**0.001**	5.1 ± 0.5	5.3 ± 0.4	0.52	5.6 ± 0.4	6.0 ± 0.3	0.06	6.3 ± 0.6	5.5 ± 0.4	**0.04**
TEAA	42.4 ± 4.5	39 ± 2.9	**0.03**	44.1 ±3.1	41.9 ± 1.5	**0.04**	37.6 ± 1.7	38.0 ± 1.5	0.90	39.0 ± 1.6	39.3 ± 1.7	0.90
Ala	8.8 ± 1.7	8.6 ± 1.0	0.42	8.9 ± 1.1	9.0 ± 0.3	0.29	8.8 ± 0.7	8.9 ± 0.5	0.72	9.0 ± 0.7	9.4 ± 1.5	0.50
Asx	8.2 ± 2.8	7.5 ± 1.8	0.53	9.6 ± 1.8	10.1 ± 0.8	0.50	11.9 ± 0.9	11.2 ± 0.8	0.55	12.1 ± 1.1	11.8 ± 0.8	0.95
Cys-Cys	1.3 ± 0.6	1.8 ± 0.4	0.30	1.0 ± 0.4	0.9 ± 0.2	0.43	1.0 ± 0.2	1.2 ± 0.2	0.28	0.4 ± 0.1	0.3 ± 0.2	0.85
Glx	10.6 ± 2.8	9.6 ± 1.8	0.30	12.2 ± 1.9	11.5 ± 0.8	0.05	12.3 ± 0.7	12.1 ± 0.7	0.50	16 ± 2.4	15.3 ± 2.0	0.80
Gly	13.8 ± 2.0	20.9 ± 1.5	**0.0001**	9.5 ± 0.8	12.2 ± 1.4	**0.0001**	11.3 ± 0.7	11.5 ± 0.5	0.43	9.1 ± 0.6	9.4 ± 0.4	0.26
Pro	4.9 ± 0.6	3.9 ± 0.3	0.77	4.5 ± 0.5	5.4 ± 0.4	**0.0001**	5.9 ± 0.3	5.8 ± 0.2	0.35	5.4 ± 0.4	5.2 ± 0.2	0.50
Ser	6.8 ± 0.9	6.1 ± 0.6	0.08	7.0 ± 0.6	6.2 ± 0.4	**0.0008**	8.1 ± 0.2	8.2 ± 0.3	0.46	7.7 ± 0.7	7.5 ± 0.5	0.95
Tyr	4.0 ± 1.5	3.5 ± 0.6	0.61	3.4 ± 1.0	2.9 ± 0.3	0.22	3.1 ± 0.4	3.2 ± 0.3	1.00	1.5 ± 0.7	1.7 ± 0.8	0.95
TNEAA	57.6 ± 4.5	61.0 ± 2.9	**0.03**	55.9 ± 3.1	58.1 ± 1.5	**0.04**	62.4 ± 1.7	62.0 ± 1.5	0.90	61.0 ± 1.6	60.6 ± 1.7	0.90

(**Bold** indicates significant difference at $p = 0.05$) * Essential amino acids

Table S2.5. Amino acid profile expressed as mg per g of dry weight (mean ± SD) of 17 amino acids in macroinvertebrates during summer and winter. TEAA = sum of essential amino acids*, TNEAA = sum of non-essential amino acids.

AAs	*Macrobrachium australiense*		*Paratya australiensis*		*Physa* sp.	
	Summer	Winter	Summer	Winter	Summer	Winter
Arg*	19.4 ± 1.0	21.3 ± 0.6	22.7 ± 1.1	30.9 ± 0.8	10.0 ± 0.7	6.0 ± 0.2
His*	8.0 ± 0.4	8.0 ± 0.2	8.0 ± 0.3	10.6 ± 0.2	3.0 ± 0.2	2.0 ± 0
Ile*	9.0 ± 0.5	9.0 ± 0.3	10.8 ± 0.5	14.5 ± 0.4	7.0 ± 0.5	4.0 ± 0.1
Leu*	17.7 ± 1.0	10.6 ± 0.4	20.9 ± 1.1	15.7 ± 0.4	13.4 ± 1.1	5.0 ± 0.2
Lys*	15.4 ± 0.9	9.0 ± 0.4	17.7 ± 1.0	15.1 ± 0.5	10.0 ± 0.8	4.0 ± 0.2
Met*	9.0 ± 0.4	6.0 ± 0.2	9.0 ± 0.4	9.0 ± 0.2	4.0 ± 0.3	2.0 ± 0
Phe*	15.5 ± 0.8	14.0 ± 0.3	15.5 ± 0.6	20.5 ± 0.4	9.0 ± 0.7	5.0 ± 0.2
Thr*	12.1 ± 0.6	9.0 ± 0.3	12.5 ± 0.6	13.9 ± 0.3	10.0 ± 0.7	6.0 ± 0.2
Val*	11.2 ± 0.6	9.0 ± 0.3	12.3 ± 0.6	14.2 ± 0.4	8.0 ± 0.6	5.0 ± 0.2
TEAA	**117.3 ± 5.7**	**95.9 ± 2.7**	**129.4 ± 6.2**	**144.4 ± 3.4**	**74.4 ± 5.6**	**39.0 ± 1.3**
Ala	15.7 ± 0.9	10 ± 0.4	16.7 ± 1	15.3 ± 0.4	10.1 ± 0.8	5.0 ± 0.2
Asx	25.1 ± 1.6	14.0 ± 0.8	31.3 ± 2.0	24.8 ± 0.8	24.5 ± 2.0	10.0 ± 0.4
Cys-Cys	6.0 ± 0.3	4.0 ± 0.2	5.0 ± 0.2	5.0 ± 0.2	4.0 ± 0.3	2.0 ± 0
Glx	35.1 ± 2.1	19.8 ± 0.9	43.4 ± 2.6	34.2 ± 1.1	29.4 ± 2.4	13.0 ± 0.5
Gly	19.8 ± 1.2	22.0 ± 0.7	13.5 ± 0.7	20.6 ± 0.6	10.0 ± 0.7	6.0 ± 0.2
Pro	10.0 ± 0.6	7.0 ± 0.3	10.9 ± 0.5	12.9 ± 0.3	8.0 ± 0.6	4.0 ± 0.1
Ser	13.1 ± 0.7	10.9 ± 0.4	14.7 ± 0.7	15.2 ± 0.4	11.5 ± 0.9	7.0 ± 0.3
Tyr	13.2 ± 0.6	10.6 ± 0.2	12.6 ± 0.6	15.5 ± 0.3	7.0 ± 0.5	4.0 ± 0.1
TNEAA	**138.0 ± 7.4**	**98.3 ± 3.7**	**148.1 ± 8.0**	**143.5 ± 3.7**	**104.5 ± 8.1**	**51.0 ± 1.8**
TAA	**255 ± 12.9**	**194 ± 6.2**	**277.5± 12.9**	**288 ± 6.9**	**179 ± 13.7**	**90 ± 3.17**

Data are not provided for *Triplectides* sp. due to lack of enough dry biomass data

Chapter Three

Spatial Variation in the Amino Acid Profile of Four Macroinvertebrate Taxa Along a Highly Polluted River

3.1 Abstract

Acid mine drainage (AMD) is one of the major environmental problems impacting aquatic ecosystems globally. We studied changes in the community composition of macroinvertebrates and amino acid (AA) profiles of dominant taxa along an AMD contamination gradient within the Dee River, Queensland, Australia to understand how AMD can affect the biomolecular composition of macroinvertebrates. Taxa richness and community composition of macroinvertebrates changed widely along the AMD gradient with significantly lower taxa richness recorded at the polluted sites compared to upstream and downstream sites. The Dipteran Families: Chironomidae and Ceratopogonidae, the Odonata Family Gomphidae, and the Coleoptera Family Dytiscidae were the only families found at all sampling sites and were used here for AA analysis. There were significant variations in the AA profiles among the studied taxa. The AA profile of each taxon also varied among upstream, polluted and downstream sites suggesting that contamination of a river system with acid mine drainage not only alters the overall macroinvertebrate community composition but also significantly influences the AA profile of organisms. This study highlights the potential of using AA profiling to study the response of aquatic organisms to contamination gradients such as those associated with AMD.[2]

[2] This chapter is under review for publication in *Environmental Pollution.*

3.2 Introduction

Acid mine drainage (AMD) from abandoned and operating mining areas is of global concern to the contamination of freshwater ecosystems. AMD is produced when sulphide-rich materials are exposed to water and oxygen, leading to the production of acidic, metal-rich leachate, which can flow into surrounding waterways. The low pH and high metal concentrations associated with AMD can severely pollute freshwater resources affecting the biodiversity of aquatic fauna (Bonilla et al., 2018). The adverse impacts of AMD on macroinvertebrate community composition is widely known, with macroinvertebrates one of the most sensitive aquatic organisms to contamination (He et al., 2015; Jones et al., 2020). While most studies have focused on the effects of AMD on macroinvertebrate communities (Gambi et al., 2020), survival and behavioural parameters (Gerhardt et al., 2005), size spectra (body mass-abundance relationships) (Pomeranz et al., 2019), very few studies have been conducted to understand the impact of AMD on the biomolecular makeup of aquatic biota (Damásio et al., 2011; Bonnail et al., 2018).

Contaminants like metals can directly affect aquatic organisms through the production of reactive oxygen species (ROS), alteration of metabolic pathways and damage to tissues, organs, and cells. Cells respond to exposure to contaminants, such as those associated with AMD through the synthesis of an array of biomolecules such as proteins and amino acids (AAs) that protect them from cellular damage (Thera et al., 2019; Lane et al., 2019). Copper (Cu), for example, can interact and cause inactivation of proteins due to its high affinity with thiol, imidazole and carboxyl groups of AAs (Filimonova et al., 2016). Changes in production of AAs have been assessed in several aquatic species and has been linked to biochemical pathways related to stress (Lane et al., 2019). For instance, Cu and lithium exposure caused the up-regulation of the free AAs: phenylalanine (Phe), leucine (Leu), lysine (Lys), glutamic acid (Glu), glycine (Gly), alanine (Ala), methionine (Met) in the water flea (*Daphnia magna*) (Lane et al., 2019). Thera et al. (2019) found a significant correlation between thiol-containing AAs: cysteine (Cys) and Met and methylmercury (MeHg) in caddisfly and stonefly tissues collected from polluted water bodies. Metals have affinities to form complexes with AAs like Cys, Met, Phe, tryptophan (Trp) (Sabullah et al., 2015). Hence, the distribution of AAs in organisms can be affected by the exposure and bioaccumulation of metals and its trophic transfer (Won et al., 2018). However, the AA profile of an aquatic organism is an important but understudied component of ecotoxicology studies (Johnson et al., 1993). Understanding how multiple stressors in the riverine environment such as those associated with AMD (metals and low pH) alter the AA

profile of macroinvertebrates may provide information on how they impact the biomolecular composition of organisms and how tolerant species survive in extremely polluted environments.

This study aimed to assess the impact of AMD on macroinvertebrate communities and the biomolecular composition (AA profile) of taxa found across sites along the contamination gradient. This study is the first to determine changes within the AA profile of macroinvertebrate taxa along an AMD contamination gradient and outlines a novel approach to assessing the impact of AMD contamination on aquatic organisms.

3.3 Materials and methods

3.3.1 Study sites

This study was conducted within the Dee River in Central Queensland, Australia. The Dee River is a tributary to the Fitzroy River and the catchment area represents 1% of the Fitzroy River catchment (Cozzolino et al., 2018). The area has a mean annual rainfall of 740 mm, estimated mean annual evaporation of about 1840 mm and temperatures ranging from 32 °C in January to 23 °C in July (Wels et al., 2004). AMD from the historic Mount Morgan mine site has been of serious environmental concern in the region for decades (Mackey, 1988; Taylor, 2004; Howse, 2007). The mine closed in 1990 after more than a century of operation (Vicente-Beckett et al., 2016a). Seven sampling sites along a 47 km section of the Dee River were chosen for this study (Figure 3.1) with details in Table S3.1. The sites were divided into three groups corresponding to contamination levels in the river. D1 and D2 were located upstream of the mine and considered as the reference sites without contamination. D3, D4 and D5 were polluted with AMD and will be referred to as the polluted sites and D6 and D7 were sites downstream of the contamination, where tributaries enter the Dee River, improving water quality (Mackey, 1988; Vicente-Beckett et al., 2016a).

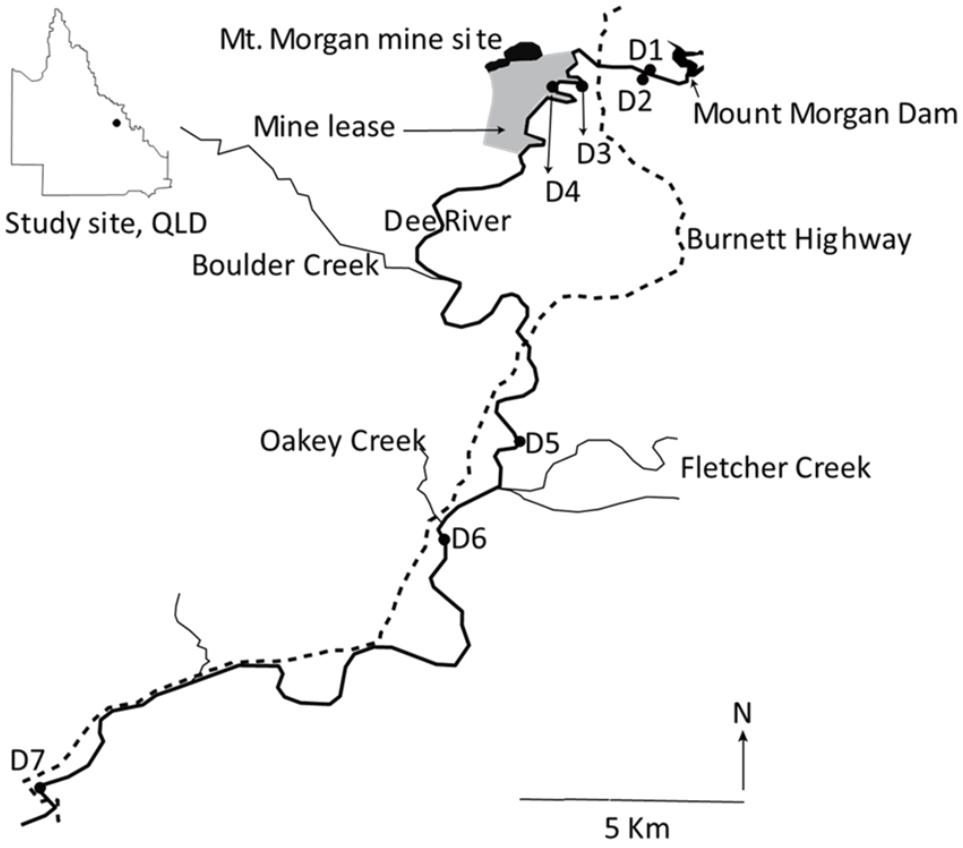

Figure 3.1. Map of Dee River in Queensland showing the seven sampling sites (D1-D7) and the location of the Mount Morgan mine site.

3.3.2 Field sampling

Macroinvertebrates were randomly sampled over 2 days in July 2018. The sweep method and a standard 250 µm mesh dip net were used to collect edge samples from a 10 m stretch of river. Macroinvertebrates were live picked in the field using the standard AusRivAS protocol (AusRivAS., 2001) and placed in glass vials containing 70% ethanol. Macroinvertebrates selected for AA analysis were also live picked and transferred into labelled plastic containers and placed on ice for transportation to the laboratory and then frozen at -80 °C until analysed.

Specimens were counted and identified to family level using the Bug Guide (Henry et al., 2009) and identifications keys (Williams, 1980; Hawking & Theischinger, 1999; Gooderham & Tsyrlin, 2002; Dean et al., 2004). The family level identification was chosen as it is often used in ecosystem health assessments to determine changes in macroinvertebrate community composition brought about by changes in water quality (AusRivAS., 2001; Svensson et al., 2018). The overall family level taxonomic richness and EPT index which represents the relative abundance of the pollution sensitive

Ephemeroptera, Plecoptera and Trichoptera (EPT) orders (Resh & Rosenberg, 1993) were also calculated for each site.

Water quality parameters were recorded at each site, including water temperature (°C), atmospheric pressure (kPa), pH, specific conductance (µS cm^{-1}), and dissolved oxygen (mg L^{-1}) (YSI Pro DSS; YSI Environmental). River water samples were also collected from all the sampling sites for the analysis of metals and dissolved organic carbon (DOC). Water samples for metal analysis were preserved in the field using 0.2% nitric acid and analyzed using inductively coupled plasma mass spectrometry (ICP-MS). Samples for dissolved organic carbon were filtered in the field using a 0.45 µm cellulose-acetate; (Bonnet Equipment) filter and measured as non-purgeable organic carbon (NPOC) using an Analytik Jena multi N/C 3100 TOC analyser.

3.3.3 Amino acid analysis

Amino acid profiles were analyzed only for families that were present at all sampling sites. These families included: Chironomidae larvae, Ceratopogonidae larvae, Dytiscidae and Gomphidae larvae. Due to sample limitation in some of the sites, one to seven individuals from each family from each site were analyzed for AA composition. Individual specimens from the family Dytiscidae and Gomphidae were homogenized with Mini beadbeater-16 (Biospec) using approximately 0.2 g of 0.5 mm diameter glass beads (Biospec) in 1 mL of Milli-Q and stored at -80 °C; 100 µL of the homogenised material was used for AA analysis representing 10% of total dry biomass. Chironomidae and Ceratopogonidae were not homogenized for AA analysis because of their small size and were instead analysed whole. Sample processing and AA analysis broadly followed that described previously by Dwyer et al. (2018) described in Section 2.3.3. List of 20 AAs measured are listed in Table S3.2 with recovery % during hydrolysis in Table S3.3.

3.3.4 Data analysis

Principal Coordinates Analysis (PCO) was performed based on the Bray-Curtis dissimilarity matrix of presence-absence data (family level identification) to determine changes in macroinvertebrate community structure across sites. A permutational multivariate dissimilarity-based Analysis of Variance (PERMANOVA) and pairwise comparisons within the PERMANOVA were used to assess and show variation in the AA profile among the four taxa and within each taxon collected from different sites based on Euclidean distances. These statistical analyses were conducted using the PERMANOVA +

V7.0.11 add-on to the Primer 7 statistical package (Anderson et al., 2008). Principal Component Analysis (PCA) plots were also used to verify and visualise difference in the AA profile among taxa and different sites (upstream, polluted and downstream) within each taxon.

The relative abundance (mol%) of individual AA was plotted as a boxplot using ggplot2 in R (Version 3.5.1) (Wickham, 2009) and statistical significances among the groups were determined by one-way ANOVA or Kruskal-Wallis H test followed by pairwise Tukey HSD test at $p < 0.05$. Pearson correlations between relative abundance (mol%) of individual AA and water quality data were analysed using "ggscatter" function of the "ggpubr" package in R. Principal Component Analysis was used to analyse the variation in water quality across the seven sites (Table S3.4) in R using FactoMineR package (Lê et al., 2008). Metals with concentrations below detection limits, temperature and dissolved oxygen (DO) data were not used for PCA analysis.

3.4 Results

3.4.1 Water quality

Average water quality parameters recorded from each site along the Dee River are summarized in Table S3.4. A clear separation among the upstream (D1 and D2), AMD polluted (D3, D4 and D5) and downstream (D6 and D7) sites can be seen in the PCA plot (Figure 3.2). 72.9% of the total variation in water quality among sites is explained by PC1 and 15.5% of the variation is explained by PC2. All of the metals (Al, As, Be, B, Cd, Co, Cu, Fe, Mn, Ni, Si, Sr and Zn) were positively correlated (> 0.8) with the PC1 and show that the polluted sites D3, D4 and D5 contain high concentrations of these metals with D4 being the most polluted site. The concentrations of Al, Cd, Co, Cu, Mn, Ni, Se and Zn were all well above the Australian and New Zealand guideline values for freshwaters (ANZG., 2018) at the polluted sites (Table S3.4). Compared to other sites, polluted sites also recorded higher specific conductance (3768 – 4205 µS cm^{-1}), lower pH (3.2 – 4.5) and lower DOC concentrations (1.3 – 1.8 mg L^{-1}), typical of acid mine drainage (Table S3.4). Upstream sites (D1 and D2) and downstream sites (D6 and D7) were similar and characterised by circumneutral pH (pH 7.1 – 7.8), lower EC (738 – 1609 µS cm^{-1}), higher DOC concentrations (2.8 – 11.6 mg L^{-1}) and lower metal concentrations.

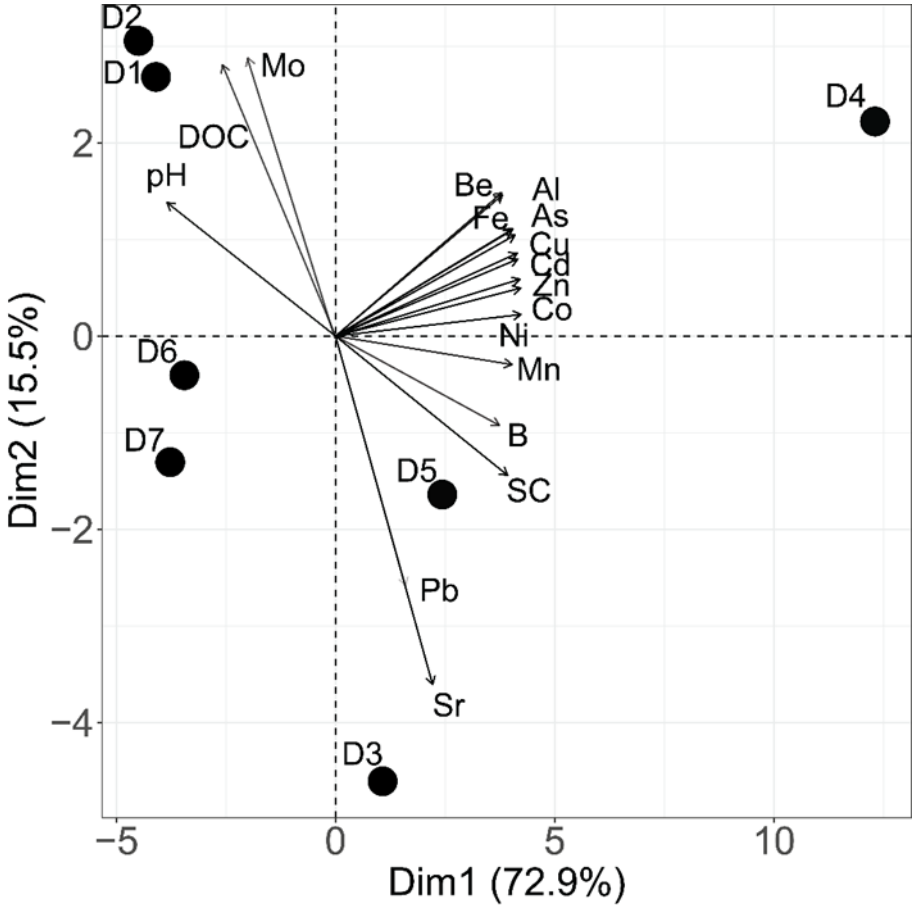

Figure 3.2. Principal component analysis of water quality parameters in the Dee River showing differences among upstream (D1 and D2), polluted (D3-D5) and downstream (D6 and D7) sites. The vectors represent the contributing parameters.

3.4.2 Macroinvertebrate community composition

Overall, 36 families from 11 orders were identified in the Dee River (Table S3.5). PCO analysis revealed that macroinvertebrate community composition changed along the Dee River in response to the AMD contamination gradient (Figure 3.3a). Taxa richness was highest at the upstream site D1 (23 taxa) and downstream site D7, gradually declining in response to AMD, with the most polluted site (D4) only containing 6 taxa (Figure 3.3b). EPT taxonomic richness was zero at the polluted sites (D3, D4 and D5) compared to a range of 1 – 3 at the upstream and downstream sites (Table S3.5). The dipteran families: Chironomidae and Ceratopogonidae, the Odonata family: Gomphidae, and the Coleoptera family: Dytiscidae were the only families found at all sampling sites and were used in this work for AA analysis.

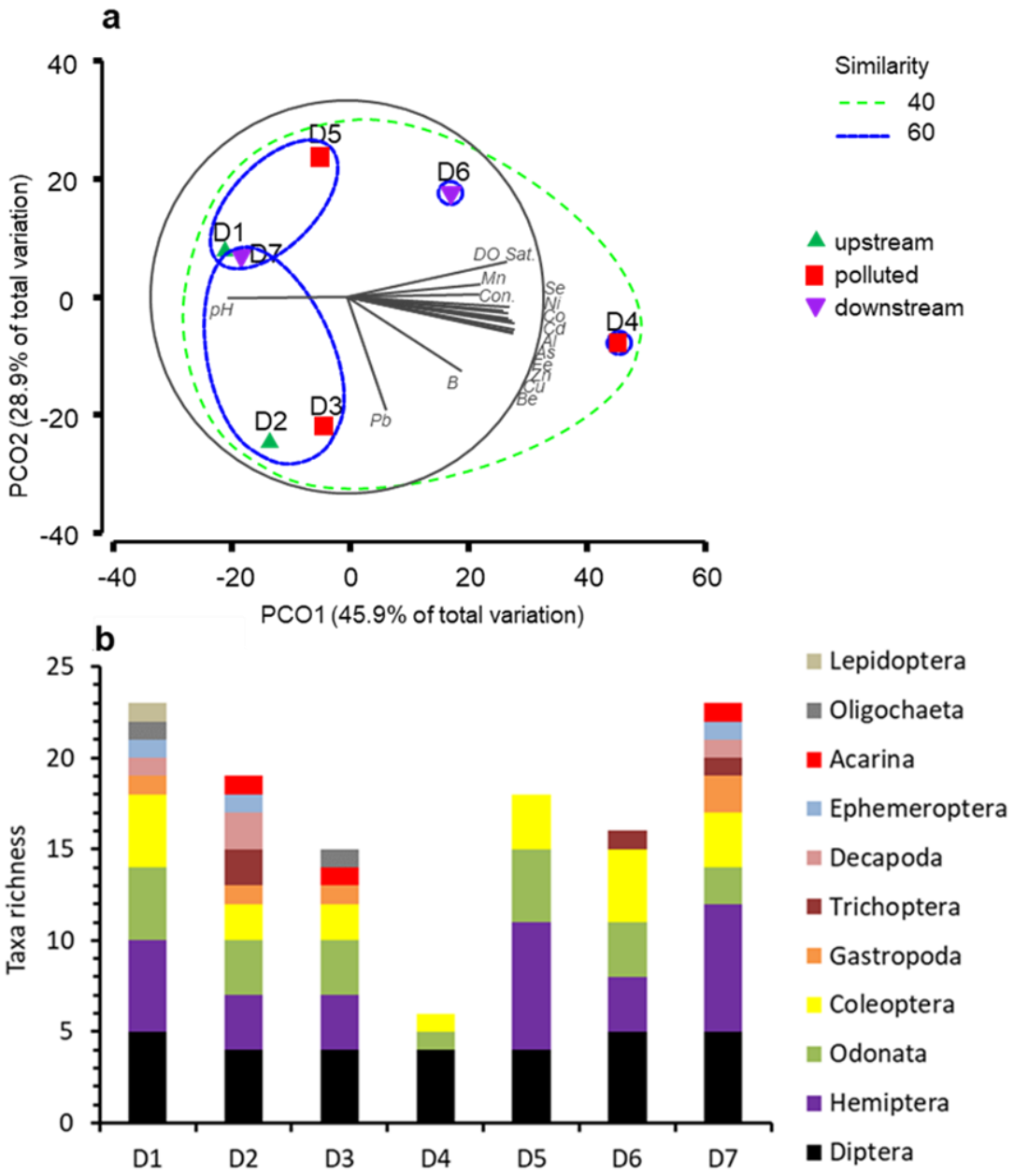

Figure 3.3. Spatial variation of macroinvertebrate communities within the Dee River, Queensland. **a**. Principal coordinate plot (PCO) based on a Bray Curtis similarity resemblance matrix on presence-absence species data showing the shift in the community composition of macroinvertebrates along the contamination gradient in the Dee River: relative macroinvertebrate taxon composition with 40% (green dash line) and 60% (blue dash line) similarities. The vectors represent the highest contributing water quality parameters corresponding to each sampling site (Pearson's correlation > 0.6). **b**. Macroinvertebrate taxa richness based on family level identification, showing the macroinvertebrate orders present at upstream sites (D1 and D2), polluted sites (D3, D4 and D5) and downstream sites (D6 and D7).

3.4.3 Amino acid profile in macroinvertebrates

Seventeen AAs were detected and quantified in every macroinvertebrate specimen. The relative abundance (mol%) of each AA obtained in each taxon are shown in Figure 3.4a (Table S3.6). Glx, Ala, Asx, and Gly were the most dominant AAs in all taxa, accounting for over 40% of the AA content. The AA profiles of all four taxa were dominated by NEAA

(Chironomidae: 57.2%, Ceratopogonidae: 57.5%, Dytiscidae: 57.0% and Gomphidae: 56.7%) compared to EAAs (Chironomidae: 42.8%, Ceratopogonidae: 42.5%, Dytiscidae: 42.9% and Gomphidae: 43.2%). Across all sites, there was a significant difference in the AA profile of the four taxa (PERMANOVA, $F_{3,117} = 27.4$, $p < 0.001$) indicating that each taxon has a distinct AA profile. The four taxa were clearly separated along dimension 1 of PCA (Figure 3.4b) that accounts for 32.2% of the variation with seven AAs: Glx, Val, Asx, Lys, Tyr, His and Met, being major AAs that separated the four taxa. Phe, Gly, Pro and Ala were major AAs that separates the four taxa along dimension 2. The dipteran families (Chironomidae and Ceratopogonidae) were relatively rich in Glx, Asx, Lys and Gomphidae were rich in Ala, Val, Pro and Tyr compared to other taxa. Further comparison of individual AAs among the taxa with one-way ANOVAs indicated that all AAs except Ile were significantly different in at least one of the taxa (Table S3.6). Interestingly, the content of Asx and Pro significantly differed among all four taxa ($p < 0.001$).

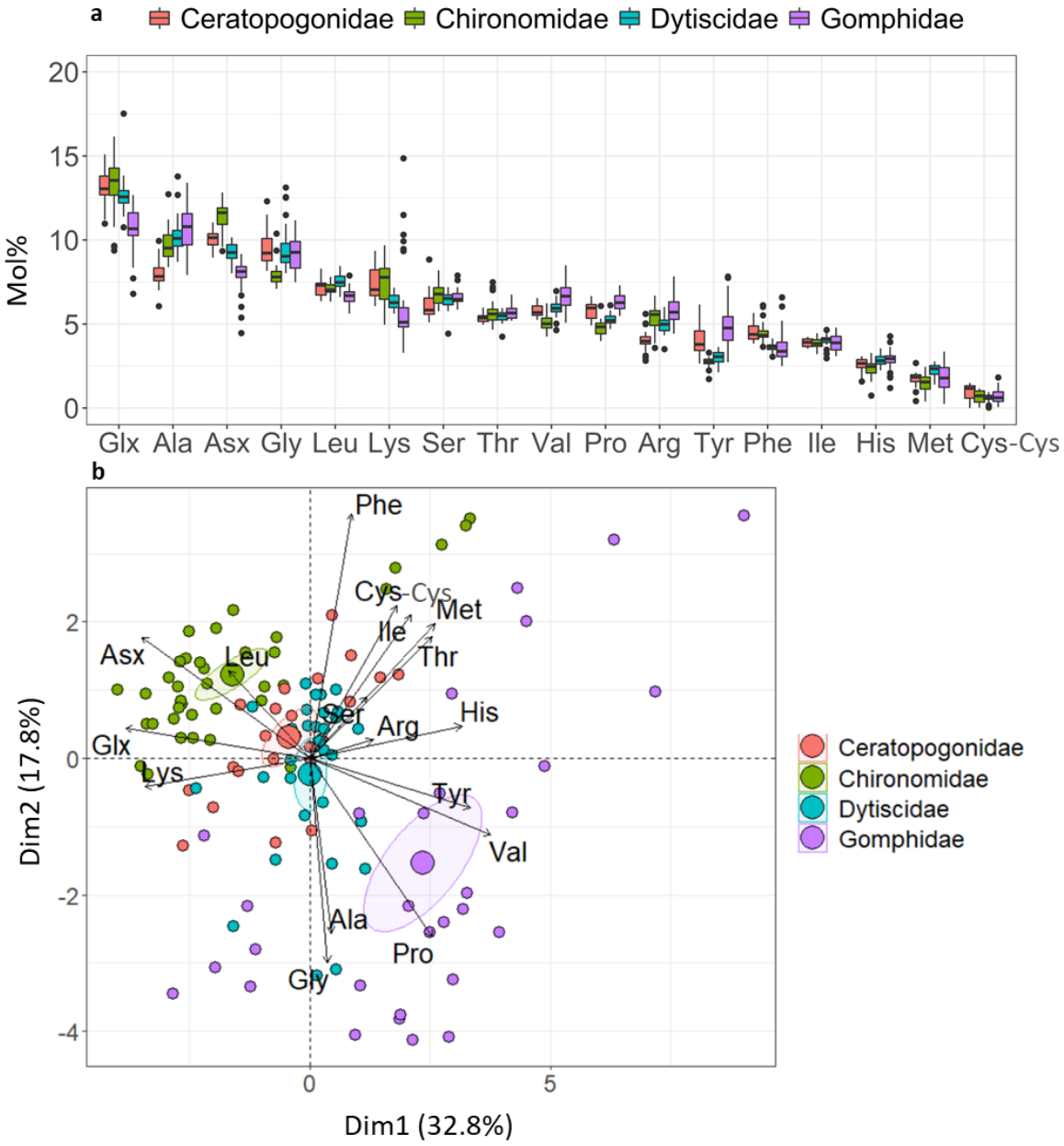

Figure 3.4. Box plot and principal component analysis (PCA) of macroinvertebrate samples based on their amino acid profile. **a**. Relative abundances (mol%) of 17 amino acids in: Ceratopogonidae, Chironomidae, Dytiscidae and Gomphidae. **b**. Biplot from PCA based on the amino acid profiles of the four macroinvertebrate taxa; each dot represents an individual specimen; group averages are shown with bigger circles with eclipses indicating 95% confidence level.

3.4.4 Spatial patterns in the amino acid profile of macroinvertebrates

Significant intraspecific spatial variations in the AA profiles of macroinvertebrates were recorded for the studied taxa (Figure 3.5, Table S3.7 and Table S3.8). The AA profile of Chironomidae significantly differed among upstream, polluted and downstream sites (Figure 3.5a: PERMANOVA, $F_{2,34} = 8.99$, $p < 0.0001$). The Kruskal-Wallis H test (Table S3.9) further revealed that 14 out of 17 AAs detected in Chironomidae varied significantly

across the studied sites. Chironomidae in polluted and downstream sites had a higher proportion of Glx, Lys, Leu and Asx but lower proportions of Phe, Val, Thr, Ser compared to the upstream sites. The highest levels of sulfur containing AAs (Cys-Cys and Met) were recorded at upstream sites, and Pro was higher at polluted sites except at D4. Furthermore, Ala was significantly lower and Arg was significantly higher at downstream sites compared to the upstream and polluted sites. No changes were observed in His, Ile and Tyr which were 3 of the five least abundant AAs in Chironomidae.

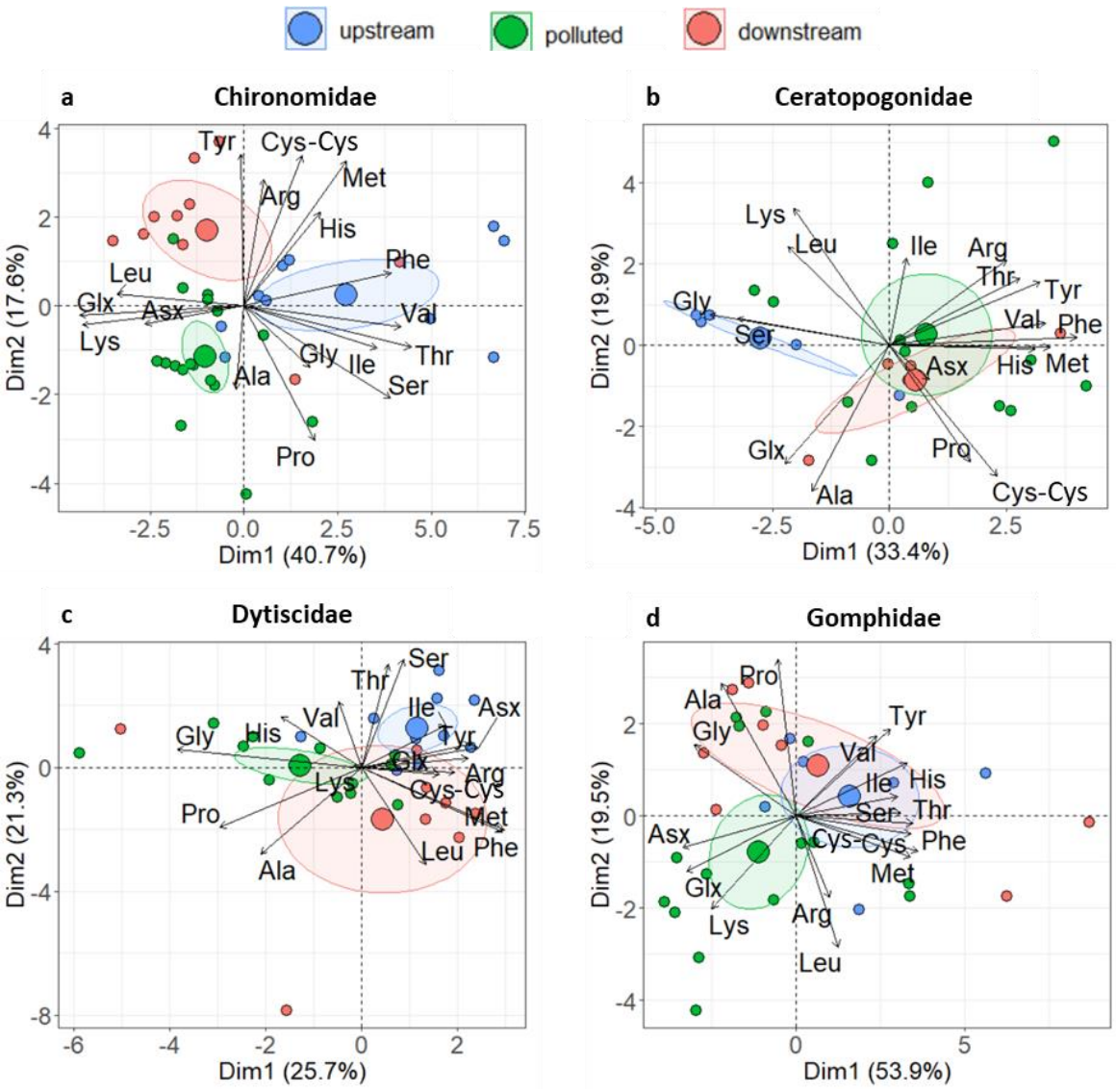

Figure 3.5. Principal component analysis (PCA) biplots for the first two components based on the amino acid profiles of macroinvertebrates collected from: upstream, polluted, and downstream sites of the Dee River, Queensland. Each dot represents an individual specimen, group averages are shown with bigger circles with 95% confidence level displayed as eclipses.

In the case of Ceratopogonidae, AA profiles of individuals collected from the upstream sites were significantly different from the polluted and downstream sites (PERMANOVA, $F_{2,21}$ = 2.15, p < 0.05), with no significant variation detected between polluted and downstream sites (Figure 3.5b; Table S3.8). Nine out of 17 AAs showed significant

variation between at least one of the sites (Table S3.10). Ceratopogonidae in downstream and polluted sites had a higher proportion of Phe, Met, Thr, Arg and Val but lower content of Gly and Ser compared to the upstream sites. Cys-Cys and Met content were higher in polluted and downstream samples compared to upstream. Like Chironomidae, there were no changes in the proportions of His, Ile and Tyr.

Dytiscidae also showed spatial variation in the AA profile with upstream sites being significantly different from polluted and downstream sites (PERMANOVA, $F_{2,27} = 2.41$, $p < 0.05$) (Figure 3.5c; Table S3.8), however, the polluted and downstream samples were similar in AA profile. Nine out of 17 AAs showed significant spatial variation (Table S3.11). A higher proportion of Gly, Ala and Pro were recorded in individuals from polluted sites. Met, Phe and Leu were recorded higher at downstream sites and Ser, Thr and Tyr were in higher abundance in taxa collected from upstream sites.

Spatial intraspecific variations in AA profile were also detected in Gomphidae with the AA profile of individuals from polluted sites significantly different from upstream sites (PERMANOVA, $F_{2,27} = 2.6$, $p < 0.05$ (Figure 3.5d; Table S3.8). Significant spatial variations were seen in 10 out of 17 AAs (Table S3.12). AA profiles of Gomphidae individuals collected from the upstream sites and downstream sites had a higher proportion of His, Ser, Thr, Cys-Cys, Tyr, Met, Val, Ile and Phe compared to the samples from the polluted site. In contrast, samples from the polluted site had a higher proportion of Asx, Glx, Gly and Lys.

For all the studied taxa, we found significantly different AA profile in specimens from polluted sites compared to the other two sites. Among the seventeen AAs analysed in this study, Glx, Ala, Cys-Cys, Met and Leu showed significant spatial variation in all the studied taxa whereas Ile was the only AA that did not change across sites within any of the studied taxa. For some AAs, significant changes between sites were only observed for one taxon, including: His which changed only in Gomphidae; Tyr changed only in Dytiscidae; and Ser and Asx which were only observed to alter in Chironomidae. Overall, we observed the strongest difference (among sites) in Glx (up to 2%) which is the most dominant AA in all four taxa and weaker differences in the less abundant AAs: Tyr, Ile and His. Even though the AA profiles of the four taxa showed significant variation between unpolluted and polluted sites, the proportions of EAA and NEAA within the taxa remained unchanged in all taxa except for Chironomidae. In the case of Chironomidae, there was a small (1%) but significant decrease in the proportion of EAAs in polluted sites compared to downstream sites (Table S3.9).

3.5 Discussion

3.5.1 Water quality

Acid-mine drainage (AMD) ascending into the Dee River from the historical Mount Morgan gold and Cu mine in Central Queensland has led to the transport and distribution of metals and low pH along a large section of the Dee River and has been a major concern in the area since 1925 (Vicente-Beckett et al., 2016a). We showed that some sites downstream from the Mount Morgan mine are still highly polluted by AMD, with elevated dissolved metal concentrations, high conductivity and low pH recorded at these sites. Comparing the recorded pH range for the polluted sites in this study with previous studies, we found an increase in the pH (3.3 – 4.5) in the polluted section compared to the reported range of 2.6 – 3.9 by Howse (2007). Improvement was also seen in the Pb concentrations (< 0.002 mg L^{-1}) with all of the measured concentrations in polluted sites being below the trigger value (0.0034 mg L^{-1}) according to ANGZ 2018 compared to previous Pb concentration of 0.1 mg L^{-1} and 0.012 mg L^{-1} reported in 1999 and 2000 (Vicente-Beckett et al., 2016b). Similarly, the maximum Al concentration recorded in our study was 120 mg L^{-1} which is extremely high compared to the trigger value of 0.0008 µg L^{-1}. However, previous studies had recorded even higher concentrations of Al, ranging from 171 – 371 mg L^{-1} (Taylor, 2004; Howse, 2007; Vicente-Beckett et al., 2016a). The concentrations of Zn (0.75 – 5.2 mg L^{-1}) was also lower compared to previously reported values of 7.38 mg L^{-1} (Vicente-Beckett et al., 2016b) and 1.36 – 5.63 mg L^{-1} (Howse, 2007). However, higher concentrations of Fe (35 mg L^{-1} at site D4) were recorded in this study compared to previous studies. By comparison, Fe concentrations in the polluted section were in the range 0.7 – 2.9 mg L^{-1} (Howse, 2007) in 1999 – 2001 and up to 12.4 mg L^{-1} in 2000 (Vicente-Beckett et al., 2016b). Changes in pH and metal concentrations over time at AMD polluted sites within the Dee River may reflect the treatment of AMD via the lime-dosing water-treatment plant built in 2006. This facility treats the AMD water within the open-pit with lime, increasing the pH from approximately 2.9 to 7.5 and precipitating out the metals, which are then returned to the pit, before releasing the treated water into the Dee River (Holland et al., 2013).

Reported concentrations for different metals in AMD polluted sites in Dee River are extremely high and greater than the average metal concentration in polluted rivers and lakes globally. For instance, Zhou et al. (2020) collated past sampling data on the concentrations of 12 metals in 168 rivers and 741 lakes across five continents (Africa, Asia, Europe, North America and South America) from 1972 – 2017 to investigate the levels of metal

contamination. The mean concentrations of Al, Co, Fe and Mn in water bodies in Asia were 3.1 ± 1.8 mg L^{-1}, 0.028 ± 0.017 mg L^{-1}, 3.1 ± 2.3 mg L^{-1} and 0.967 ± 0.533 mg L^{-1}, respectively which were highest among five continents. The highest Zn concentrations occurred in water bodies in Europe with a mean value of 1.3 ± 0.97 mg L^{-1}. Concentrations of Al and Co in polluted sections of the Dee River were more than 35 times higher, Fe was more than 10 times higher, Mn was 25 times higher and Zn was at least 4 times greater than those reported by Zhou et al. (2020). Hence, the current study makes a useful contribution to the literature on the impact of extreme environmental stress on community composition and AA profiles of freshwater organisms.

3.5.2 Macroinvertebrate community changes

We showed a clear change in the macroinvertebrate communities at AMD polluted sites compared to the upstream and downstream sites, with reduced taxa richness and EPT richness recorded in polluted sites. Upstream sites consisted of a diverse macroinvertebrate community consisting of 23 families from 11 orders whereas only 6 families belonging to 3 orders were found at the most polluted site. Indeed, the EPT index was zero for the 3 polluted sites and the site D4 was composed of only three tolerant orders of Diptera, Coleoptera and Odonata. Of the macroinvertebrates, Chironomidae, Ceratopogonidae, Dytiscidae and Gomphidae were most tolerant to AMD contamination and the only taxa found at all seven sites. The abundance of Diptera (mostly chironomids) and loss of sensitive taxa have previously been reported for sites polluted by AMD globally (Howse, 2007; Mocq & Hare, 2018). For instance, Howse (2007) found Chironomids were the most abundant species in the AMD polluted Dee River and questioned how they were able to survive such extreme contamination. Ceratopogonidae and Gomphidae are generally acidophilic and have been reported to increase in abundance during acidification of waterways (Sommer & Horwitz, 2001). Dytiscidae has also been reported to be tolerant to AMD (Last, 2001). Due to the changes in community structure, AMD polluted waterways also see a shift in their overall food web and this has been reported in several studies (Hogsden & Harding, 2012; Hogsden et al., 2013).

3.5.3 Amino acid profiles of macroinvertebrate taxa

Amino acids are biomolecules that can be transferred up the food web and play a part in determining an organism's response to contaminants such as metals. Our study found that each taxon contains a distinct AA profile. For instance, Chironomidae and Ceratopogonidae are rich in Glx, Asx, Lys and Lue, whereas, Gomphidae are rich in Val. Distinct AA profiles

of macroinvertebrate taxa have also been reported in previous studies (Dwyer et al., 2018; Vesterinen et al., 2020; Thera et al., 2020). The distinct AA profile of each taxon suggests that AA profiles in organisms are under homeostatic control (Moura et al., 2013).

Our results also showed that the four studied tolerant taxa were dominated by NEAA and were relatively poor in EAA content, with EAAs making up 43% of the AA profile in Ceratopogonidae, Chironomidae and Dytiscidae and 44% in Gomphidae. These values were low compared to that reported for other macroinvertebrates families like Gammaridae (46%), Trichoptera (50%), and Ephemeroptera (47%) (Kolmakova et al., 2013). Hence, AMD contamination driven changes in community composition of macroinvertebrates may limit the supply of EAAs to consumers. Although caution must be taken when analysing such results in AMD polluted rivers as fish are often missing and generally replaced by other invertebrates as top predators (Hogsden & Harding, 2012). Leucine, Lys, Val and Thr were the most dominant EAAs in the studied insects in our study. This is consistent with a previous study that showed higher content of these AAs in insects from Nigeria (Oibiokpa et al., 2018). Oibiokpa et al. (2018) also found that Met, Ile and His were present in lower amounts compared to other EAAs which is similar to our results which determined that these three AAs are the major limiting AAs in the four studied taxa. Other studies have also stated that insect taxa are low in Met and Cys content, but high in Lys and Thr (Xiaoming et al., 2010; Schabel, 2010). Glx, Ala, Gly and Asx were the most dominant AAs in all the studied taxa in this study. Oibiokpa et al. (2018) reported that glutamic acid was the most abundant NEAAs in four species of insects (*Gryllus assimilis* (Cricket), *Melanoplus foedus* (grasshopper), *Macrotermes nigierensis* (termite), and *Cirina forda* (moth caterpillar)).

3.5.4 Effect of AMD contamination on macroinvertebrate amino acid profiles

For the four taxa that were found at all sites, the AA profiles differed depending on whether they were collected from upstream, downstream, or polluted sites. This might indicate that macroinvertebrates in polluted sites alter their biochemical properties so that they can function in such a polluted environment. Organisms show responses to environmental stress at biochemical and physiological levels (Lane et al., 2019). The observed changes in the AA profile in our study might be the result of i) alteration to the protein structure ii) changes in the up and/or down-regulation of certain proteins iii) a change in the amount of free AAs (Janssens et al., 2009; Hussain et al., 2016; Hussain et al., 2018). Previous studies have reported a change in the level of certain AAs and proteins in different benthic macroinvertebrates due to metal stress (Johnson et al., 1993; Lane et al., 2019). Certain

AAs such as sulfur containing AAs are involved in antioxidant activities during metal stress. Moreover, they help in synthesis of intracellular antioxidants like glutathione and metalloproteins during stressed conditions that help in metal chelation and detoxification.

In this study we found some common AAs that responded to the AMD contamination gradient, however, results across the taxa were inconsistent. For instance, the content of two sulphur-rich AAs: Cys-Cys and Met, showed spatial variation in all the studied taxa. Strong negative correlations between the content of Cys-Cys (Figure S3.1) and Met (Figure S3.2) in Chironomidae and dissolved metal concentrations were found, with the strongest relationship shown for Mn (R = -0.71, $p < 0.0001$). Cys-Cys and Met content in Dytiscidae and Gomphidae were also negatively correlated with most of the metals. However, in Ceratopogonidae, Cys-Cys and Met content increased in polluted sites compared to upstream. Exposure to metal contamination may cause an increasing demand for sulphur-containing AAs in macroinvertebrates. Previous studies have reported increases in Cys and Met in response to metal contamination given their relationship with metallothioneins (MTs). Metallothioneins are Cys-rich proteins that bind to metals through metal-thiolate bonds and plays a role in protection against metal toxicity (Kemp et al., 2017; Man et al., 2019). However decreased MTs production might be a mechanism of tolerance to high metal stress which may allow certain organisms to accumulate lower amounts of toxic metals (Byrne et al., 2012). This might be the reason that Cys-Cys and Met content in our study showed a negative relationship with metal concentrations in the water in Chironomidae, Dytiscidae and Gomphidae. Other AAs that varied with sites in all studied taxa are Glx and Leu. Glx content in chironomids from polluted sites and downstream sites increased by around 2% compared to upstream sites, with a strong positive correlation shown between strontium and negative correlation with concentration of dissolved organic carbon (Sr: R = 0.51, p = 0.001, DOC: R = -0.57, $p < 0.001$ (Figure S3.3). However, no such correlation was observed in other taxa. The lowest Leu contents were recorded at upstream sites for Chironomidae and Gomphidae, but this was not found for Ceratopogonidae and Dytiscidae. The inconsistent responses in AA profiles of studied taxa might indicate differences in adaptation mechanisms and stress response in macroinvertebrates during extreme environmental conditions.

Site-specific variation in the AA profile of these macroinvertebrates may also be due to other factors as well. Firstly, we cannot rule out a change in the species within a taxon present at each site along the AMD contamination gradient. This could also explain shifts in AA composition, as taxa were only identified to family. Identification of specimens via

DNA barcoding was trialled however was not successful due to technical challenges such as acidic pH and metal interference with the recovery of DNA in specimens from AMD polluted sites (Fortin et al., 2004; Henneberger et al., 2006). Another possible explanation is a change in the food resources available at each site, given taxa receive EAAs from their diet. For instance, in Chironomidae, the proportion of EAAs decreased significantly at polluted sites compared to the upstream and downstream sites. Even though no spatial variations in the overall proportions of EAAs and NEAAs were observed for the other three taxa, a significant reduction was observed for specific EAAs between polluted and upstream sites, including: Thr, Tyr, Arg and Met for Dytiscidae; and Val in the case of Gomphidae. Chironomidae and Ceratopogonidae are mostly detritivores and filter collectors, feeding on fine particulate organic matter. The lower proportions of certain EAAs in Chironomidae and Ceratopogonidae could be due to less productive basal resources available at the AMD polluted sites due to slow microbial processing of organic matter and decrease in algal diversity (Hogsden & Harding, 2012; Hogsden et al., 2013). This might have also had a cascading effect on the diet quality of predators such as Gomphidae and Dytiscidae in the river resulting in the changes in the AA profile of these predators. However, limited studies have been conducted to understand the role of diet on defining the AA profile of organisms, with contrasting reports existing in the literature, with some studies showing a correlation between the AA profile of prey items and the consumer (Reed & D'Abramo, 1989; Binoy et al., 2012), while others suggest that diet has no influence in defining AA profile of organisms (Brückner et al., 2017).

3.6 Conclusion

AMD within the highly polluted Dee River adversely impacts the community composition and AA profiles of macroinvertebrates. The AA profiles of macroinvertebrates were also shown to be taxa specific with a higher relative abundance of NEAAs over EAAs suggesting that shifts in community composition are likely to cause changes in the AA nutritional landscape available to higher predators. The AA profiles in macroinvertebrates showed detectable variations along the AMD contamination gradient with changes in the relative abundance of AAs depending on the taxa. The relative abundance of the most abundant AAs (Glx, Ala), sulfur-rich AAs (Cys-Cys and Met) and Leu varied across the sites in all studied taxa, but such variation was not observed in regards to the low abundant AAs such as Ile, His. The current study provides the first report of changes in the AA profiles of aquatic macroinvertebrates along a contamination gradient. Changes in community composition and AA profiles of specific taxa due to contamination is likely to

have cascading effects across the food web and requires further study. Overall, this study highlights the potential of using AA profiling to study the responses of aquatic invertebrates to contamination.

3.7 Acknowledgements

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3.9 Supplementary materials

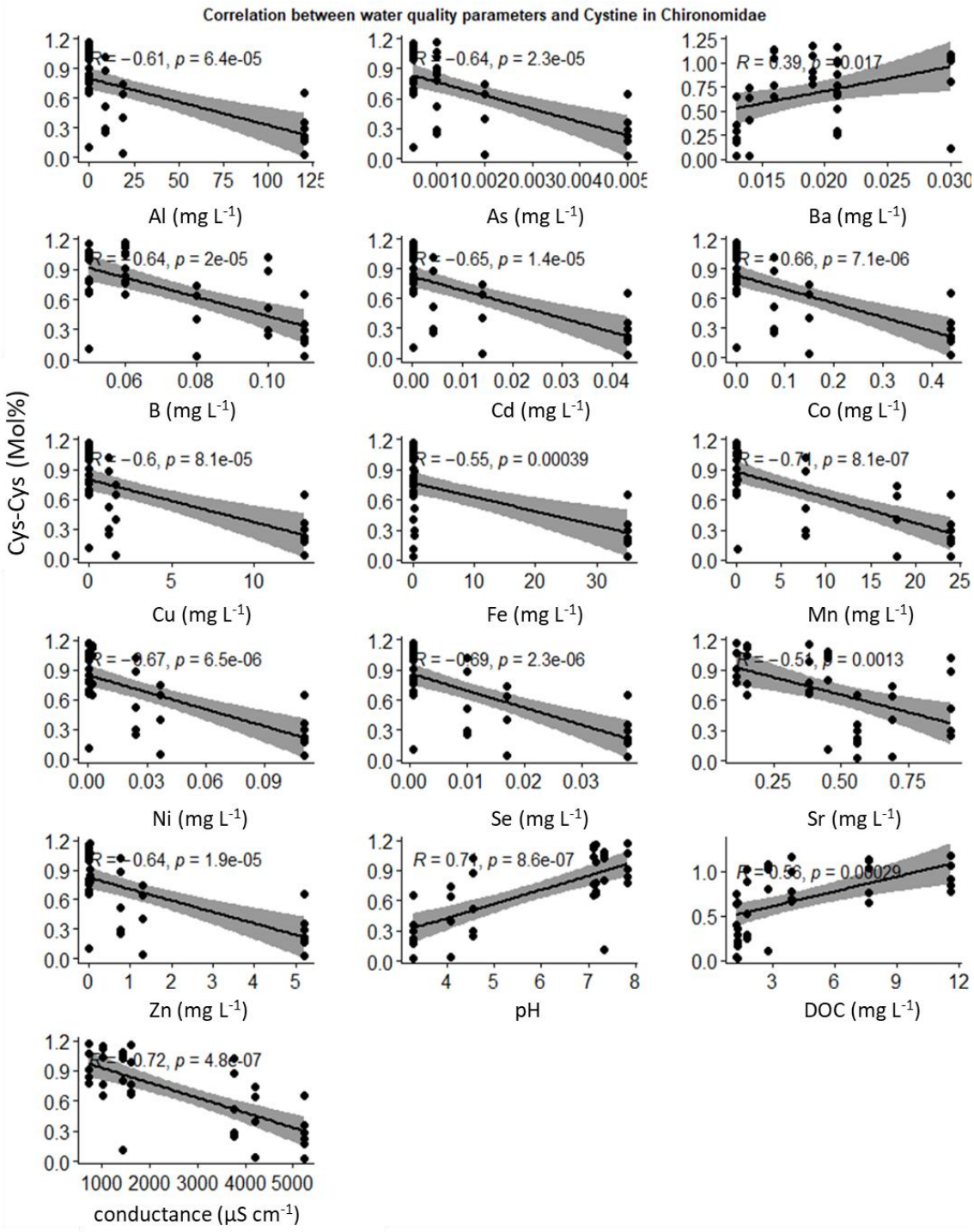

Figure S3.1. Pearson correlation of cystine content (mol%) in Chironomidae with dissolved metal concentrations and water quality parameters.

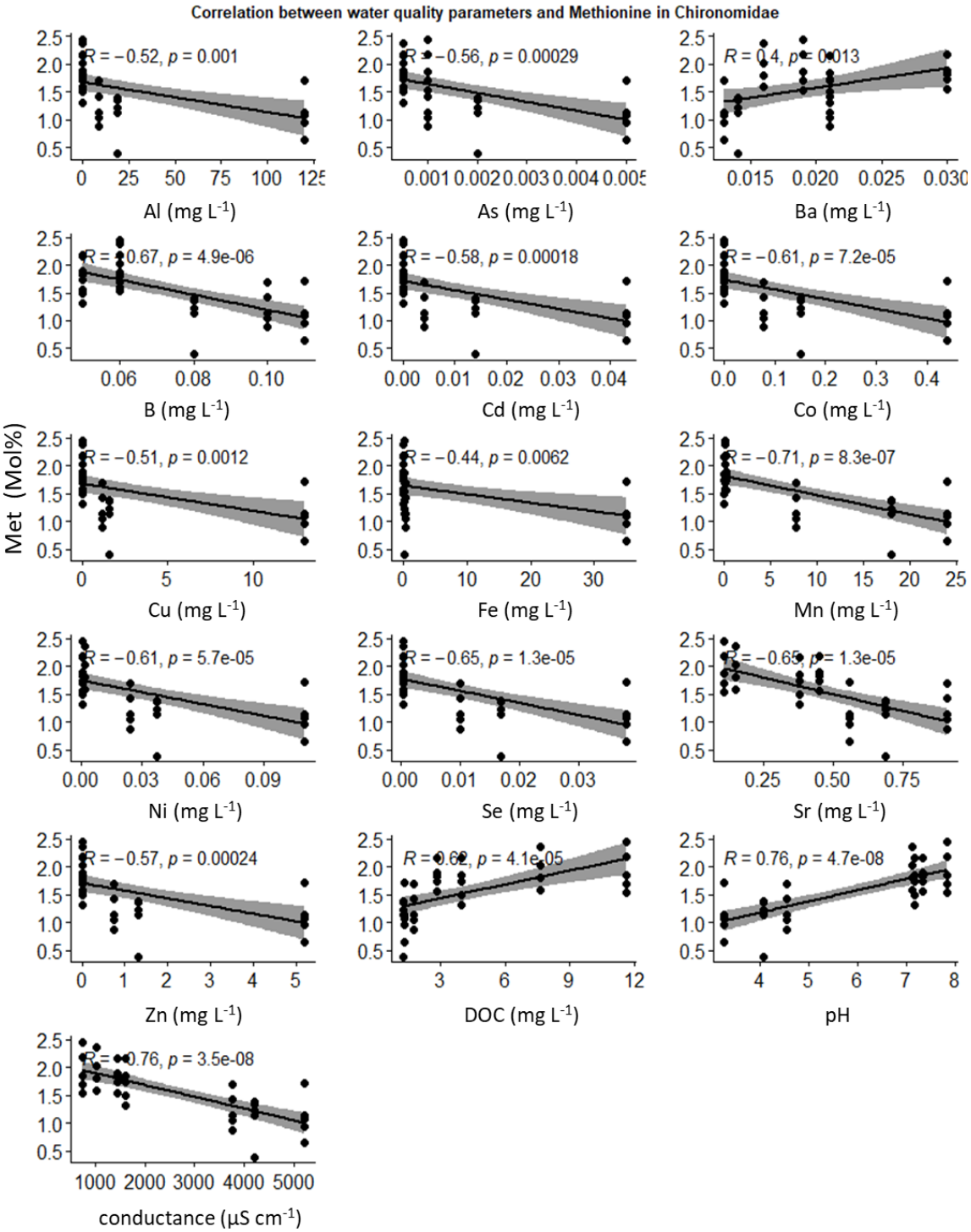

Figure S3.2. Pearson correlation of methionine content (mol%) in Chironomidae with dissolved metal concentrations and water quality parameters.

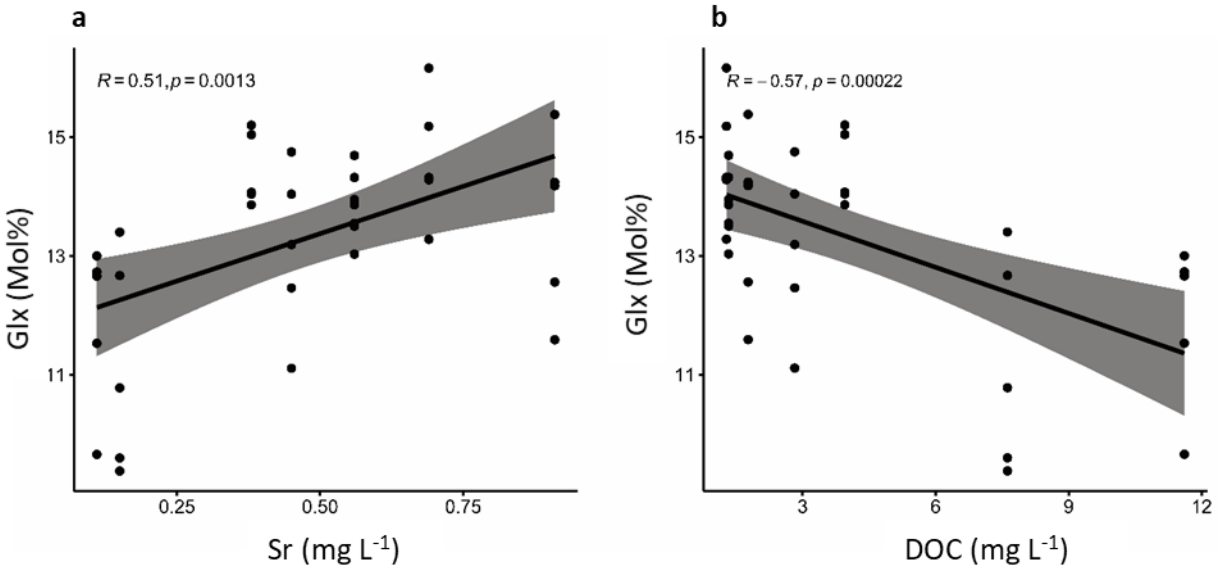

Figure S3.3. Pearson correlation between Glx content (mol%) in Chironomidae and the levels of: (a) strontium and (b) dissolved organic carbon, in Dee River Queensland. Glx = glutamic acid + glutamine.

Table S3.1. Details of the sampling sites along the Dee River, Queensland, Australia.

Site Name	Latitude	Longitude	Distance* (km)	Description of site
D1	23°38'37.0"S	150°23'51.7"E	1.2	Upstream of mine
D2	23°38'33.6"S	150°23'46.7"E	1.3	Upstream of mine
D3	23°38'45.5"S	150°23'00.3"E	4.4	Polluted site
D4	23°38'56.5"S	150°22'39.9"E	5.2	Polluted site
D5	23°72'51.5"S	150°37'17.6"E	25	Polluted site
D6	23°46'18.2"S	150°21'10.4"E	28	Downstream from Fletcher creek confluence
D7	23°51'04.2"S	150°16'00.5"E	47	Downstream from Oakley creek confluence

* from the Mt. Morgan Dam

Table S3.2. Amino acid properties, including: polymerised molecular weight (MW.poly), abbreviated amino acid identifier (AA.ID.short) and essential and non-essential amino acids (EAA/NEAA) based on Li et al., (2009). (ASX = asparagine + aspartic acid; GLX = glutamine + glutamic acids).

AA.ID	MW.poly	AA.ID.short	EAA/NEAA
Histidine	137.14	His	Essential
Asparagine	114.11	Asn	Non-Essential
Arginine	156.19	Arg	Essential
Serine	87.08	Ser	Non-Essential
Glutamine	128.14	Gln	Non-Essential
Glycine	57.05	Gly	Non-Essential
Aspartate	115.09	Asp	Non-Essential
Glutamate	129.12	Glu	Non-Essential
Threonine	101.11	Thr	Essential
Cystine	102.15	Cys	Non-Essential
Alanine	71.09	Ala	Non-Essential
Proline	97.12	Pro	Non-Essential
Lysine	128.17	Lys	Essential
Tyrosine	163.18	Tyr	Non-Essential
Methionine	131.19	Met	Essential
Valine	99.14	Val	Essential
Isoleucine	113.16	Ile	Essential
Leucine	113.16	Leu	Essential
Phenylalanine	147.18	Phe	Essential
Tryptophan	186.21	Trp	Essential
ASX	115.09	Asx	Non-Essential
GLX	129.12	Glx	Non-Essential

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Table S3.3. Percentage recovery of 16 amino acids (AAs) from bovine insulin with 6 N HCl containing 0.02% phenol hydrolysis (note: methionine (Met) is not present in bovine insulin), Recovery percentage (R%).

AA	His	Arg	Ser	Gly	Asx	Glx	Thr	Cys-Cys	Ala	Pro	Lys	Tyr	Val	Ile	Leu	Phe
R%	37	86	90	96	80	76	91	28	74	77	76	58	46	26	57	57

Table S3.4. Water quality and dissolved metal concentrations at seven sampling sites along the Dee River during the sampling period (July 2018). Values in bold indicate the concentration is outside the 95% protection guideline values according to Australian and New Zealand Guidelines for Fresh and Marine water quality (ANZG., 2018).

	Upstream		Polluted			Downstream		Guideline value
	D1	D2	D3	D4	D5	D6	D7	
Temperature (°C)	12.1	14.4	19.1	17.9	10.5	13.4	17.4	
DO (% Saturation)	**24.9**	**41.4**	**66.3**	83.8	**72.1**	**78.8**	**49.8**	80 – 90%
Specific conductance (µS/cm)	1026	738	**3768**	**5225**	**4205**	**1609**	1444	1500
pH	7.12	7.83	**4.54**	**3.27**	**4.07**	7.16	7.32	6.5 – 9
DOC (mg L^{-1})	7.62	11.61	1.77	1.33	1.28	3.95	2.82	
Dissolved metals (mg L^{-1})								
Aluminium	<0.01	0.02	**9.0**	**120**	**19**	<0.01	<0.01	0.0008 for pH <6.5 0.055 for pH >6.5
Antimony	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.009
Arsenic	<0.001	0.001	0.001	0.005	0.002	<0.001	<0.001	0.024
Barium	0.016	0.019	0.021	0.013	0.014	0.021	0.030	
Beryllium	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001	
Boron	0.06	0.06	0.10	0.11	0.08	0.05	0.05	0.37
Cadmium	<0.0002	<0.0002	**0.0041**	**0.043**	**0.014**	<0.0002	<0.0002	0.0002
Chromium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.0033
Cobalt	<0.001	<0.001	**0.077**	**0.44**	**0.15**	<0.001	<0.001	0.001
Copper	**0.003**	**0.002**	**1.2**	**13**	**1.6**	**0.005**	**0.002**	0.0014
Iron	<0.01	0.11	0.34	35	0.15	<0.01	<0.01	
Lead	<0.001	<0.001	0.002	0.001	<0.001	<0.001	<0.001	0.0034
Manganese	0.061	0.036	**7.7**	**24**	**18**	0.022	0.21	1.9
Mercury	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0006
Molybdenum	0.004	0.003	<0.001	<0.001	<0.001	<0.001	<0.001	0.034
Nickel	0.002	<0.001	**0.024**	**0.11**	**0.037**	<0.001	<0.001	0.011
Selenium	<0.001	<0.001	0.010	**0.038**	**0.017**	<0.001	<0.001	0.011
Silver	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.00005
Strontium	0.15	0.11	0.91	0.56	0.69	0.38	0.45	
Thallium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.00003
Tin	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Titanium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	
Vanadium	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.006
Zinc	0.005	**0.012**	**0.75**	**5.2**	**1.3**	0.006	0.003	0.008

Table S3.5. Presence/absence of macroinvertebrate taxa, taxa richness, Ephemeroptera, Plecoptera and Trichoptera (EPT) index from seven sites along the Dee River, Queensland, Australia. The four taxa used for AA analysis are highlighted in bold.

	Upstream		Polluted			Downstream	
Taxa	D1	D2	D3	D4	D5	D6	D7
Ancylidae			1				
Planorbidae	1	1					1
Physidae							1
Oligochaeta	1		1				
Acarina		1	1				1
Atyidae	1	1					1
Parastacidae		1					
Haliplidae	1						
Dytiscidae	1	1	1	1	1	1	1
Gyrinidae					1		
Hydrophilidae		1	1			1	1
Hydraenidae	1				1	1	1
Staphylinidae						1	
Scirtidae	1						
Tipulidae				1		1	
Culicidae					1		
Ceratopogonidae	1	1	1	1	1	1	1
Tabanidae			1				1
Stratiomyidae	1						
Chironomidae	1	1	1	1	1	1	1
Caenidae	1	1					1
Veliidae	1				1		1
Gerridae					1		
Nepidae							1
Corixidae	1	1	1		1	1	1
Notonectidae	1	1	1		1		1
Pleidae	1	1	1		1		1
Hydrometridae					1	1	1
Naucoridae	1				1	1	1
Pyralidae	1						
Isostictidae	1	1					
Protoneuridae	1		1		1		
Aeshnidae		1	1				
Gomphidae	1	1	1	1	1	1	1
Libellulidae	1				1	1	1
Hydroptilidae		1					
Leptoceridae		1				1	1
Taxa Richness	23	19	15	6	18	16	23
EPT index	1	3	0	0	0	1	2

Table S3.6. The relative abundances (mol%) of individual amino acids of macroinvertebrate taxa (mean ± SD). Letters indicate significant differences between taxa.

Amino acids	Ceratopogonidae	Chironomidae	Dytiscidae	Gomphidae
Ala	8.0 ± 0.82^{a}	9.7 ± 0.9^{c}	10 ± 1.10^{bc}	11.0 ± 1.40^{b}
Arg	4.0 ± 0.64^{b}	5.3 ± 0.79^{a}	4.9 ± 0.49^{a}	5.8 ± 0.73^{c}
Asx	10.0 ± 0.53^{a}	11.0 ± 0.8^{b}	9.3 ± 0.52^{c}	7.8 ± 1.00^{d}
Cys-Cys	0.9 ± 0.48^{a}	0.67 ± 0.36^{b}	0.63 ± 0.22^{b}	0.68 ± 0.45^{ab}
Glx	13.0 ± 1.00^{a}	13.0 ± 1.6^{a}	13 ± 1.10^{a}	11.0 ± 1.40^{b}
Gly	9.5 ± 1.10^{a}	7.9 ± 0.61^{b}	9.5 ± 1.40^{a}	9.1 ± 1.00^{a}
His	2.6 ± 0.41^{ab}	2.4 ± 0.5^{a}	2.9 ± 0.32^{b}	2.9 ± 0.61^{b}
Ile	3.9 ± 0.23^{a}	3.9 ± 0.31^{a}	4 ± 0.35^{a}	3.9 ± 0.50^{a}
Leu	7.2 ± 0.49^{a}	7.1 ± 0.37^{a}	7.5 ± 0.48^{b}	6.7 ± 0.48^{c}
Lys	7.4 ± 0.96^{a}	7.4 ± 1.2^{a}	6.4 ± 0.46^{ab}	6.2 ± 2.60^{b}
Met	1.7 ± 0.45^{a}	1.5 ± 0.48^{ac}	2.2 ± 0.37^{b}	1.9 ± 0.80^{ad}
Phe	4.5 ± 0.58^{a}	4.4 ± 0.54^{a}	3.6 ± 0.24^{b}	3.6 ± 0.96^{b}
Pro	5.8 ± 0.54^{a}	4.8 ± 0.44^{b}	5.3 ± 0.32^{c}	6.3 ± 0.49^{d}
Ser	6.2 ± 0.83^{a}	6.8 ± 0.68^{b}	6.4 ± 0.52^{ab}	6.6 ± 0.44^{ab}
Thr	5.4 ± 0.24^{a}	5.7 ± 0.66^{b}	5.4 ± 0.33^{ab}	5.7 ± 0.41^{b}
Tyr	3.9 ± 0.81^{b}	2.7 ± 0.29^{a}	3.0 ± 0.40^{a}	4.8 ± 1.30^{c}
Val	5.8 ± 0.35^{a}	5.1 ± 0.49^{b}	5.9 ± 0.46^{a}	6.7 ± 0.81^{c}
EAA (mol%)	42.5 ± 2.0	42.8 ± 1.5	43.0 ± 2.0	43.3 ± 2.6
NEAA (mol%)	57.5 ± 2.0	57.2 ± 1.5	57.0 ± 2.0	56.7 ± 2.6

Means sharing similar letter in a row are statistically not significant ($p > 0.05$)

Table S3.7. Statistical results of permutational multivariate analysis of variance (PERMANOVA) conducted for detecting a difference in amino acid profiles of each taxon among upstream, polluted and downstream sites (levels = 3) along the Dee River, Queensland, Australia. (df = degree of freedom, SS = sum of squares, MS = mean sum of squares, Pseudo -F = F value by permutation, boldface indicates statistical significance with $p < 0.05$, *p*-values based on 9999 permutations (perms)).

	Source	*df*	SS	MS	Pseudo-F	*p*	perms
Chironomidae	Sites	2	114.54	57.27	8.99	**< 0.0001**	9949
	Residuals	34	216.63	6.38			
	Total	36	331.17				
Ceratopogonidae	Sites	2	30.00	15.00	2.15	**0.029**	9934
	Residuals	21	146.28	6.96			
	Total	23	176.28				
Dytiscidae	Sites	2	28.95	14.48	2.41	**0.019**	9934
	Residuals	27	161.59	5.98			
	Total	29	190.54				
Gomphidae	Sites	2	88.19	44.09	2.60	**0.028**	9910
	Residuals	27	457.2	16.93			
	Total	29	545.4				

Table S3.8. Permutational multivariate analysis of variance (PERMANOVA) pairwise comparisons for the amino acid profiles of the four macroinvertebrate taxa, using upstream, polluted and downstream as fixed factors.

Resemblance worksheet
Chironomidae Amino acid profile
Data type: Distance
Selection: All
Standardise: Samples by Total
Resemblance: D1 Euclidean distance
Sums of squares type: Type III (partial)
Fixed effects sum to zero for mixed terms
Permutation method: Permutation of residuals under a reduced model
Number of permutations: 9999

Factors

Name	Abbrev.	Type	Levels
Sites	Si	Fixed	3

PAIR-WISE TESTS

Term 'Si'

Groups	*t*	*p* (perm)	Unique perms
polluted, down stream	1.90	0.0036	9924
polluted, upstream	3.62	0.0002	9933
downstream, upstream	3.03	0.0011	9424

Resemblance worksheet
Name: Ceratopogonidae Amino acid profile
Data type: Distance
Selection: All
Standardise: Samples by Total
Resemblance: D1 Euclidean distance
Sums of squares type: Type III (partial)
Fixed effects sum to zero for mixed terms
Permutation method: Permutation of residuals under a reduced model
Number of permutations: 9999

Factors

Name	Abbrev.	Type	Levels
Sites	Si	Fixed	3

PAIR-WISE TESTS

Term 'Si'

Groups	*t*	*p* (perm)	Unique perms
polluted, downstream	0.84	0.6493	6686
polluted, upstream	1.78	0.0145	6663
downstream, upstream	1.88	0.0376	126

Resemblance worksheet
Name: Dytiscidae amino acid profile
Data type: Distance
Selection: All
Standardise: Samples by Total
Resemblance: D1 Euclidean distance
Sums of squares type: Type III (partial)
Fixed effects sum to zero for mixed terms
Permutation method: Permutation of residuals under a reduced model
Number of permutations: 9999

Factors

Name	Abbrev.	Type	Levels
Sites	Si	Fixed	3

PAIR-WISE TESTS

Term 'Si'

Groups	*t*	*p* (perm)	Unique perms
upstream, downstream	1.62	0.019	8893
upstream, polluted	1.92	0.0083	9852
downstream, polluted	1.17	0.213	9544

Resemblance worksheet
Name: Gomphidae amino acid profile
Data type: Distance
Selection: All
Standardise: Samples by Total
Resemblance: D1 Euclidean distance
Sums of squares type: Type III (partial)
Fixed effects sum to zero for mixed terms
Permutation method: Permutation of residuals under a reduced model
Number of permutations: 9999

Factors

Name	Abbrev.	Type	Levels
Sites	Si	Fixed	3

PAIR-WISE TESTS

Term 'Si'

Groups	*t*	*p* (perm)	Unique perms
upstream, downstream	0.81	0.4898	5070
upstream, polluted	1.91	0.0305	9678
downstream, polluted	1.65	0.0506	9850

Table S3.9. Mean (± SD) amino acid profile (mol%) of Chironomidae collected from seven sites along the Dee River, Queensland. AAs in bold are essential (EAAs) and the remainder non-essential (NEAAs). Amino acids that significantly varied among 7 sites determined using One-Way ANOVA or Kruskal Wallis's H test are marked as ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). N = no of individuals analysed.

	Upstream		Polluted			Downstream	
Site	D1	D2	D3	D4	D5	D6	D7
N	5	5	5	7	5	5	5
Ala*	10.0 ± 2	10.0 ± 1.0	9.3 ± 0.5	10.0 ± 0.4	9.6 ± 0.5	8.9 ± 0.1	9.2 ± 0.8
Arg*	5.3 ± 0.6	5.2 ± 1.0	5.0± 0.9	4.7 ± 0.6	5.4 ± 0.3	6.1 ± 0.4	5.9 ± 0.3
Asx*	10.0 ± 0.5	11.0 ± 1.0	12.0 ± 0.3	12.0 ± 0.4	11.0 ± 1.0	11.0 ± 0.7	12.0 ± 0.6
Cys-Cys**	0.94 ± 0.2	0.95 ± 0.2	0.59 ± 0.3	0.27 ± 0.2	0.44 ± 0.3	0.85 ± 0.2	0.81 ± 0.4
Glx***	11.0 ± 2	12.0 ± 1.0	14.0 ± 2	14.0 ± 0.6	15.0 ± 1.0	14.0 ± 0.6	13.0 ± 1.0
Gly***	8.1 ± 0.2	7.9 ± 0.4	8.9 ± 1.0	7.4 ± 0.3	7.4 ± 0.2	7.8 ± 0.2	7.9 ± 0.2
His	2.7 ± 0.5	2.5 ± 0.5	2.1 ± 0.5	2.5 ± 0.4	1.9 ± 0.7	2.2 ± 0.4	2.7 ± 0.2
Ile	4.1 ± 0.4	3.9 ± 0.3	3.7 ± 0.3	3.9 ± 0.2	4.0 ± 0.3	3.7 ± 0.2	3.9 ± 0.4
Leu*	6.7 ± 0.3	7.0 ± 0.4	7.2 ± 0.4	7.1 ± 0.1	7.2 ± 0.3	7.6 ± 0.1	7.0 ± 0.2
Lys**	5.8 ± 0.9	6.4 ± 1.0	7.5 ± 1.0	8.1 ± 0.6	8.2 ± 0.4	8.5 ± 0.9	6.9 ± 1.0
Met***	2.0 ± 0.3	1.9 ± 0.4	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.7 ± 0.3	1.8 ± 0.2
Phe***	5.2 ± 0.9	4.6 ± 0.5	4.0 ± 0.3	4.5 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	4.6 ± 0.2
Pro*	4.8 ± 0.4	4.8 ± 0.3	5.1 ± 0.6	4.8 ± 0.3	5.1 ± 0.2	4.2 ± 0.1	4.5 ± 0.5
Ser**	7.6 ± 0.6	7.2 ± 0.6	7.2 ± 0.5	6.6 ± 0.4	6.5 ± 0.3	6.0 ± 0.2	6.5 ± 0.7
Thr*	6.6 ± 0.9	6.1 ± 0.7	5.6 ± 0.4	5.5 ± 0.3	5.4 ± 0.2	5.0 ± 0.4	5.6 ± 0.5
Tyr	2.7 ± 0.3	2.8 ± 0.2	2.4 ± 0.4	2.7 ± 0.2	2.8 ± 0.2	2.8 ± 0.1	2.8 ± 0.4
Val*	5.7 ± 0.5	5.2 ± 0.5	4.8 ± 0.5	4.9 ± 0.2	5.1 ± 0.4	4.8 ± 0.1	5.1 ± 0.6
EAAs*	43.9 ± 2.5	42.8 ± 2.1	41.3 ± 0.8	42.3 ± 0.6	42.4 ± 1.2	43.8 ± 0.5	43.5 ± 0.9
NEAAs*	56.1 ± 2.5	57.2 ± 2.1	58.7 ± 0.8	57.7 ± 0.6	57.6 ± 1.2	56.2 ± 0.5	56.5 ± 0.9

Table S3.10. Mean (± SD) amino acid profile (mol%) of Ceratopogonidae collected from seven sites along the Dee River, Queensland. AAs in bold are essential (EAAs) and the remainder non-essential (NEAAs). Amino acids that significantly varied among 7 sites determined using One-Way ANOVA or Kruskal Wallis's H test are marked as ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). N = no of individuals analysed.

	Upstream		Polluted			Downstream	
Site	D1	D2	D3	D4	D5	D6	D7
N	3	2	5	7	2	1	4
Ala*	8.3 ± 0.1	8.0 ± 0.5	7.2 ± 0.7	8.3 ± 0.8	7.7 ± 0.2	7.4 ± 0	8.6 ± 1.0
Arg	3.4 ± 0.4	4 ± 0.04	4.4 ± 1.0	4.1 ± 0.2	3.3 ± 0.6	4.5 ± 0	4.2 ± 0.6
Asx	10.0 ± 0.5	9.3 ± 0.2	9.8 ± 0.6	10 ± 0.4	10 ± 0.5	9.7 ± 0	10.0 ± 0.5
Cys-Cys***	0.28 ± 0.4	1.1 ± 0.4	0.48 ± 0.2	1.1 ± 0.3	1.4 ± 0.04	1.4 ± 0	1.3 ± 0.1
Glx*	14.0 ± 0.4	13.0 ± 0.3	12.0 ± 0.9	13.0 ± 0.6	14.0 ± 2.0	13 ± 0	13.0 ± 0.7
Gly*	11.0 ± 0.5	10.0 ± 0.9	9.8 ± 2	8.7 ± 0.5	8.5 ± 0.4	10 ± 0	9.3 ± 0.4
His	2.4 ± 0.4	2.1 ± 0.7	2.4 ± 0.6	2.8 ± 0.2	2.9 ± 0.3	2.6 ± 0	2.6 ± 0.3
Ile	4.0 ± 0.2	3.8 ± 0.3	3.9 ± 0.3	3.9 ± 0.3	3.7 ± 0.1	3.8 ± 0	3.9 ± 0.2
Leu*	7.3 ± 0.08	7.3 ± 0.04	7.7 ± 0.5	7.0 ± 0.4	6.5 ± 0.2	6.7 ± 0	7.2 ± 0.4
Lys***	8.6 ± 0.3	7.3 ± 0.4	8.3 ± 1.0	6.7 ± 0.4	7.1 ± 1.0	7.0 ± 0	6.9 ± 0.2
Met*	0.86 ± 0.4	1.9 ± 0.04	1.9 ± 0.5	1.8 ± 0.3	1.9 ± 0.2	1.9 ± 0	1.9 ± 0.2
Phe	4.1 ± 0.3	4.1 ± 0.3	4.5 ± 0.5	4.8 ± 0.6	4.8 ± 1.0	4.3 ± 0	4.7 ± 0.5
Pro***	5.1 ± 0.2	6.6 ± 0.1	5.3 ± 0.3	6.2 ± 0.2	5.9 ± 0.9	6.2 ± 0	5.9 ± 0.1
Ser	6.9 ± 0.3	6.3 ± 1.0	6.7 ± 1.0	5.8 ± 0.2	6.2 ± 0.2	6.4 ± 0	5.5 ± 0.3
Thr***	5.0 ± 0.09	5.1 ± 0.04	5.5 ± 0.3	5.4 ± 0.2	5.6 ± 0	5.5 ± 0	5.3 ± 0.09
Tyr	3.0 ± 0.5	4.0 ± 0.8	4.4 ± 1.0	3.9 ± 0.6	4.4 ± 0.9	3.8 ± 0	3.8 ± 0.9
Val	5.5 ± 0.1	5.8 ± 0.3	5.8 ± 0.2	6.0 ± 0.3	6 ± 0.7	5.6 ± 0	5.8 ± 0.5
EAAs	41.3 ± 0.5	41.4 ± 1.3	44.4 ± 3.5	42.3 ± 1.1	41.8 ± 1.5	41.88	42.5 ± 1.8
NEAAs	58.7 ± 0.5	58.6 ± 1.3	55.6 ± 3.5	57.7 ± 1.1	58.2 ± 1.5	58.11	57.5 ± 1.8

Table S3.11. Mean (± SD) amino acid profile (mol%) of Dytiscidae collected from seven sites along the Dee River, Queensland. AAs in bold are essential (EAAs) and the remainder non-essential (NEAAs). Amino acids that significantly varied among 7 sites determined using One-Way ANOVA or Kruskal Wallis's H test are marked as ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). N = no of individuals analysed.

	Upstream		Polluted			Downstream	
Site	D1	D2	D3	D4	D5	D6	D7
N	5	5	7	2	3	5	3
Ala*	9.3 ± 0.7	9.9 ± 0.3	11.0 ± 1	11 ± 0.9	10.0 ± 0.5	10.0 ± 0.6	11.0 ± 2
Arg*	5.5 ± 0.3	5.0± 0.2	4.6 ± 0.6	4.6 ± 0.3	4.7 ± 0.5	5.2 ± 0.5	4.9 ± 0.3
Asx	9.8 ± 0.2	9.2 ± 0.5	9.1 ± 0.7	9.1 ± 0.3	8.9 ± 0.6	9.3 ± 0.2	9.4 ± 0.6
Cys-Cys*	0.7 ± 0.1	0.65 ± 0.1	0.39 ± 0.2	0.73 ± 0.1	0.72 ± 0.1	0.8 ± 0.3	0.56 ± 0.1
Glx*	13.0 ± 0.7	12.0 ± 0.5	13.0 ± 2	12 ± 0.7	12.0 ± 1	12 ± 0.9	13 ± 0.4
Gly	8.9 ± 0.2	9.2 ± 1	11.0 ± 1	8.6 ± 0.09	10.0 ± 2	9.4 ± 2	8.9 ± 0.7
His	3.0 ± 0.3	2.9 ± 0.3	2.9 ± 0.3	2.7 ± 0.2	2.6 ± 0.4	3.0 ± 0.4	2.5 ± 0.1
Ile	4.0 ± 0.2	4.2 ± 0.1	3.8 ± 0.3	4.5 ± 0.2	4.0 ± 0.1	4.1 ± 0.2	3.8 ± 0.8
Leu*	7.2 ± 0.4	7.5 ± 0.2	7.4 ± 0.5	8.0 ± 0.5	7.6 ± 0.4	7.5 ± 0.4	8.4 ± 0.1
Lys	6.1 ± 0.2	6.2 ± 0.4	6.5 ± 0.6	6.4 ± 0.3	6.7 ± 0.5	6.5 ± 0.4	6.4 ± 0.6
Met**	2.2 ± 0.3	2.4 ± 0.2	1.9 ± 0.3	2.4 ± 0.08	2.2 ± 0.3	2.3 ± 0.5	2.8 ± 0.03
Phe	3.6 ± 0.1	3.7 ± 0.2	3.5 ± 0.3	3.7 ± 0.06	3.5 ± 0.1	3.6 ± 0.3	4.0 ± 0.1
Pro*	4.9 ± 0.1	5.2 ± 0.2	5.5 ± 0.2	5.2 ± 0.1	5.4 ± 0.2	5.3 ± 0.3	5.4 ± 0.7
Ser	6.9 ± 0.2	6.6 ± 0.2	6.5 ± 0.3	6.5 ± 0.1	6.7 ± 0.07	6.0 ± 0.3	5.5 ± 0.9
Thr***	5.7 ± 0.2	5.5 ± 0.2	5.6 ± 0.2	5.4 ± 0.1	5.4 ± 0.2	5.3 ± 0.2	4.9 ± 0.5
Tyr***	3.4 ± 0.2	3.4 ± 0.2	2.5 ± 0.3	3.0 ± 0.3	3.0 ± 0.4	3.0 ± 0.2	3.0 ± 0.3
Val	5.8 ± 0.2	6.2 ± 0.3	5.9 ± 0.6	6.1 ± 0.1	5.9 ± 0.1	6.0 ± 0.4	5.6 ± 0.9
EAAs	43.1 ± 0.7	43.4 ± 0.5	42.0 ± 1.3	43.9 ± 0.8	42.5 ± 0.2	43.5 ± 1.1	43.3 ± 2.6
NEAAs	56.9 ± 0.7	56.6 ± 0.5	57.9 ± 1.3	56.1 ± 0.8	57.5 ± 0.2	56.5 ± 1.2	56.7 ± 2.6

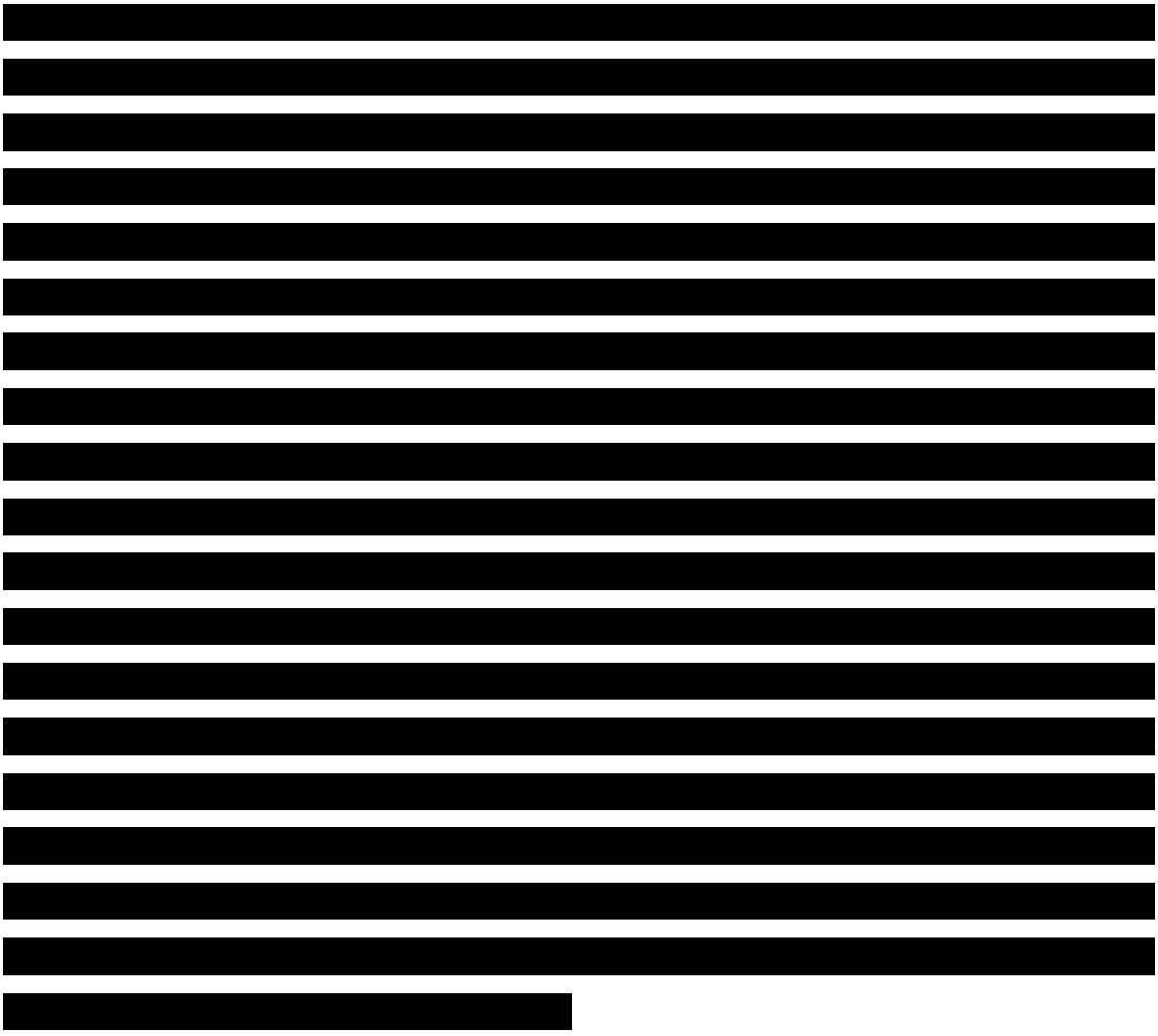
Table S3.12. Mean (± SD) amino acid profile (mol%) of Gomphidae collected from seven sites along the Dee River, Queensland. AAs in bold are essential (EAAs) and the remainder non-essential (NEAAs). Amino acids that significantly varied among 7 sites determined using One-Way ANOVA or Kruskal Wallis's H test are marked as ($p < 0.001$***, $p < 0.01$**, $p < 0.05$*). N = no of individuals analysed.

	Upstream		Polluted			Downstream	
Site	D1	D2	D3	D4	D5	D6	D7
N	6	1	7	1	7	4	4
Ala*	10.0 ± 0.8	12.0	10.0 ± 0.9	12.0	10.0 ± 1	12.0 ± 0.8	10.0 ± 2.0
Arg	5.9 ± 0.5	5.2	5.7 ± 1.0	5.4	6.1 ± 0.5	5.8 ± 0.8	5.7 ± 0.8
Asx	7.3 ± 1	8.1	8.4 ± 0.2	8.2	8.0 ± 0.8	8.1 ± 0.4	6.8 ± 2.0
Cys-Cys**	0.93 ± 0.2	0.78	0.22 ± 0.2	0.08	0.85 ± 0.5	0.53 ± 0.05	1.1 ± 0.6
Glx*	10.0 ± 1	10.0	12.0 ± 0.9	11.0	11.0 ± 1	11.0 ± 0.7	9.0 ± 2.0
Gly**	8.4 ± 0.7	9.6	9.5 ± 0.4	10.0	8.7 ± 0.9	11.0 ± 0.5	8.5 ± 1.0
His*	3.1 ± 0.3	3.1	2.2 ± 0.7	3.0	3.0 ± 0.4	2.8 ± 0.1	3.4 ± 0.8
Ile	4.2 ± 0.3	4.0	3.6 ± 0.4	3.5	3.8 ± 0.7	3.6 ± 0.2	4.3 ± 0.1
Leu*	6.8 ± 0.4	6.3	6.8 ± 0.3	6.4	6.8 ± 0.5	5.9 ± 0.2	6.8 ± 0.5
Lys*	4.9 ± 0.6	5.1	9.4 ± 3	5.2	5.6 ± 2	5.7 ± 0.7	4.5 ± 0.9
Met**	2.3 ± 0.4	1.7	1.1 ± 0.6	1.1	2.4 ± 0.6	1.2 ± 0.2	2.4 ± 1.0
Phe*	3.9 ± 0.7	3.5	3.2 ± 0.3	3.4	3.9 ± 0.6	2.7 ± 0.2	4.7 ± 2.0
Pro	6.4 ± 0.3	6.6	6.1 ± 0.5	7.3	6.2 ± 0.5	6.5 ± 0.5	6.3 ± 0.6
Ser	6.7 ± 0.3	6.4	6.4 ± 0.2	6.8	6.6 ± 0.4	6.6 ± 0.3	7.1 ± 0.8
Thr	5.9 ± 0.3	5.6	5.4 ± 0.3	5.5	5.6 ± 0.4	5.6 ± 0.2	6.3 ± 0.6
Tyr	5.3 ± 1	5.6	3.7 ± 0.9	4.4	5.0 ± 2.0	4.6 ± 0.7	5.8 ± 1.0
Val*	7.5 ± 0.7	6.8	6.4 ± 0.7	6.8	6.1 ± 0.6	6.3 ± 0.6	7.2 ± 0.6
EAAs	44.6 ± 2.1	41.39	43.8 ± 1.2	40.17	43.4 ± 2.5	39.5 ± 1.0	45.3 ± 5.3
NEAAs	55.4±2.1	58.60	56.2 ± 1.2	59.82	56.6 ± 2.5	60.5 ± 1.0	54.7 ± 5.3

Chapter Four

Changes to the Amino Acid Profile and Proteome of the Tropical Freshwater Microalga *Chlorella* sp. in Response to Copper Stress

4.1 Abstract



[3] This chapter is submitted for publication in *Environmental Science and Technology.*

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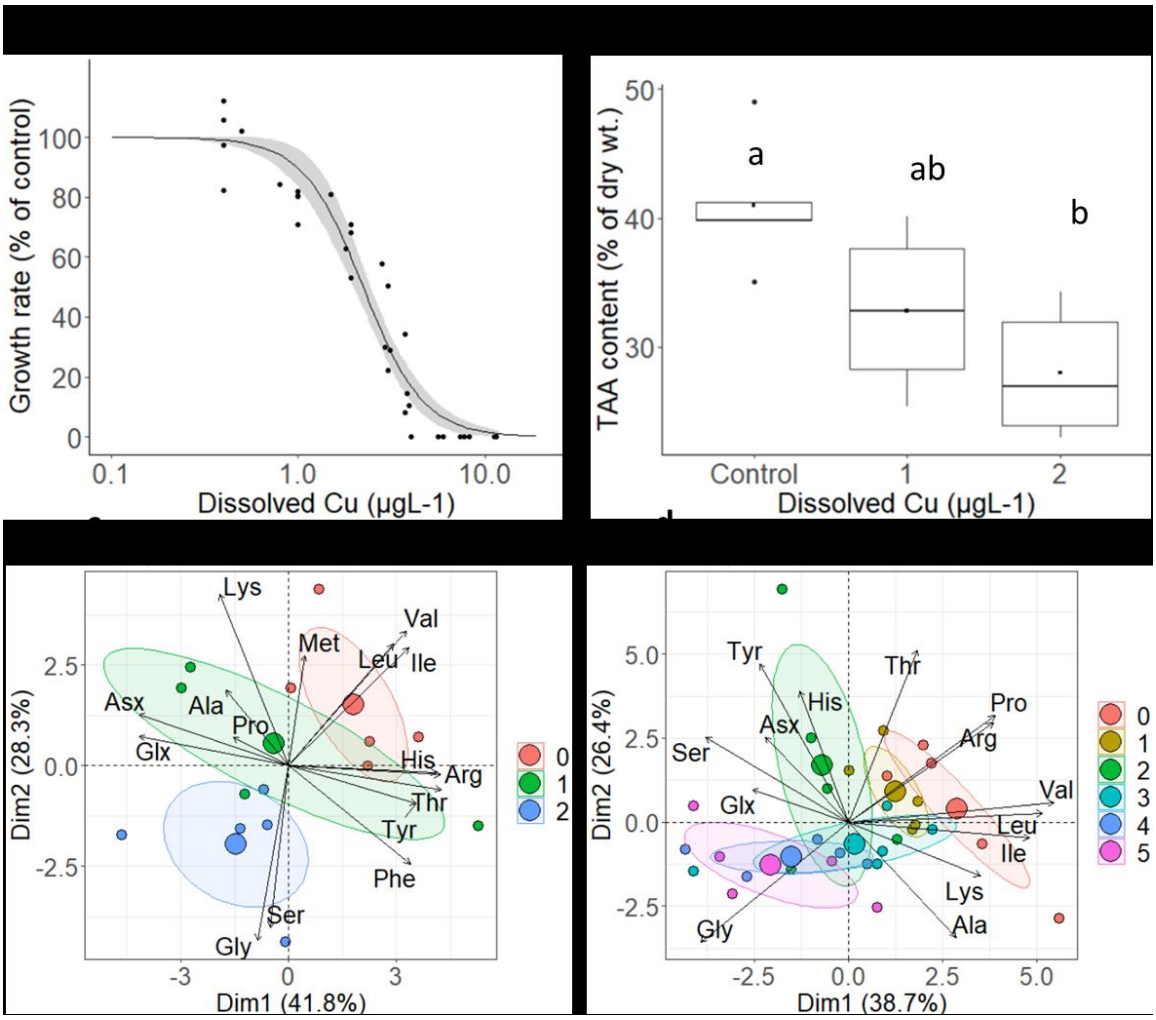

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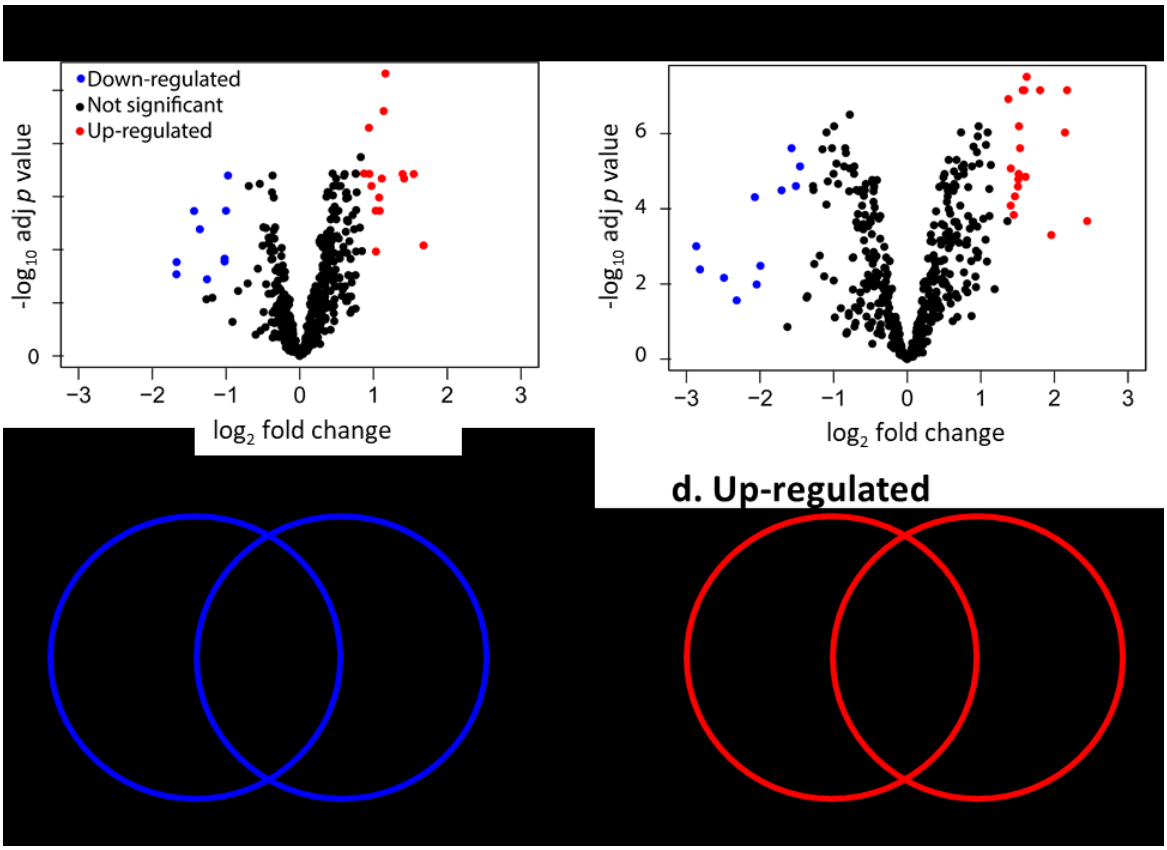

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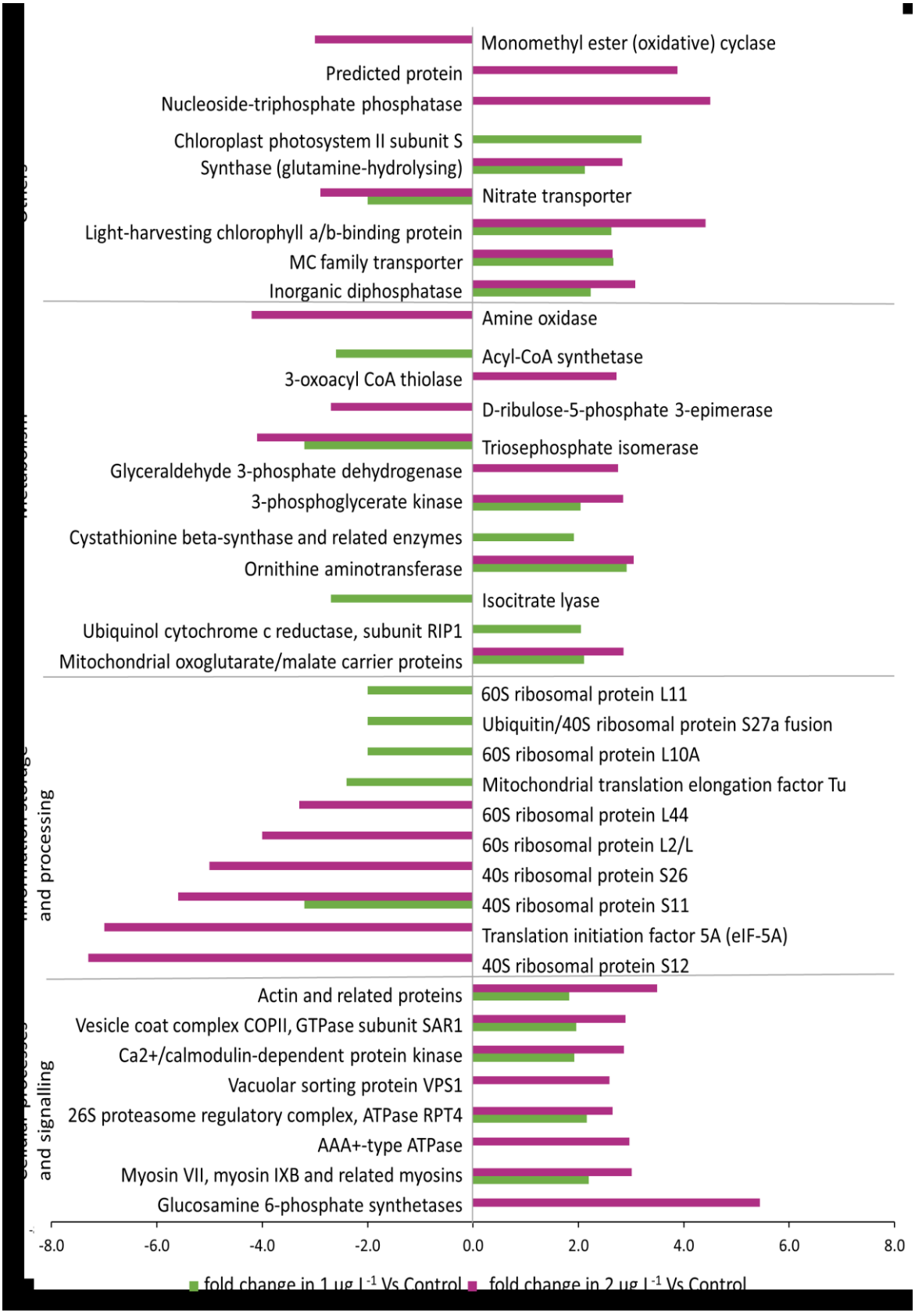

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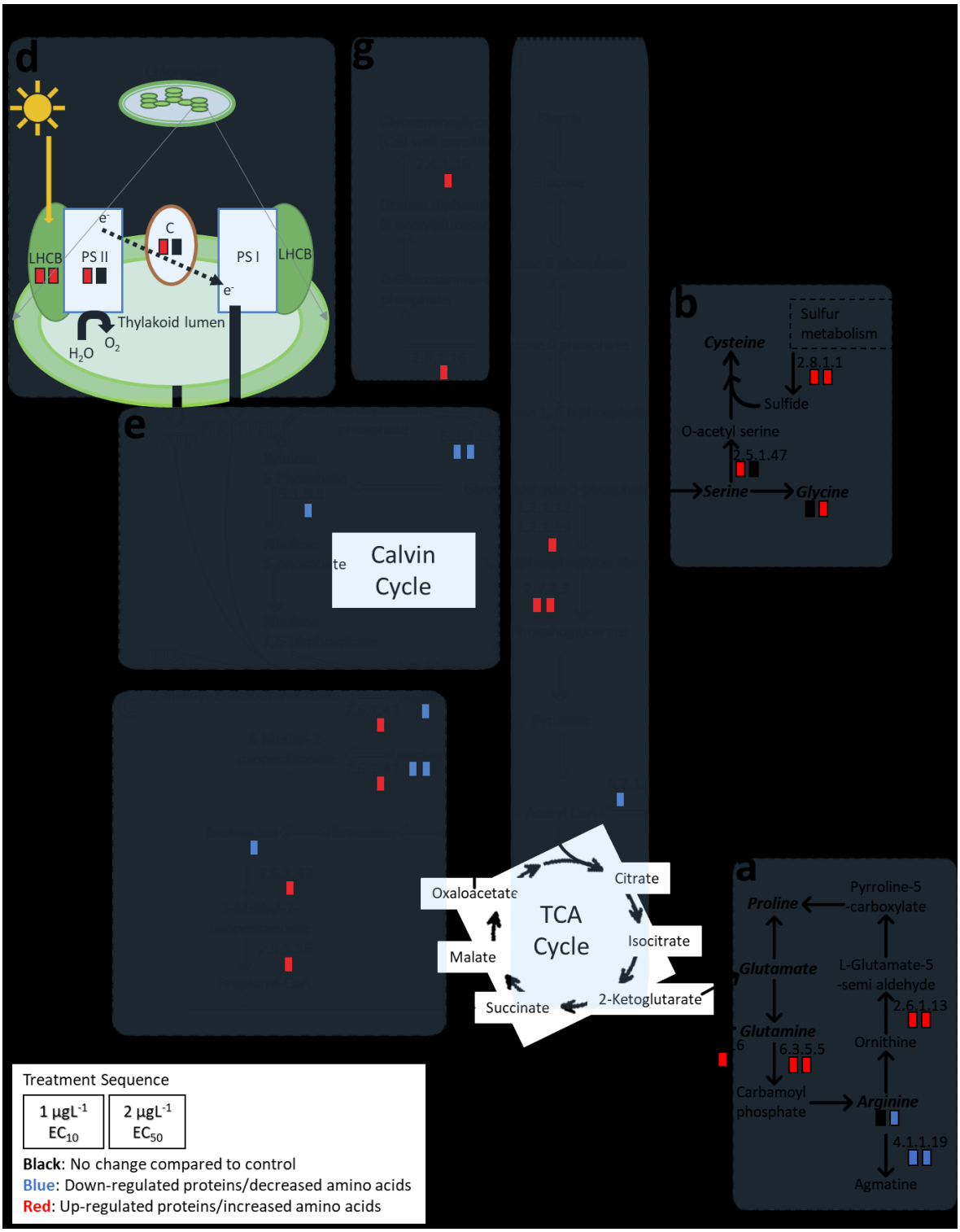

Xxxxxx 4.4. Xxxxxxx xxxxxxx xxxxxxxxxx xxx xxxxxx xx 1 µx X^{-1} xxx 2 µx X^{-1} xxxxxx (Xx) xx xxx xxxxx xxxx (XXx), xxxxxx xxx xxxxxxxxxxxx xxxxxxxxxx xx *Xxxxxxxx* xx. Xxxxxxx xxxx xxxx xxxxxxxxxxxxx xxxxxxxxx xxx xxxxxxx xx xxxxx XX xxxxxx xxxxx xx xxx Xxxxx Xxxxxxxxxxxx xx Xxxxx xxx Xxxxxxx (XXXX) xxxxxxx xxxxxxxx. Xxxxx xxxxx xxx xxxxxxxxx xx xxxx. Xxx xxx xxxxx XX xxxxxx, XXx xx xxxxxxx xxxxxxxx xxxxxxxxxxxx xxxxxxxxx xxxxxx xx Xx xxxxxxx xxxxxxx xxxxxxxx xx xxxxxxx, xxxx xxxx xxxxxxxx xxxxxxxxxxxx xxxxxxxxx xxxxxx xxx xxxxx xxxx xxxxxxxx xx xxxxxxxxxxx xxxxxxxxxx xxxxxxx xxxxxxx xxx xxxxxxx xxxxxxx. Xxx xxx xxxxxxxxxxxx xx xxxxxx xxx xxxx xx xxxxxxx xxx Xxxxxxxxxxxxx Xxxxx X4.7. XXXX = xxxxx-xxxxxxxxxx xxxxxxxxxx x/x xxxxxxx xxxxxx, XX XX = Xxxxxxxxxx xxxxxxxxxx XX, XX X = Xxxxxxxxxx xxxxxxxxxx X, X = Xxxxxxxxx, x^{-} = xxxxxxx, xxxxxx xxxxx xxxxx xxxxxxxxx xxxxxxxx xxxxxxxx xxxxx. Xxxxxxx **x – x** xxxxxxxxx xx xxxxxxx xxxxxxx xx xxx xxxxxxx xxxxxxxxx xx xxxx xxxx.

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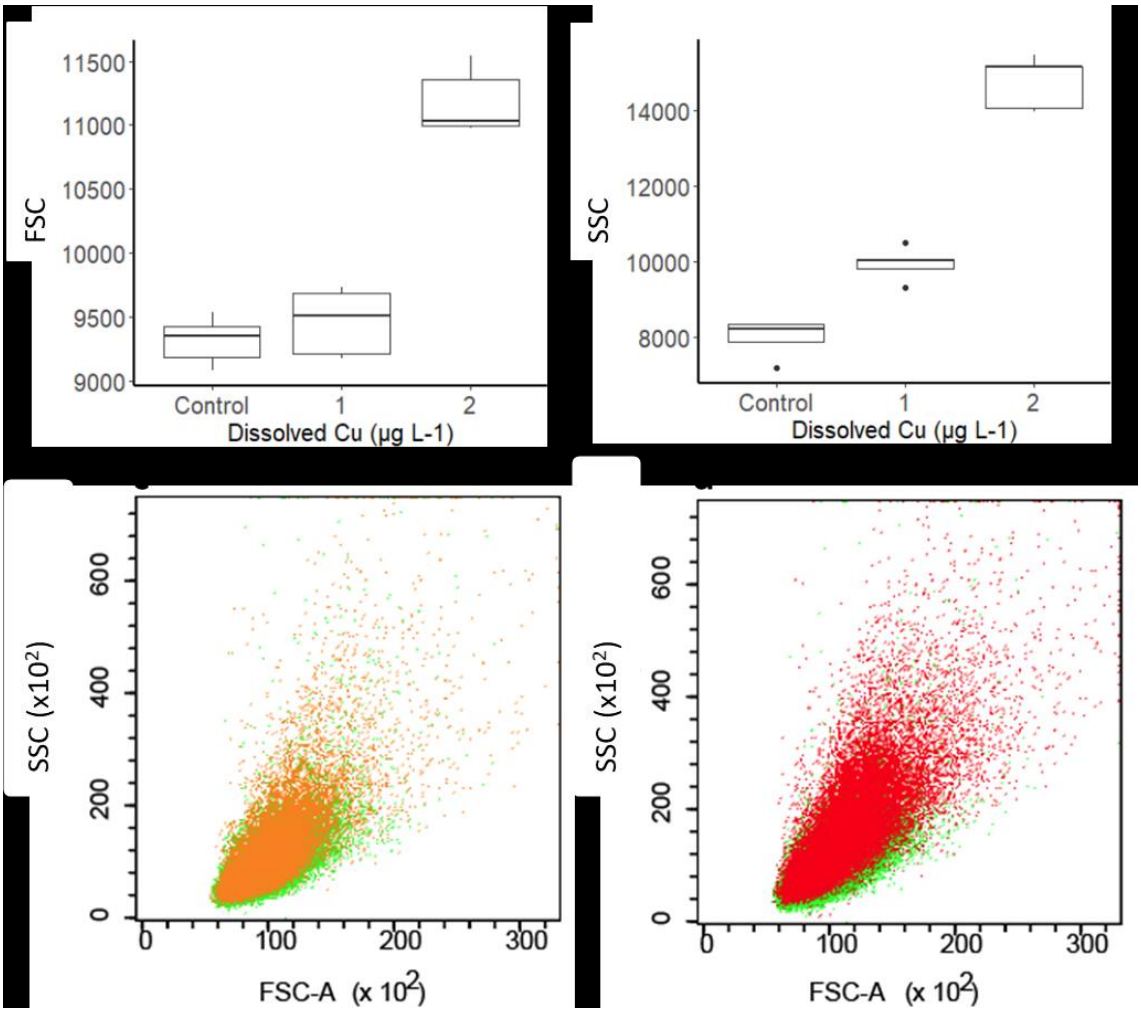

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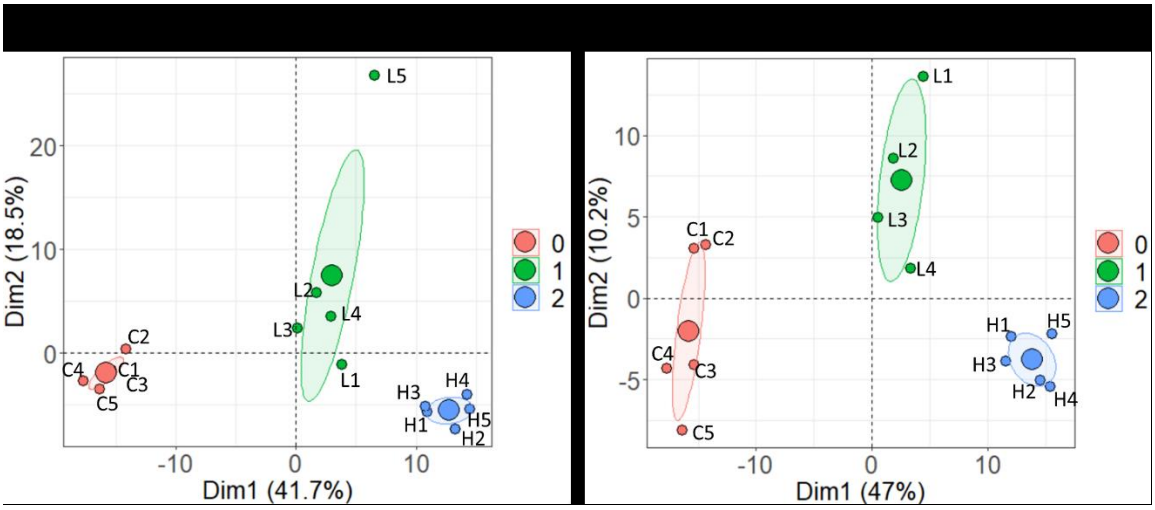
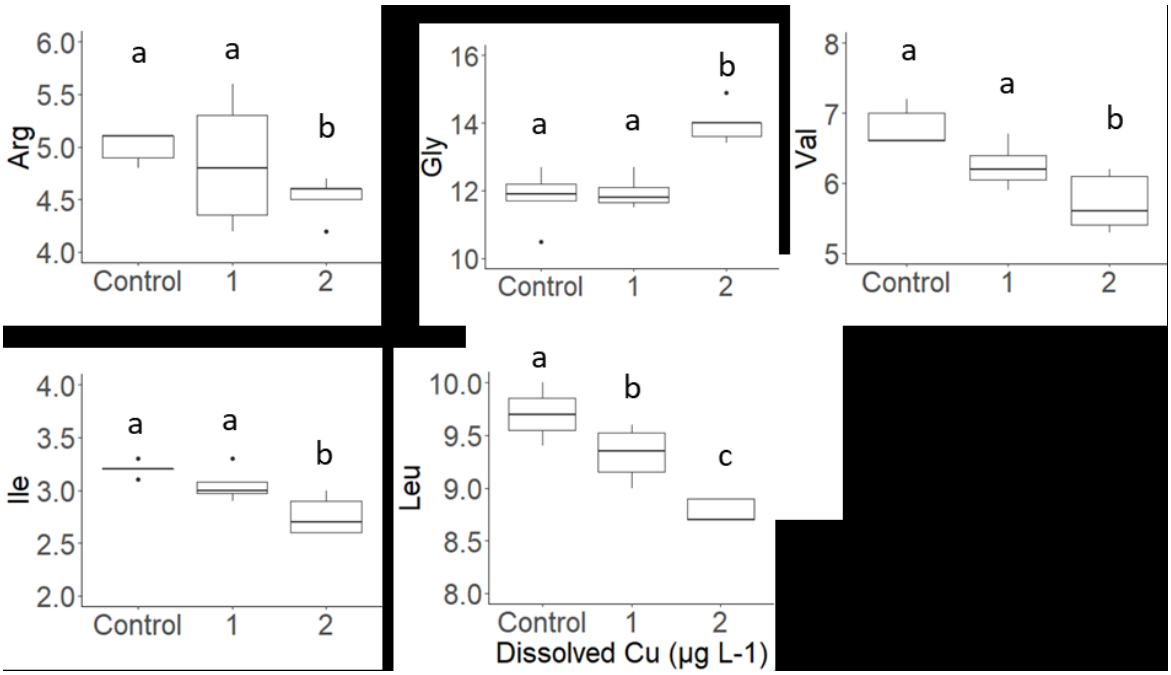


Xxxxxx X4.2. Xxxxxxxxx xxxxxxxxx xxxxxxxx (XXX) xxxx. **x**. xxxxxxx xxx xxxxxxxxxx xx xxxxxxxxxx xxxxxxx: 0 µx X^{-1} (Xxx: X1-X5), XX_{10}: 1 µx X^{-1} (Xxxxx: X1-X5), xxx XX_{50}: 2 µx X^{-1} (Xxxx: X1-X5) xxxxx xx xxx xxxxxxxxx xxxxxxx xxxx xxxxxx X5 xx xxx xxxxxxx. **x**. XXX xxxx xxxxx xx xxx xxxxxxxxx xxxxxxx xx *Xxxxxxxx* xxxxxxx xx 1 xxx 2 µx X^{-1} xxx xxxxxxxxx Xx xxxxxxxxxx xxxxxxx xxxxxx X5.



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Xxxxx X4.1. Xxxxxx xxx Xxxxxxxx'x Xxxxxx (XX) (2/5 xxxxxxxx) xxxxx xxxxxxx xxxxx.

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Xxxxx X4.2. Xxxxxxxxxxxxx (xx X^{-1}) xx xxxxxxxxx xx xxxxxxxxx xxxxxxxxx xxxxxx xxxx xxx Xx xxxxxxxx xxxxxxxx xxxxxxxx xxxx XXXXX. (2002).

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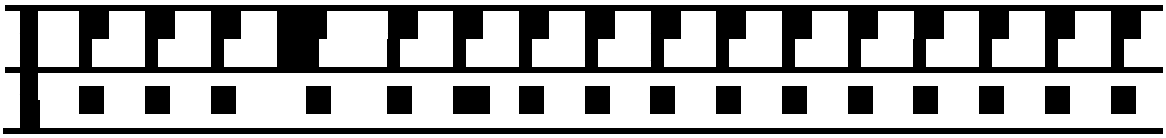

Xxxxx X4.4. Xxxxx xxxx xxxxxxx (xxxxxxxxx xx xxxxxxxx xxxxxxxxx xx xxx %) xx *Xxxxxxxxx* xx. xxxxx 72 x xxxxxxxx xx xxx xxxxxxxxx Xx xxxxxxxxxxxxxx. Xxxxxxxxx xxxxxxx xx x xxx xxxxxxxx xxxxxxxxxxx xxxxxxxxxxx xx xxxxxxxx xxxxxxxxx xx XX xxxxx xxxxxxxxxx.

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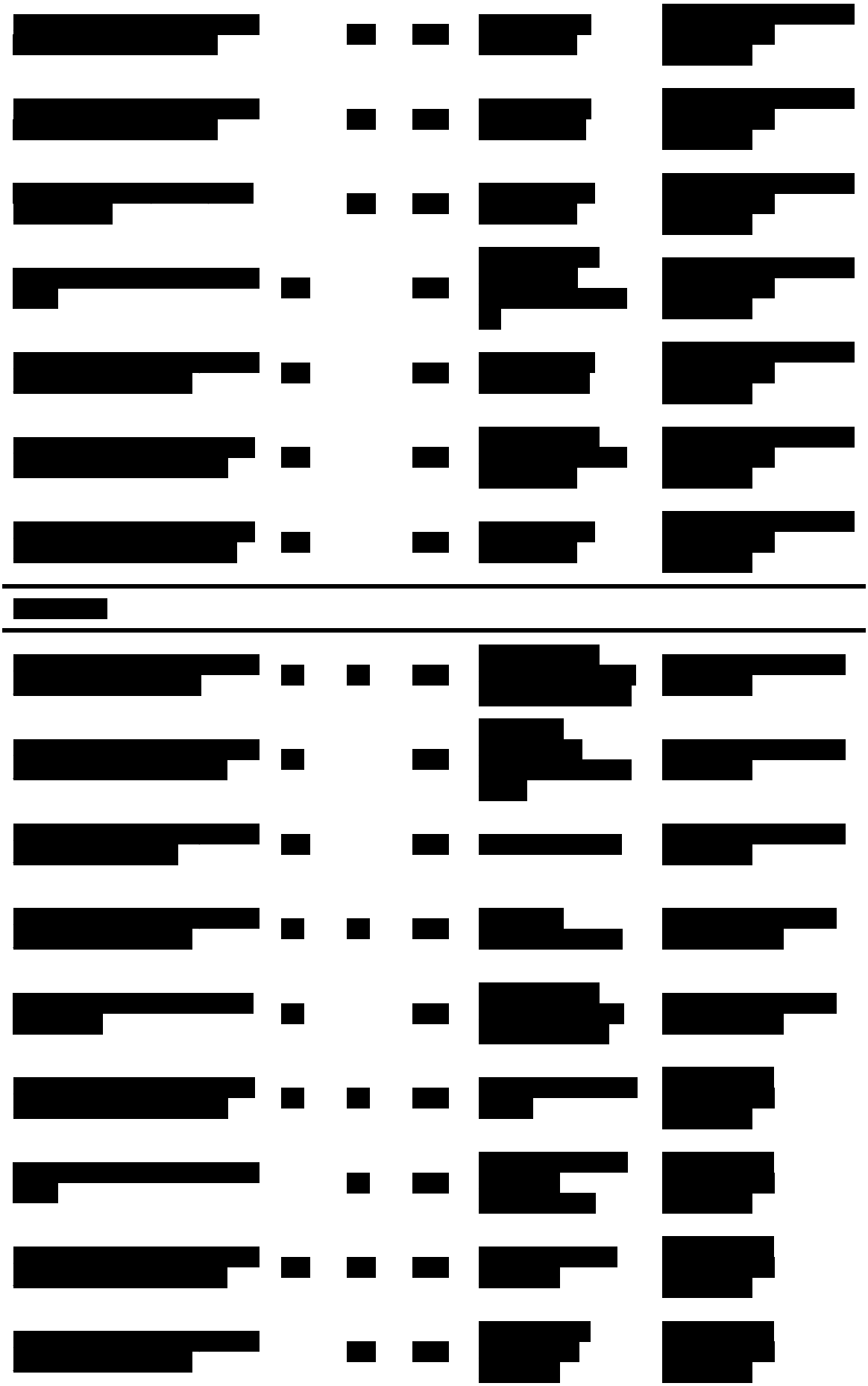
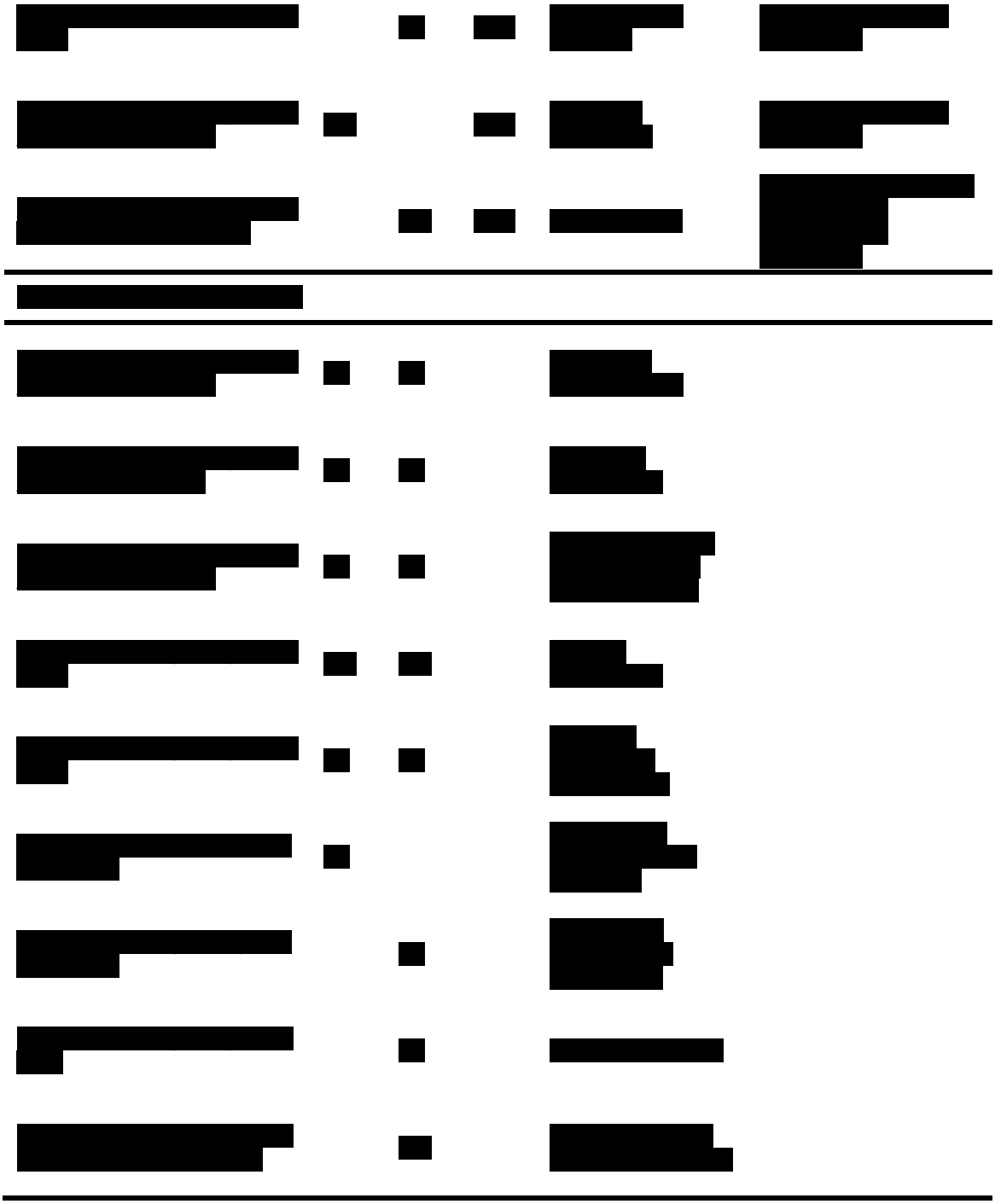
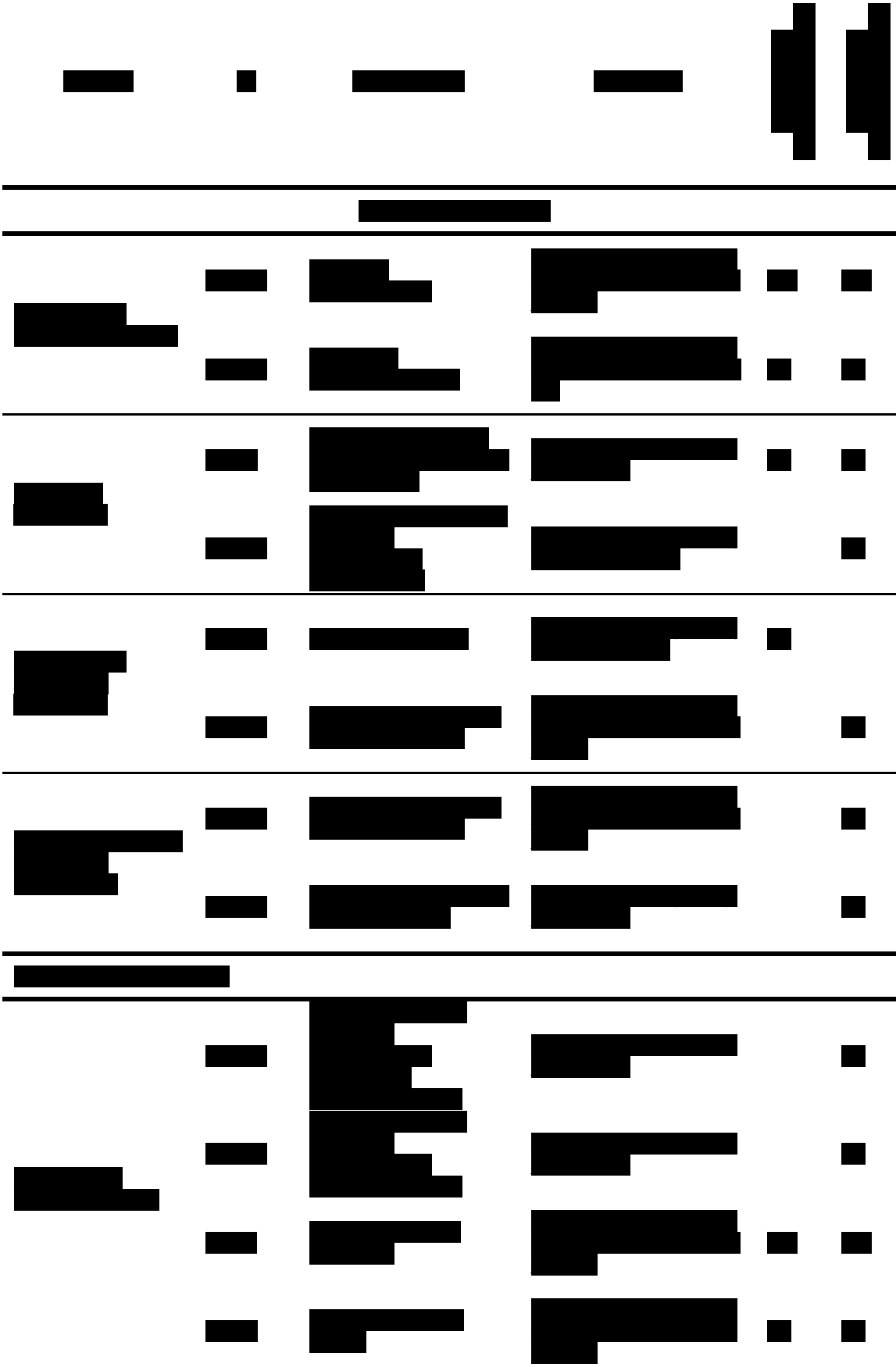
Xxxxx X4.5. Xxxxxxxxxxx xxxxxxx xx xxxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxx xx xxxxxxxx (XXXXXXXXX) xxx xxxxxxxx xxxxxxxxxxx xxxxxxxxx xxx xxxxxxxxx x xxxxxxxxxx xx xxxxx xxxx xxxxxxxx xx *Xxxxxxxx* xx. xxxxxxx xx xxxxxxxxx Xx xxxxxxxxxxxxxx xxx 72 x (*xx* = xxxxxx xx xxxxxxx, XX = xxx xx xxxxxxx, XX = xxxx xxx xx xxxxxxx, Xxxxxx -X= X xxxxx xx xxxxxxxxxx, xxxxxxxx xxxxxxxxx xxxxxxxxxxx xxxxxxxxxxx xxxx $x < 0.05$, *x*-xxxxxx xxxxx xx 999 xxxxxxxxxxxx (xxxxx)).

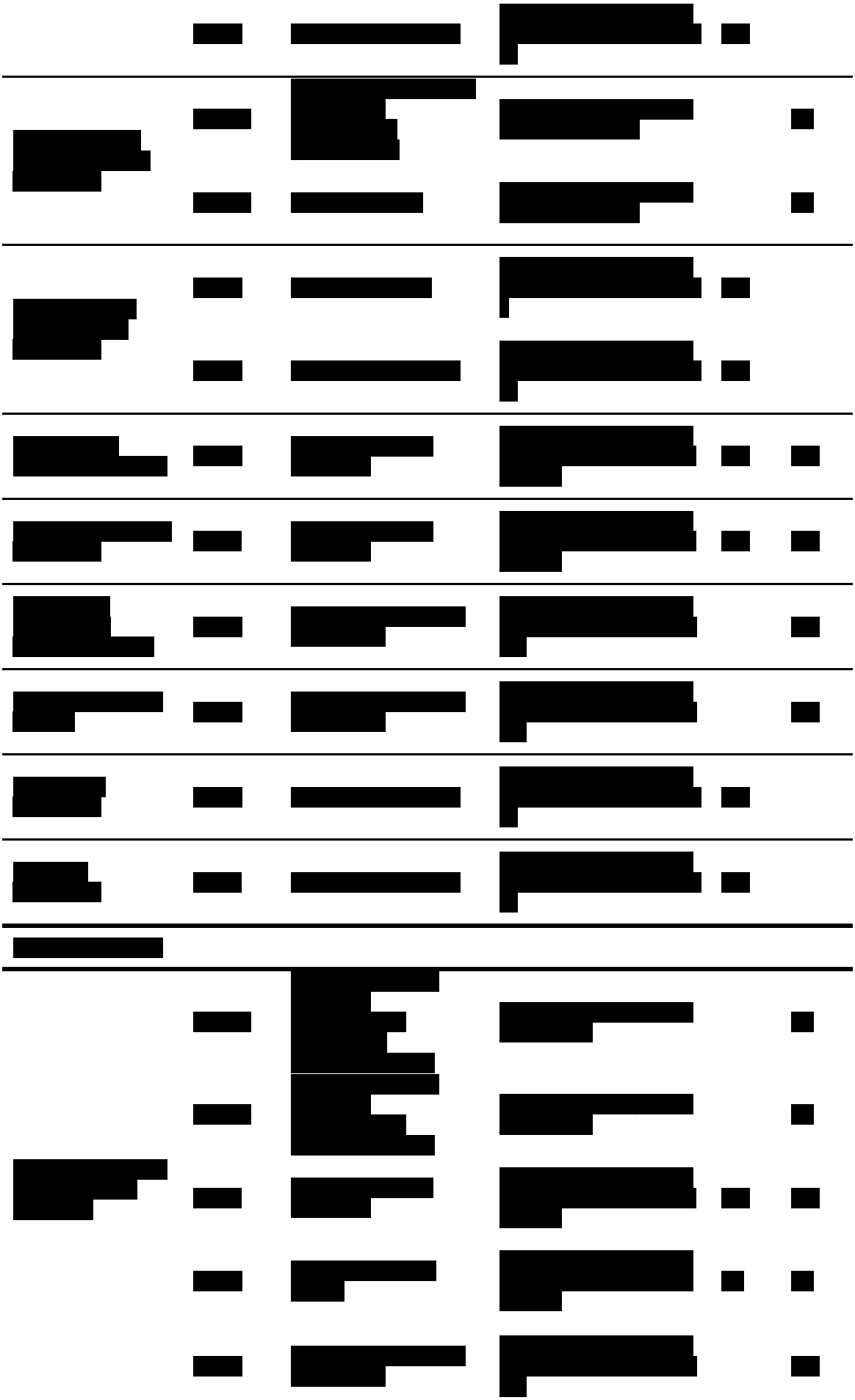
Xxxxx X4.6. Xxxxxxxxxx xxxxxxxxxxxxxx xx xxxxxxxx xx *Xxxxxxxx* xx. xxxxx XxXxxxxxxx Xxxxxxxxxx Xxxxxx (XXX) xxxxxxx xxxxxxxxxxxx xxxxxxxxx xx Xx xxxxxxx xxxxxxx xxxxxxxx xx xxxxxxx.





Xxxxx X4.7. Xxx Xxxxx Xxxxxxxxxxxx xx Xxxxx xxx Xxxxxxx (XXXX) xxxxxxx xxxxxxxx xx xxxxxxxxxxxxxx xxxxxxxxx xxxxxxxx xx *Xxxxxxxx* xx. xx xxxxxxxx xx xxx xxxxxxxxx Xx xxxxxxxxxx. xx = xxxxxxxxx xxxxxxxxx xxxxxxx xxxxxxxxxxx xxxxxxx.





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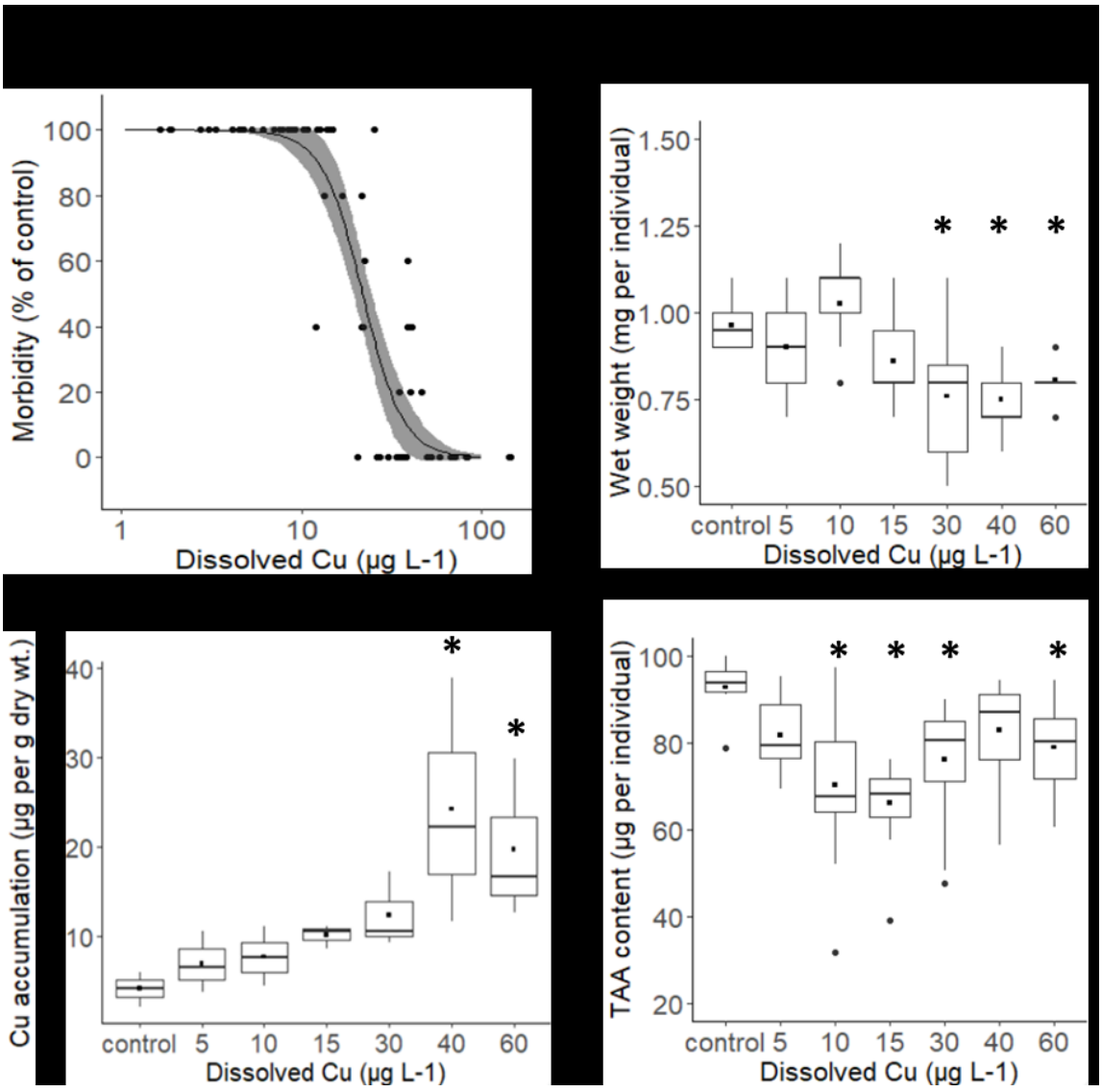

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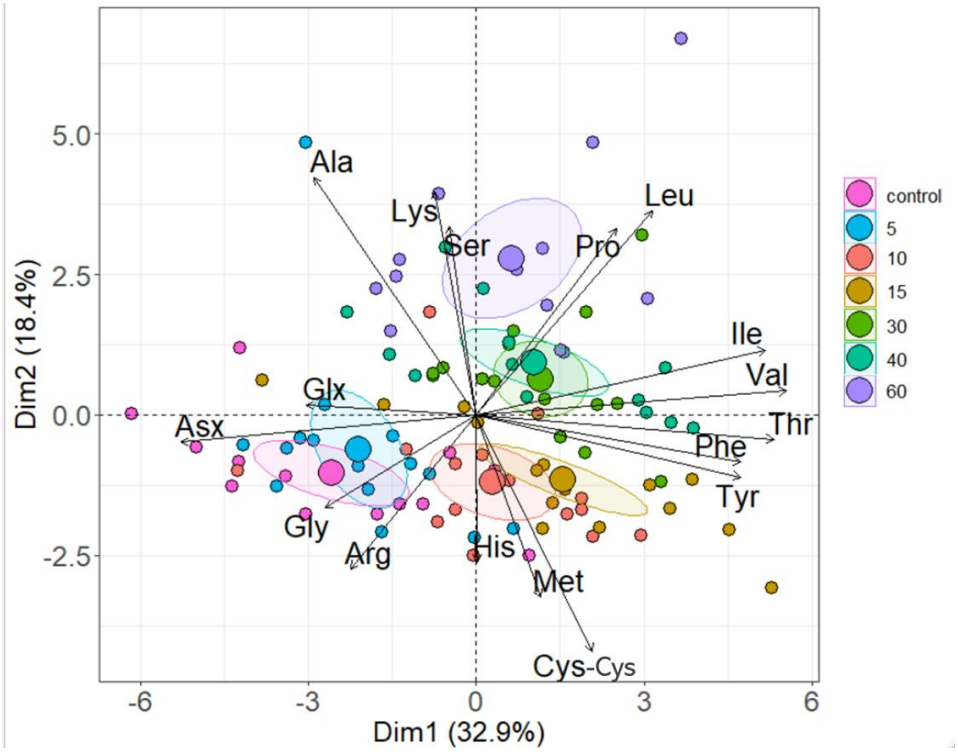

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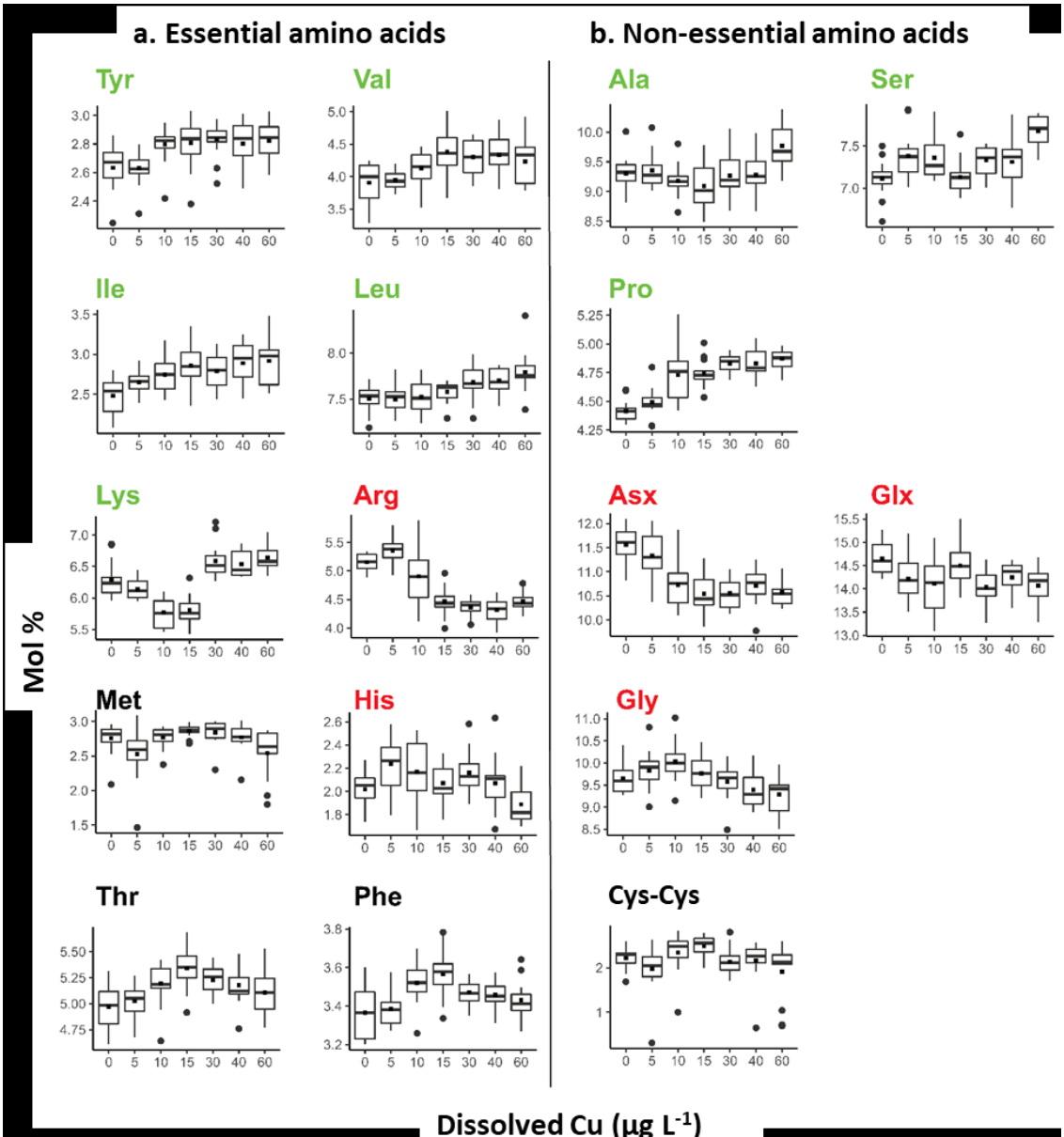
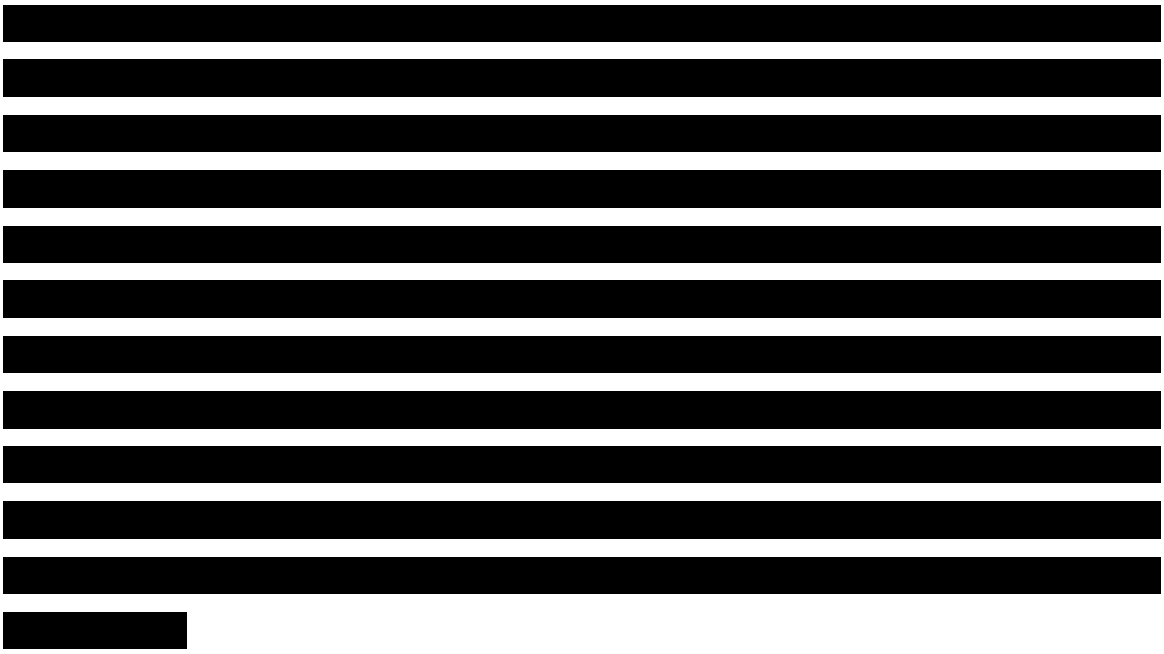


Xxxxxx 5.3. Xxxxxxxxx xx xxx xxxxxxxx xxxxxxxxx (xxx%) xx xxxx xxxxx xxxx x. xxxxxxxxx xxx x. xxx-xxxxxxxxx xx xxxxxx xx Xxxxxx-xxxxxxx Xxxxxxx xxxxxxx xx xxxxxxxxx xxxxxx (Xx) xxxxxxxxxxxxx xxxx 96 x. Xxxxx xxxx xxxxxx xxxxxxxxxx xx xxxxx xxxx xxxxxxxxxx xxxxxxxxx; xxx xxxx xxxxxxxxx xxxxxxxxx xxx xxxxx xxx xx xxxxxxxxxx xxxxxxxxxx xxxx xxxxxxxx Xx xxxxxxxxxxx.



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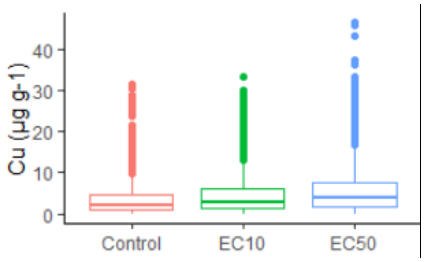
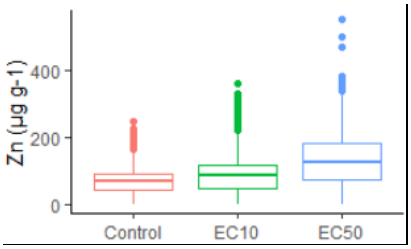
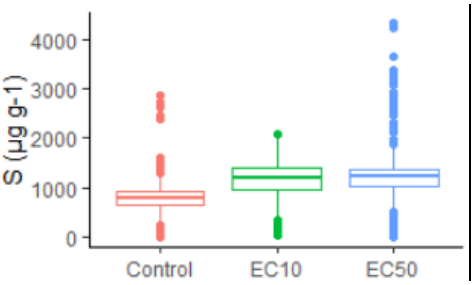
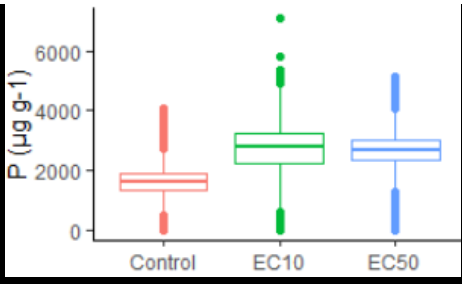
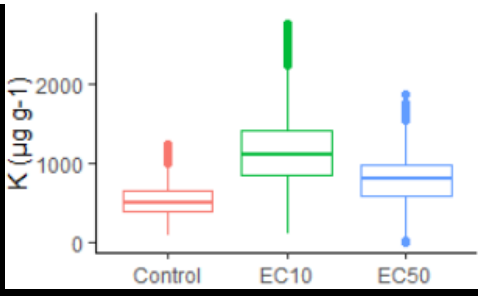
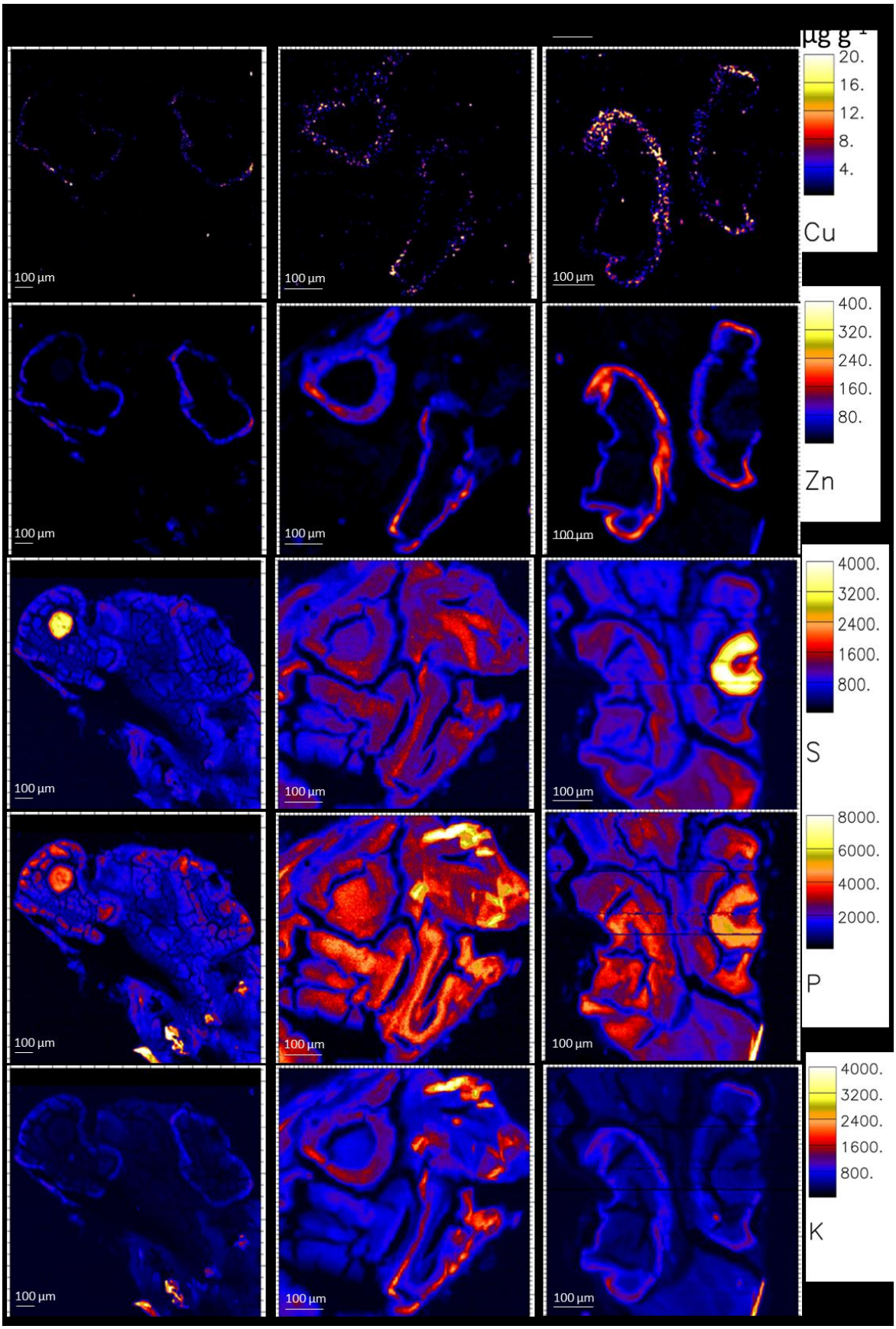
15 µx Xx X^{-1} (XX_{10}) xxx 30 µx Xx X^{-1} (XX_{50}) Xx xxxxxxxx. Xx = xxxxxx, Xx = xxxx, X = xxxxxx, X = xxxxxxxxxx, X = xxxxxxxxx.



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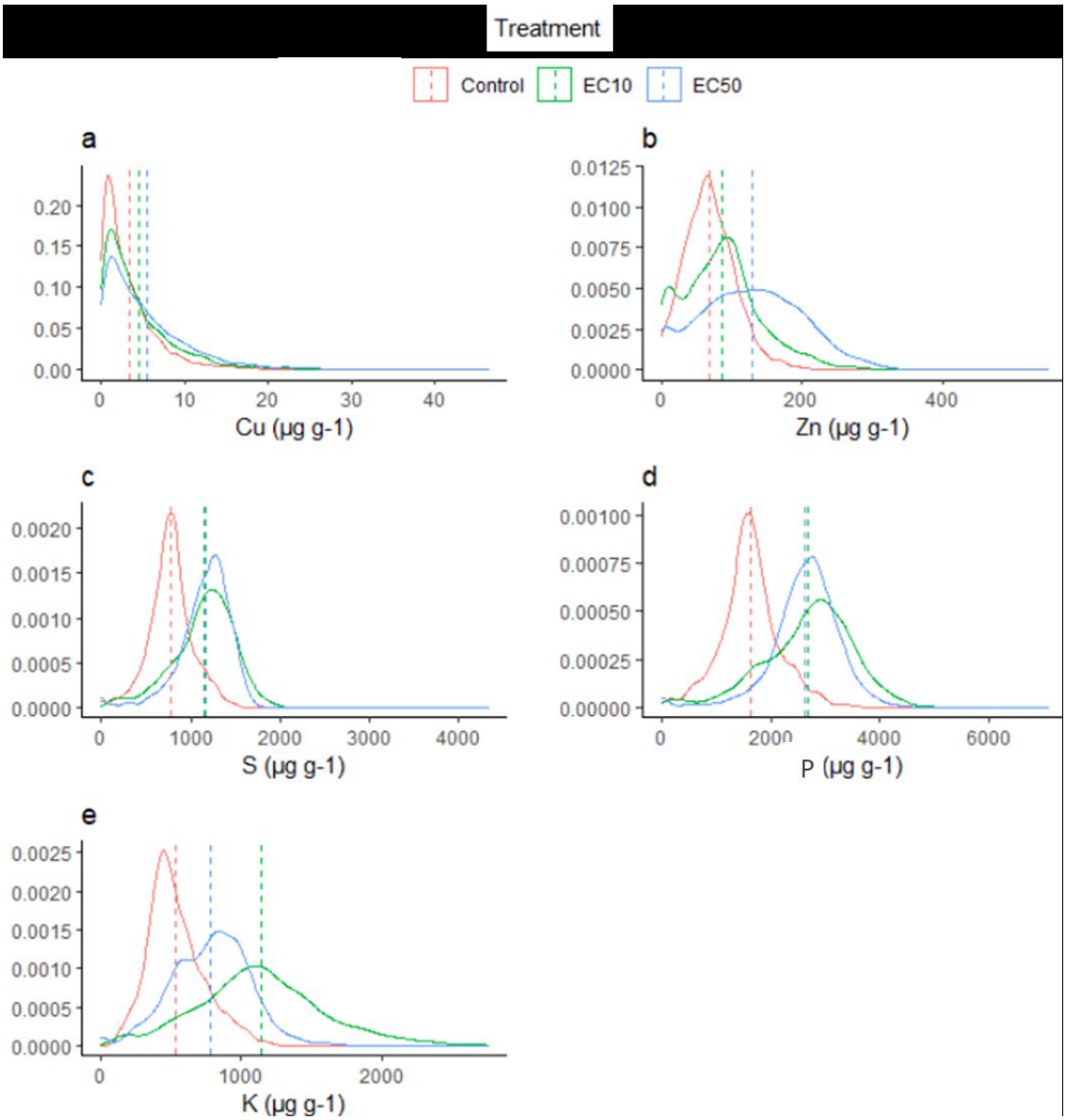

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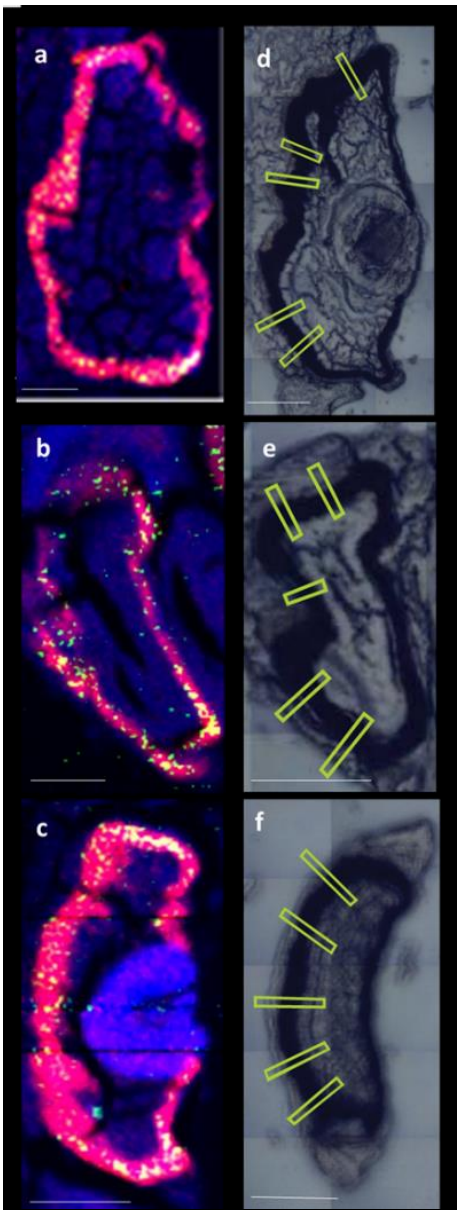

Xxxxxx 5.6. Xxxxx-xxxxxxx XXX xxxxxx (Xx (xxx), Xx (xxxxx) xxx X (xxxx)) xxxxxxxx xxxxx Xxxxxx Xxxxxxx X-xxx Xxxxxxxx (XXXX) xxxxxxxx xx Xxxxxx-xxxxxxx Xxxxxxx xxxxxx xxx xxxxxx xxxxxxx xx: x. Xxxxxxx (0 µx Xx X^{-1}), x. XX_{10} (15 µx Xx X^{-1}) xxx x. XX_{50} (30 µx Xx X^{-1}) Xx xxxxxxxxxxxxxx xxx 96 x xxxxx xxxxxxxx. Xxxxx xx: x. Xxxxxxx, x. XX_{10} xxx x. XX_{50} xxx xxx xxxxxxxxxxxxx xxxxxxxxxxxxxxxx xx xxx xxxxxxxx (xxxxxxxx xx xxx xxxxxxxx

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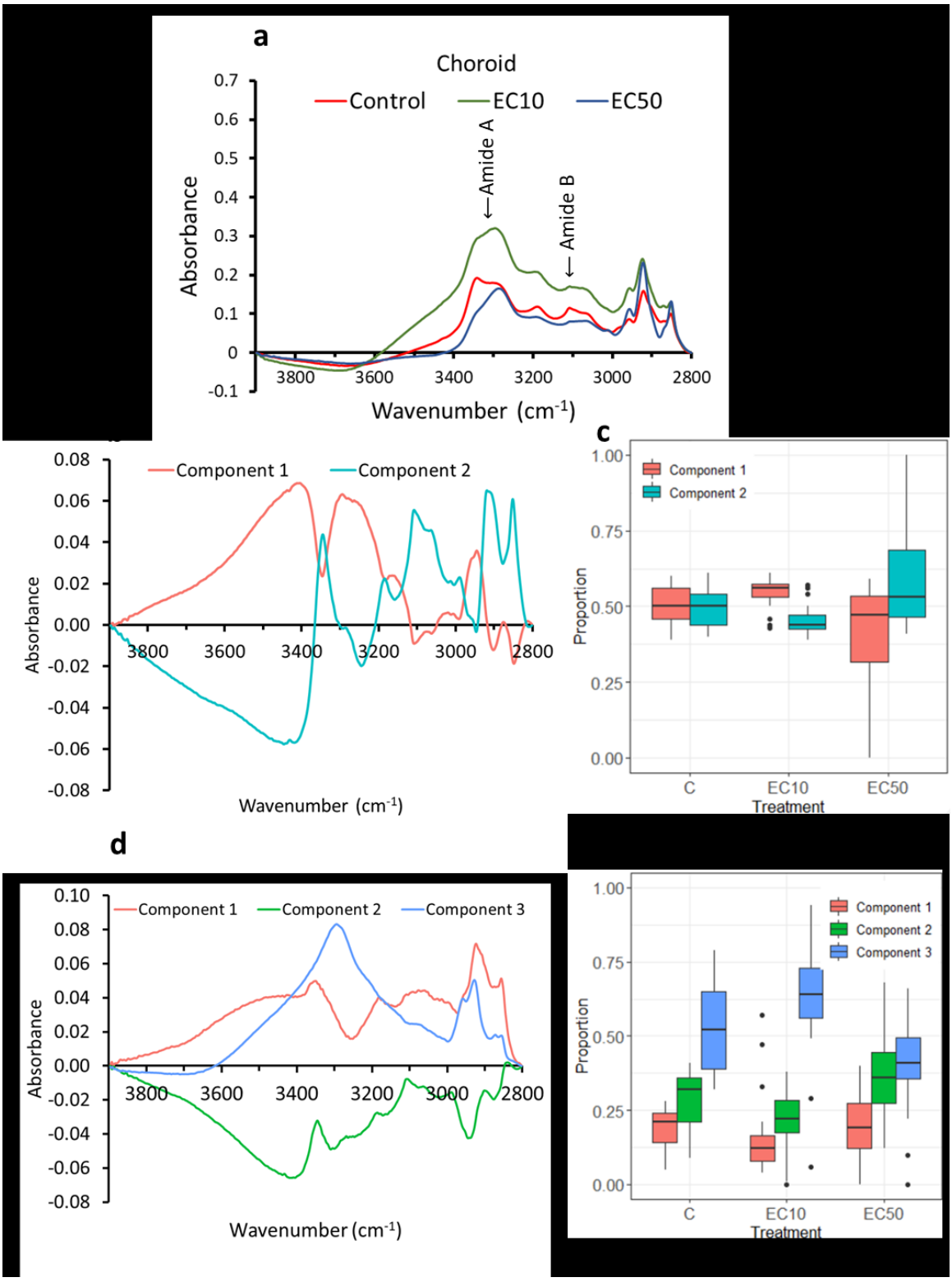

Xxxxxx 5.7. Xxxxxxx-xxxxxxxxx xxxxxxxx xxxxxxxxxx xxxxxxx (XXXX) xxx xxxxxxxxxxxx xxxxx xxxxxxxxxx (XXX) xxxxxxx xxx xxx 3900 – 2800 xx^{-1} xxxxxx xx xxxxxxx xxxxx xx Xxxxxx-xxxxxxx Xxxxxxx (XXX); **x**. Xxxxxxx XXXX xxxxxxx xxxxxxxxx xxxx xxxxxxx xx xxxxxxx, XX_{10} xxx XX_{50} Xx xxxxxxx XXX; **x** xxx **x**. 2-xxxxxxxxx xxxxxxx xxx 3-xxxxxxxxx xxxxxxx, xxxxxxxxxxx, xxxxxxxx xxxx XXX xxxxxxxx; **x** xxx **x**. Xxxxxxxx xxxxxxx xx xxxxxxxxxx xx xxxx xxxxxxxx xxx xxx 2 xxx 3 xxxxxxxxx xxxxxx, xxxxxxxxxxx.

Xxxxx 5.2. Xxxx xxxxxxxxxxx xxx Xxxxxxx-xxxxxxxxx xxxxxxxx xxxxxxxxxx xxxxxxx xxxxxx xxxxxxx xxxxxxx (Xxxxx, 2007; Xxxx & Xx, 2007; Xxxxx xx xx., 2008; Xxxxxxx-Xxxxxxx xx xx., 2012; Xxxxxxxxxxxx xx xx., 2017).

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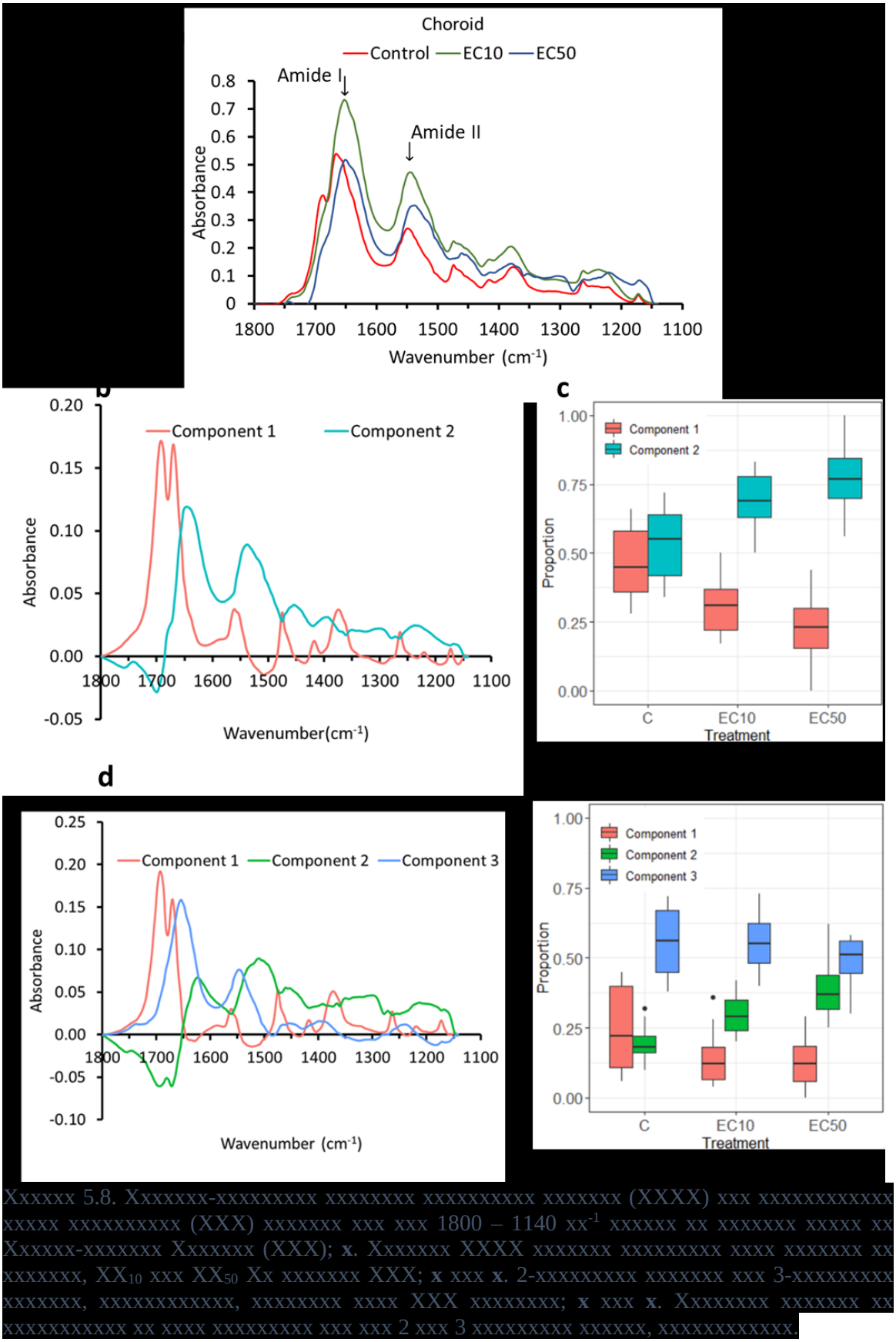

Xxxxxx 5.8. Xxxxxxx-xxxxxxxxx xxxxxxxx xxxxxxxxxx xxxxxxx (XXXX) xxx xxxxxxxxxxxx xxxxx xxxxxxxxxx (XXX) xxxxxxx xxx xxx 1800 – 1140 xx^{-1} xxxxxx xx xxxxxxx xxxxx xx Xxxxx-xxxxxxx Xxxxxxx (XXX); **x**. Xxxxxxx XXXX xxxxxxx xxxxxxxxx xxxx xxxxxxx xx xxxxxxx, XX$_{10}$ xxx XX$_{50}$ Xx xxxxxxx XXX; **x** xxx **x**. 2-xxxxxxxxx xxxxxxx xxx 3-xxxxxxxxx xxxxxxx, xxxxxxxxxxx, xxxxxxxx xxxx XXX xxxxxxxx; **x** xxx **x**. Xxxxxxxx xxxxxxx xx xxxxxxxxxx xx xxxx xxxxxxxxx xxx xxx 2 xxx 3 xxxxxxxxx xxxxxx, xxxxxxxxxxx.

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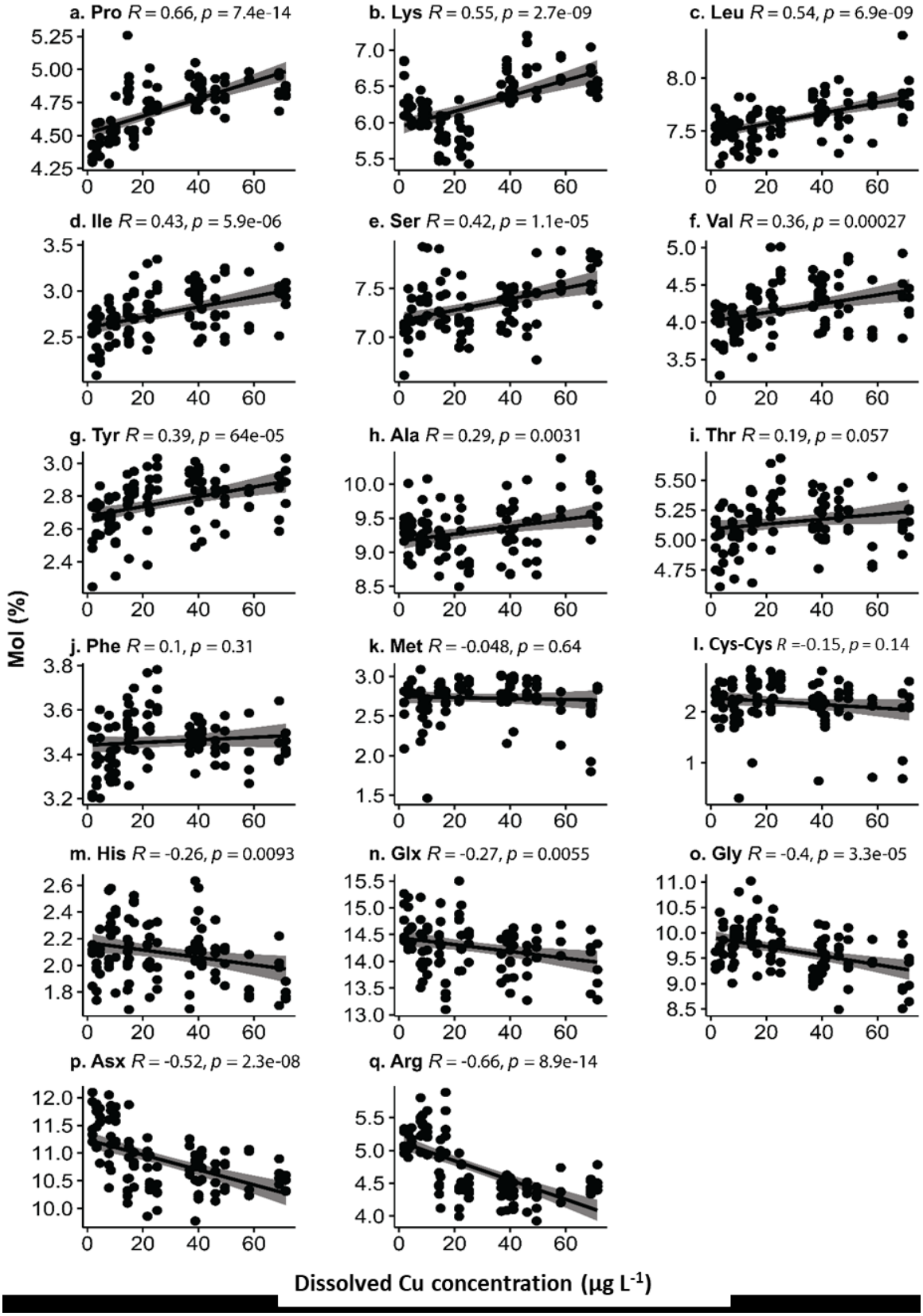

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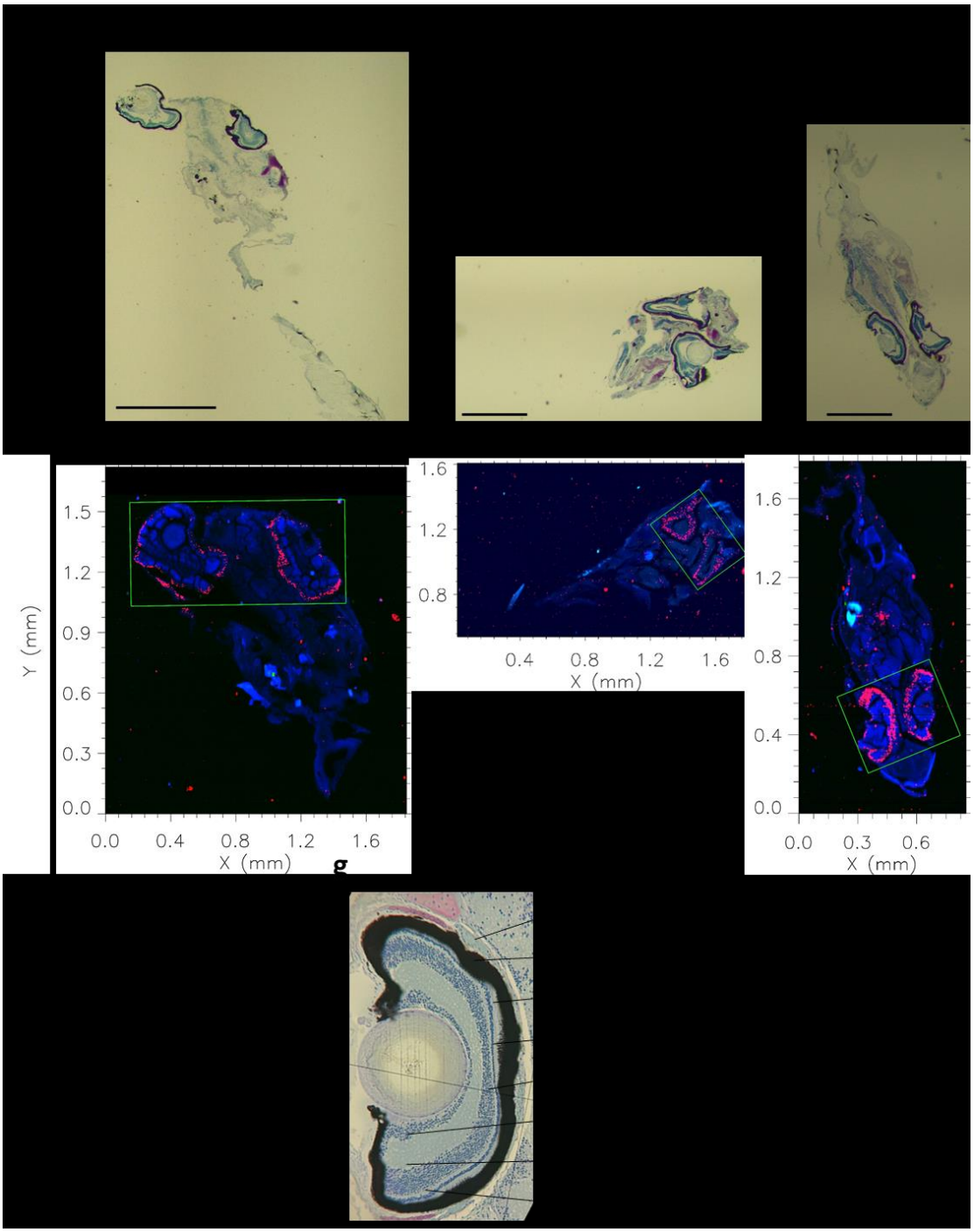
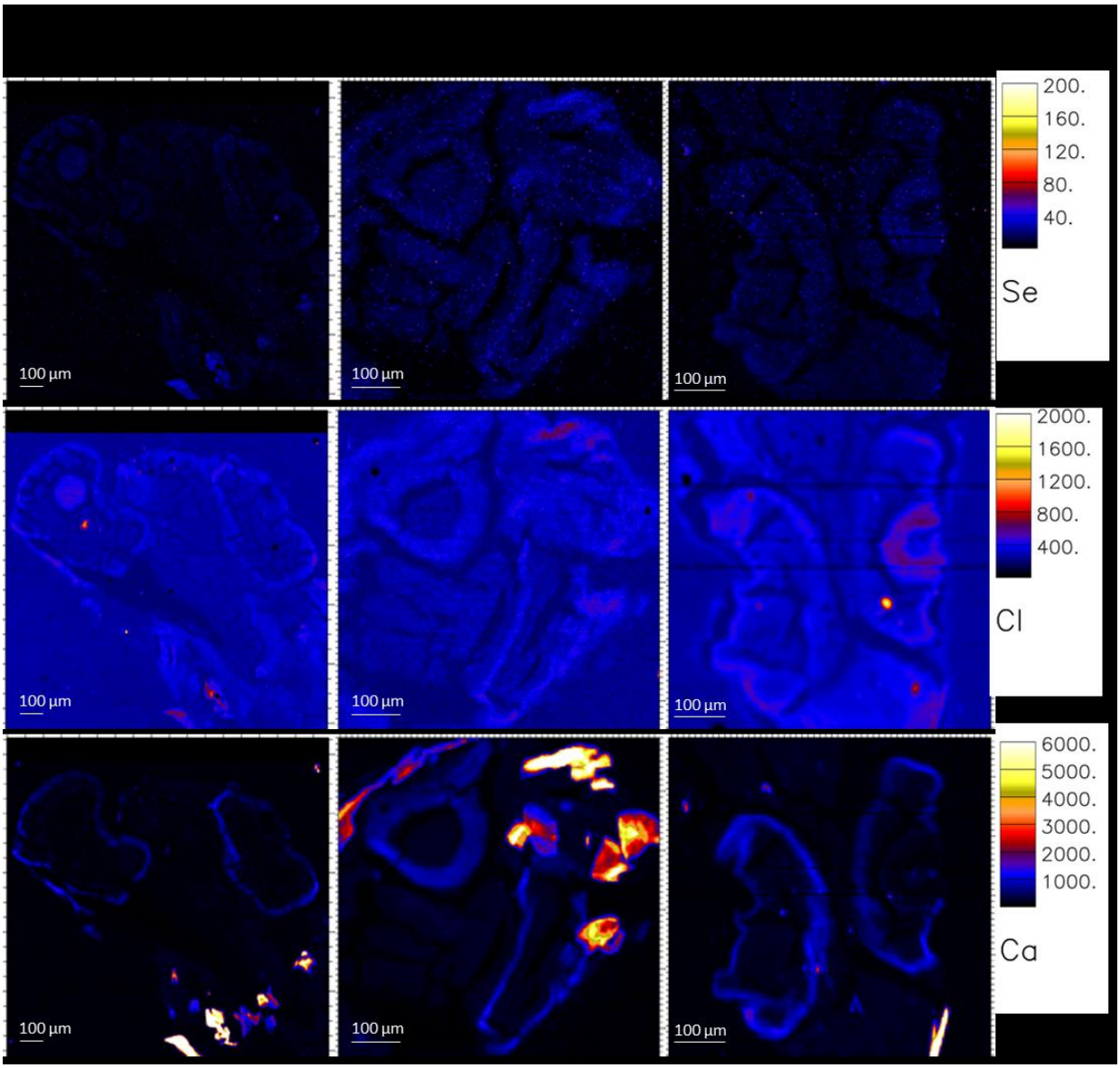
Xxxxx X5.1. Xxxxxxx xxxxxxxxxx xxxxxxx xxx xxxxxxx xxxxxxxx (xxx%) xx xxxx xx 17 xxxxx xxxxx xx Xxxxxx-xxxxxxx Xxxxxxx xxxxxx xxx xxxxxxx Xx xxxxxxxxxxxxx.



Xxxxxx X5.2. Xxxxxxx xx Xxxxxx-xxxxxxx xxxxxxx xxxxxx xxxxxxxx xx XXX xxxxxxxx (Xxxxxxx Xxxxxxx Xxxxxxxxxx) xxx xxxxxxx xxxx xxxxxxxxx xxxx xxxxxxx xx **x**. Xxxxxxx (xx xxxxx Xx) **x**. XX_{10} xxx **x**. XX_{50}; **x-x** xxxxx xxx xxxxxxxxxxx xx Xx (xxx) xx xxxxxxxxxx xxx xxxxxxx xxxxxxxx xx Xxxxxx xxxxxxx X-xxx xxxxxxxx (XXXX) xx 30 µx xxxxxxxx xxx xxxxxxxx xx xxx xxxxxxxxxxx xxxxxxxx (x-x) xxx xxxx xxxxxxxxx. **x**. Xxxxxxxxx xx Xxxxxx-xxxxxxx Xxxxxxx xxx xxxxxx (Xxxxxxx) xxxxxxxx xxxx xxxxxxxxxx xxxxxxxxxxx xxx xxxxxxx xxxx xxxxxxxxx xxxx. Xxxxxxxxx xxxxxxxxxxxx xxxx xxxxxxxxx xxxx xxx xxxxxxxxxxx xxxxxx xxxxxxx xx x. Xxxxxxx (1.3 xx×0.5 xx), x. XX_{10} (0.6 xx×0.4 xx) xxx x. XX_{50} (0.5 xx×0.4 xx). Xxx = Xx, Xxxx = Xx xx Xxxxxx x-x.



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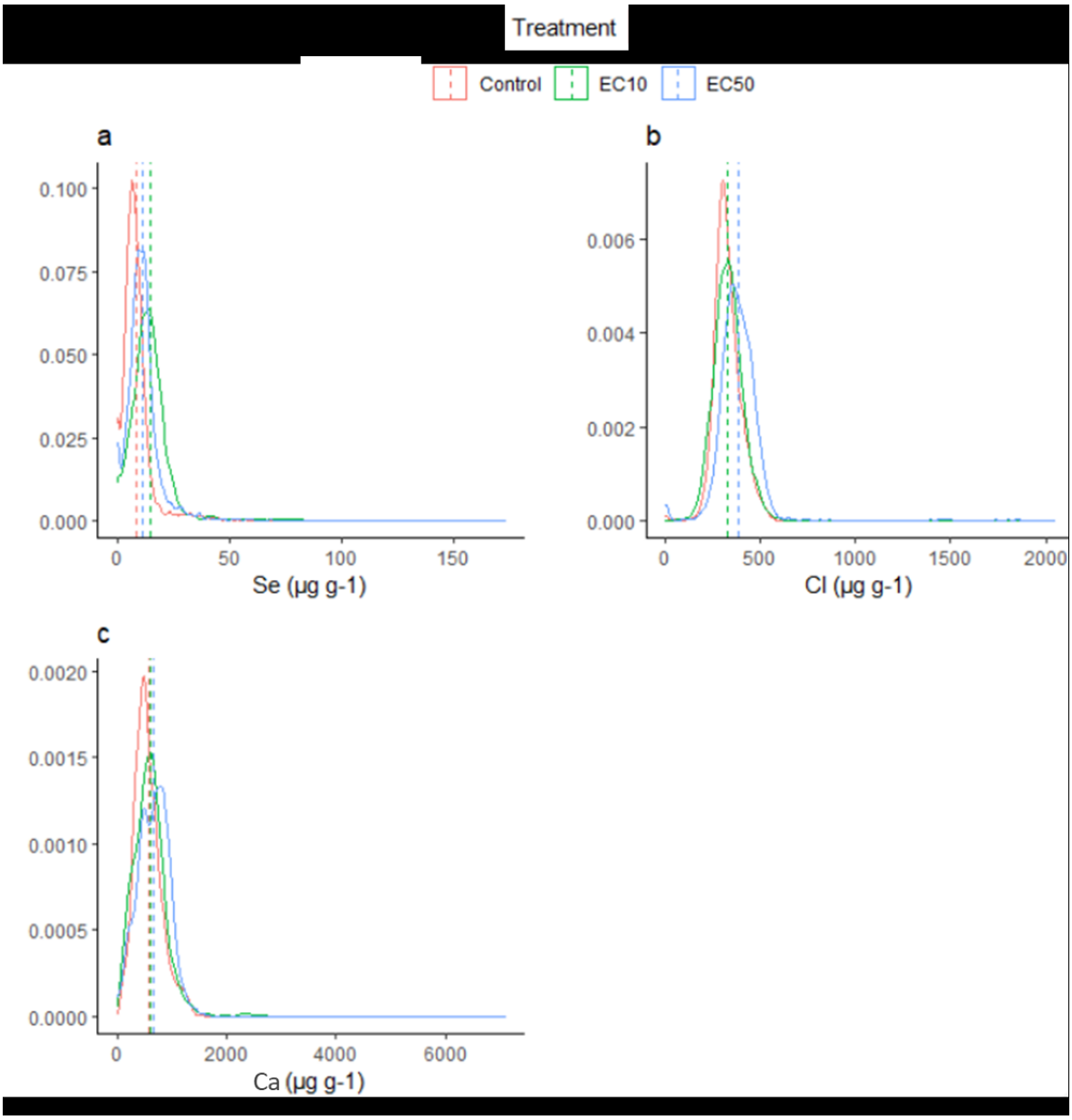

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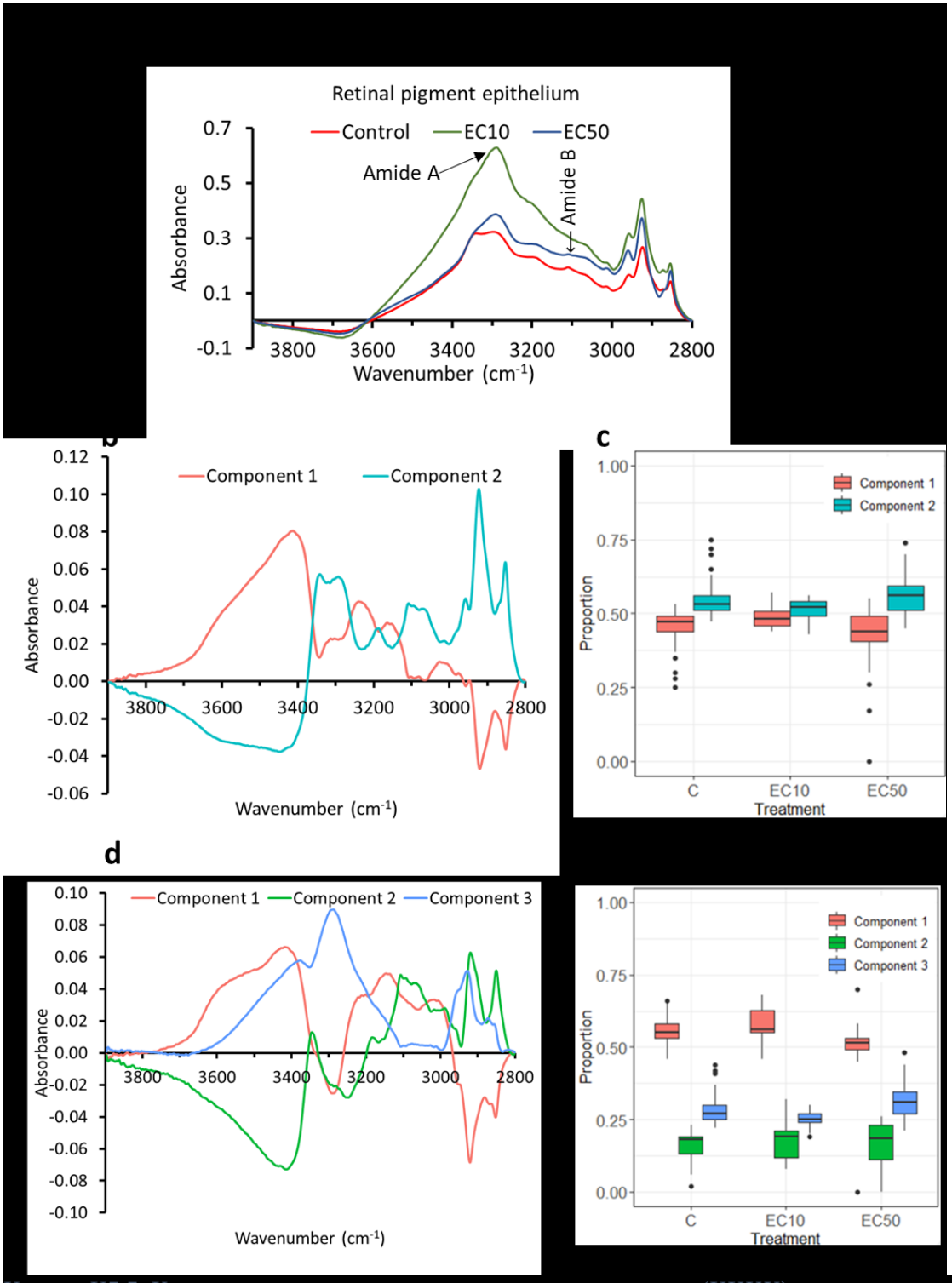

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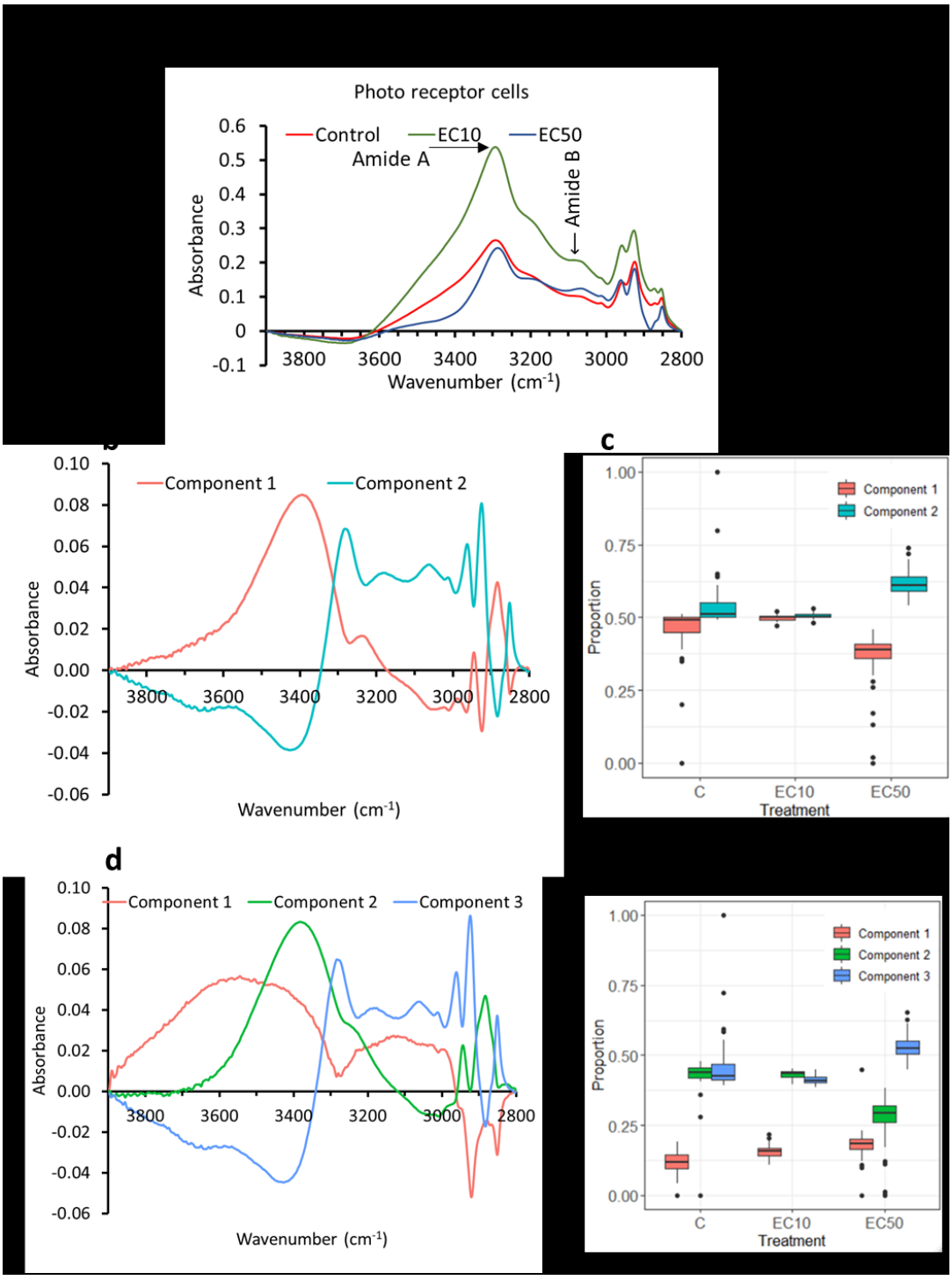

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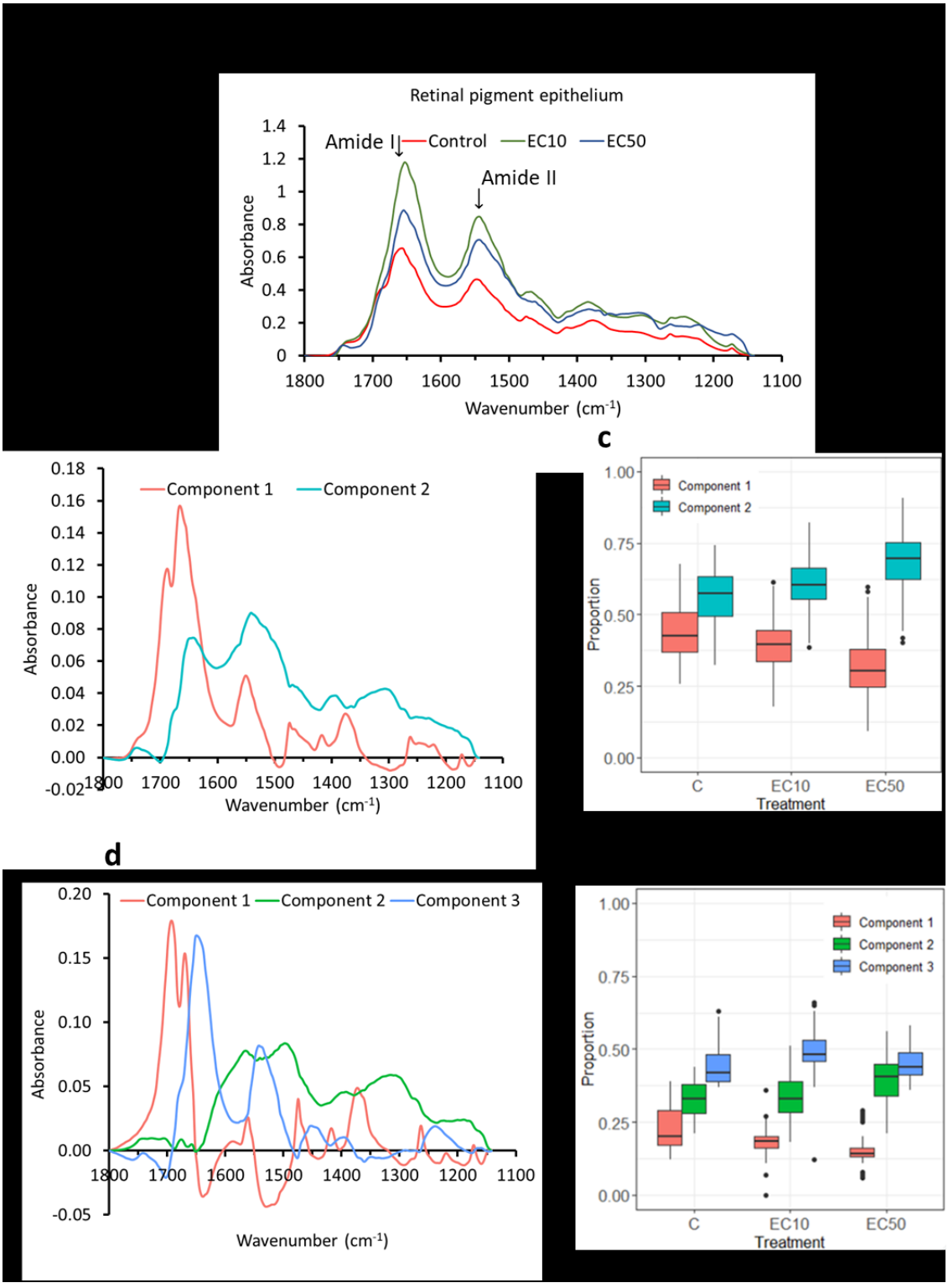

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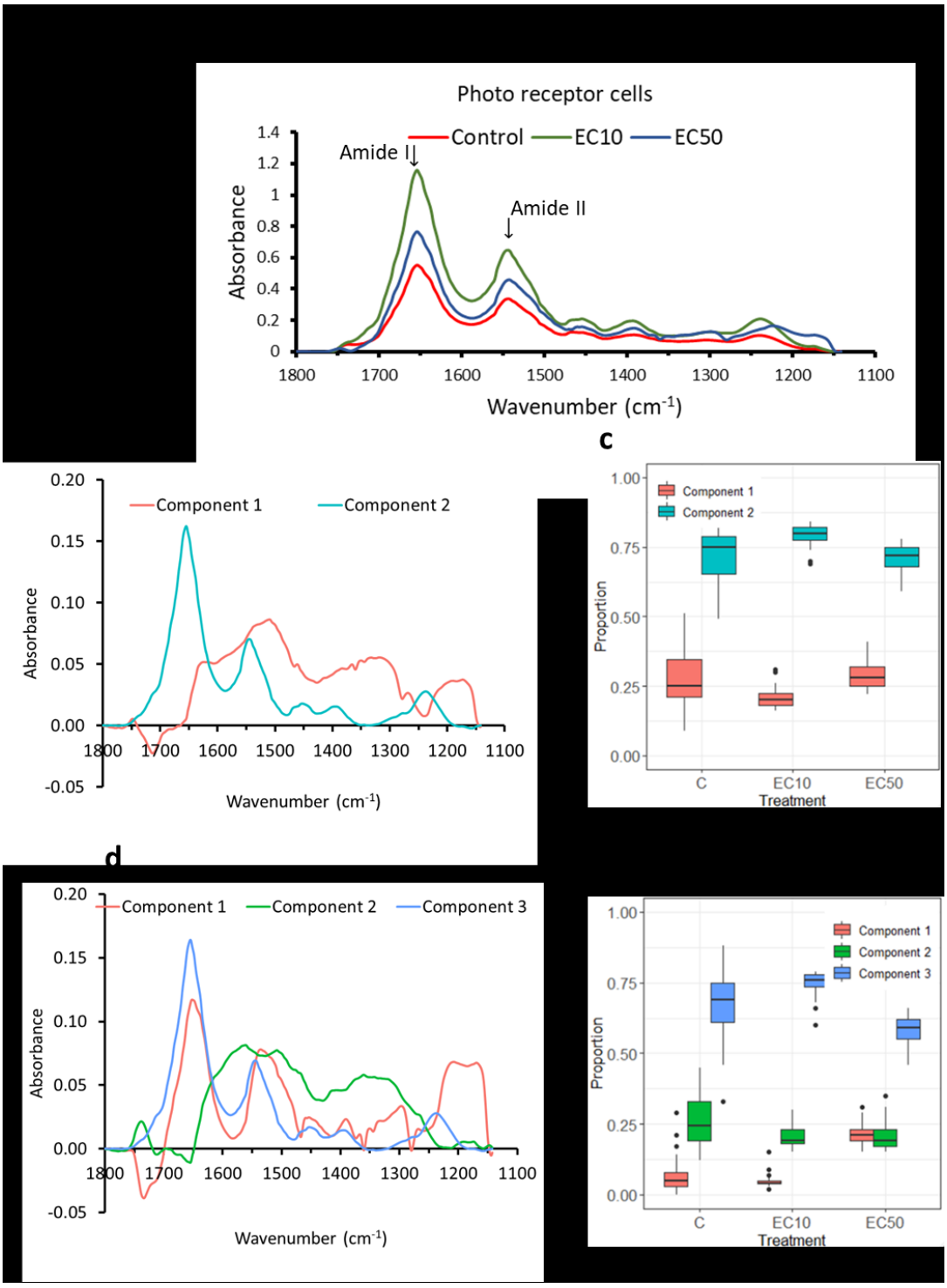
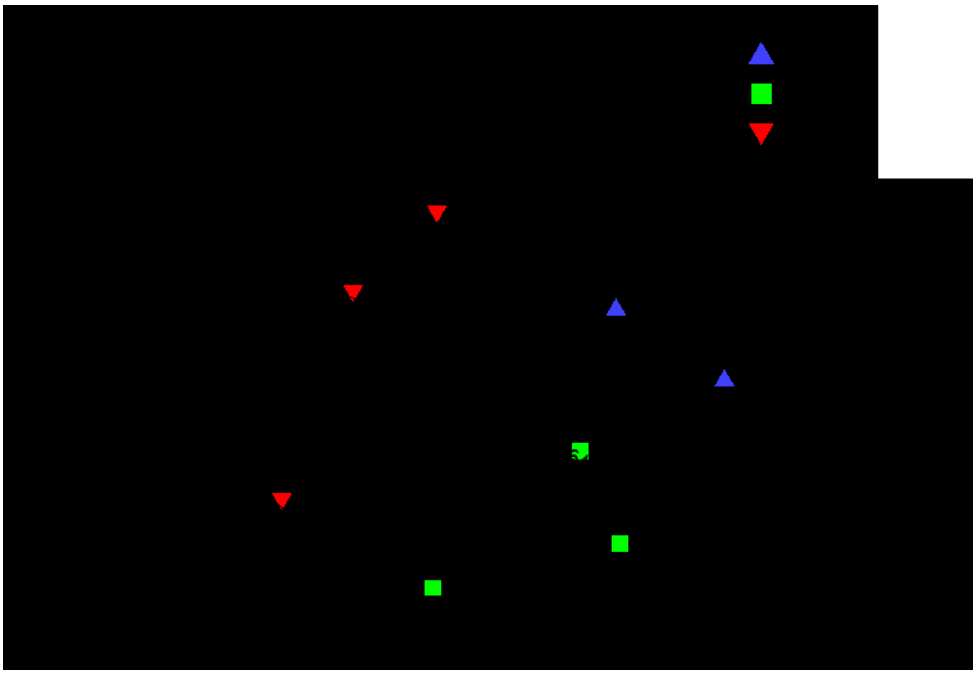


Xxxxx X5.8. Xxxxxx-xxxxxxxx xxxxxxx xxxxxxxxx xxxxxxx (XXXX) xxx xxxxxxxxxxx xxxxx xxxxxxxxx (XXX) xxxxxxx xxx xxx 1800 – 1140 xx^{-1} xxxxxx xx xxxxx xxxxxxx xxxxx (XXX) xx Xxxxxx-xxxxxxx Xxxxxxx (XXX); **x**. Xxxxxxx XXXX xxxxxxx xxxxxxxxx xxxx XXX xx xxxxxxx, XX_{10} xxx XX_{50} Xx xxxxxxx Xxxxxx-xxxxxxx Xxxxxxx (XXX); **x** xxx **x**. 2-xxxxxxxxx xxxxxxx xxx 3-xxxxxxxxx xxxxxxx, xxxxxxxxxxx, xxxxxxx xxxx XXX xxxxxxx; **x** xxx **x**. Xxxxxxx xxxxxxx xx xxxxxxxxxx xx xxxx xxxxxxxx xxx xxx 2 xxx 3 xxxxxxxx xxxxxx, xxxxxxxxxxx.



Xxxxxx X5.9. Xxxxxxxxx xxxxxxxxx xxxxxxxx (XXX) xxxx xxxxx xx xxx xxxxxxxxx xxxxx xxxxxxxx xxxxxxxxxxxxx (xXXX xxxxx) xx xxxxxxxx xx Xxxxxx-xxxxxxx xxxxxxx-*Xxxxxxxx xxxxxxxx* xxxxxx xxxxxxx xxx xxxxxxxxxx xx xxxxxxxxxx xxxxxxx, XX_{10}, xxx XX_{50} xxxxx xx xxx xxxxxxxxx xxxxxxx. Xxxxxxx xxx xxxxx xxxx xxx xxx xxxxxxxx xxxx xxxxxxxxxxx xxxxxxxxxxx > 0.6. Xxxxxxx xx xxxxxxx xxxx xxx xxxxx xx Xxxxxxxxxxxx Xxxxx X5.8.

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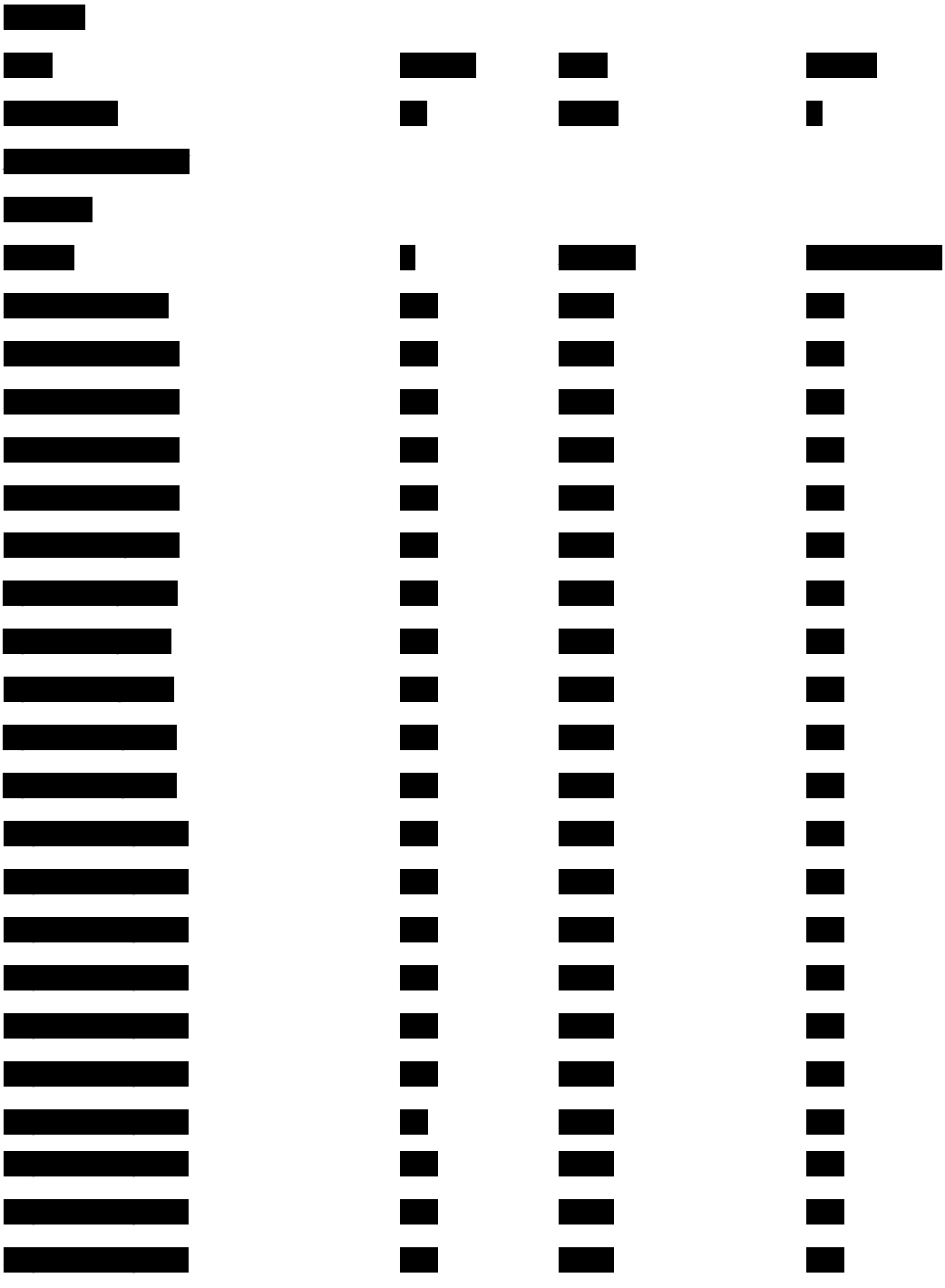
Xxxxx X5.5. Xxx xxxx ± XX xxxxxxxx xxxxxxxxx (xxx%) xx 17 xxxxx xxxxx xxxxxxxxxx xx Xxxxxx Xxxxxxx Xxxxxxx xxxxxx xxxxxxx xx xxxxxxxxx Xx xxxxxxxxxxxxxx xxx 96 x. Xxxxx xxxxx xx xxxx xxx xxxxxxxxx (XXXx) xxx xxx xxxxxxxxx xxx-xxxxxxxxx (XXXXx). Xxxxx xxxxx xxxx xxxxxxxxxxxx xxxxxx xxxxx xxxxxxxxxx xxxxxxxxxx xxxxx Xxx-Xxx XXXXX xxxxxxxx xx Xxxxx XXX xxx xxxxxx xx ($x < 0.0001$***, $x < 0.001$**, $x < 0.01$*, $x < 0.05$•). x = xx xx xxxxxxxxxx xxxxxxxx.

Xxxxx X5.6. Xxxxxxxxxxxx xxxxxxxxxxx xxxxxxx xx xxxxxxx (XXXXXXXX) xxxxxxx xxxxxxxxxx xxx xxxxx xxxx xxxxxxx xx xxxxxx xx Xxxxx-xxxxxxx Xxxxxxx xxxxxxx xx xxxxxxxxx Xx xxxxxxxxxxxxx xxx 96 xxxxx.



Xxxxx X5.7. Xxxxxx xx xxxxxxx xxxxxxxx xxxxx Xxxxxxx-xxxxxxxxx xxxxxxxx xxxxxxxxx xxxxxxxxx xxxx xxx xxxxxxxxx xxxxxx xx xxxxxxx xxxxxx xxxxxx xxxxxxx, XX_{10} xxx XX_{50} Xx xxxxxxxxx xxxxxx.

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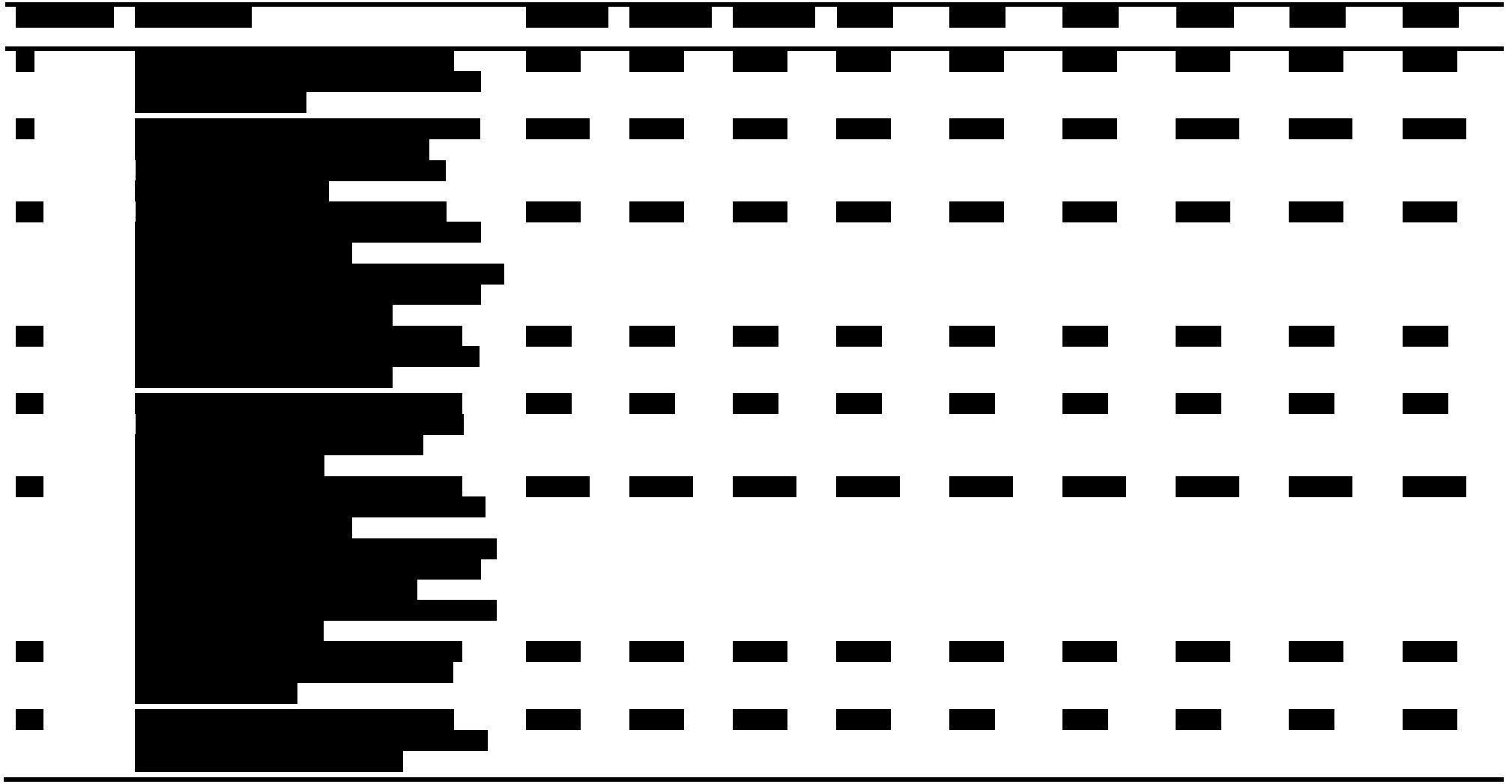
Xxxxx X5.8. Xxxxxxxxx (XXXX xxxxx) xx xxxxxxxxxx xxxxxxxx xx xxxxxx xx Xxxxxx-xxxxxxx xxxxxxx-*Xxxxxxxx xxxxxxxx* xxxxxxxx xx Xxxxxxxxx xxxxx xxxxxxxx xxxxxxxxxxxxx (xXXX) xxxxxx.



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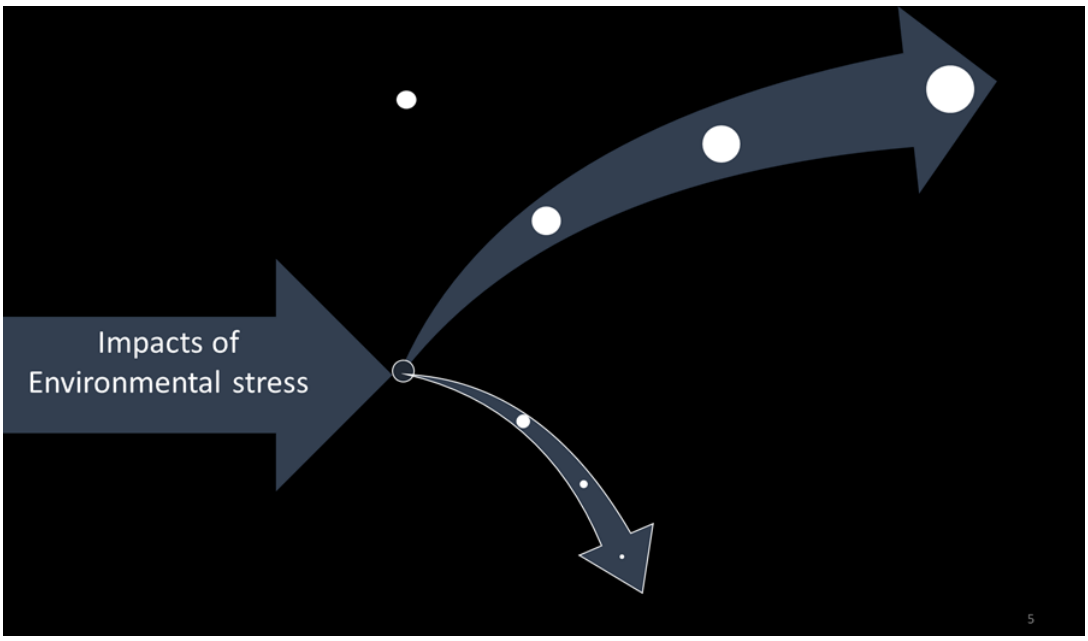

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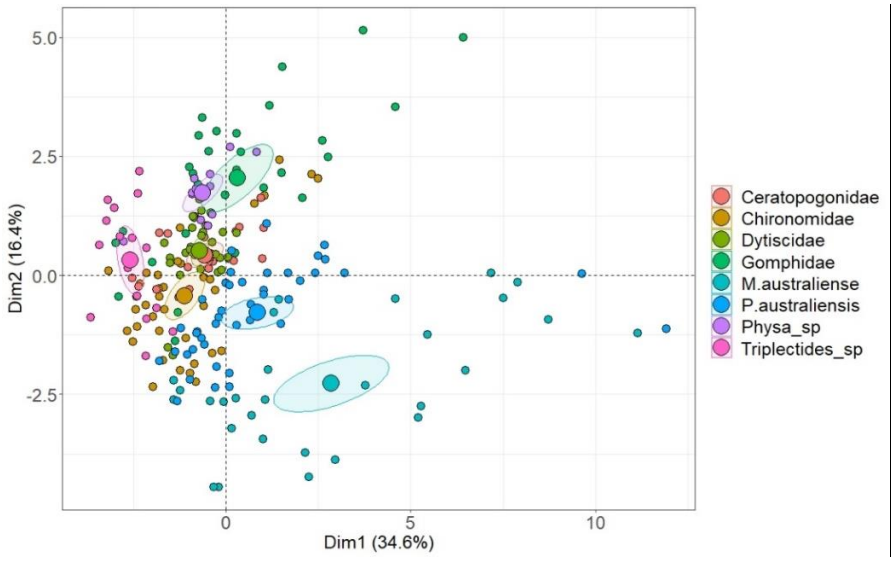
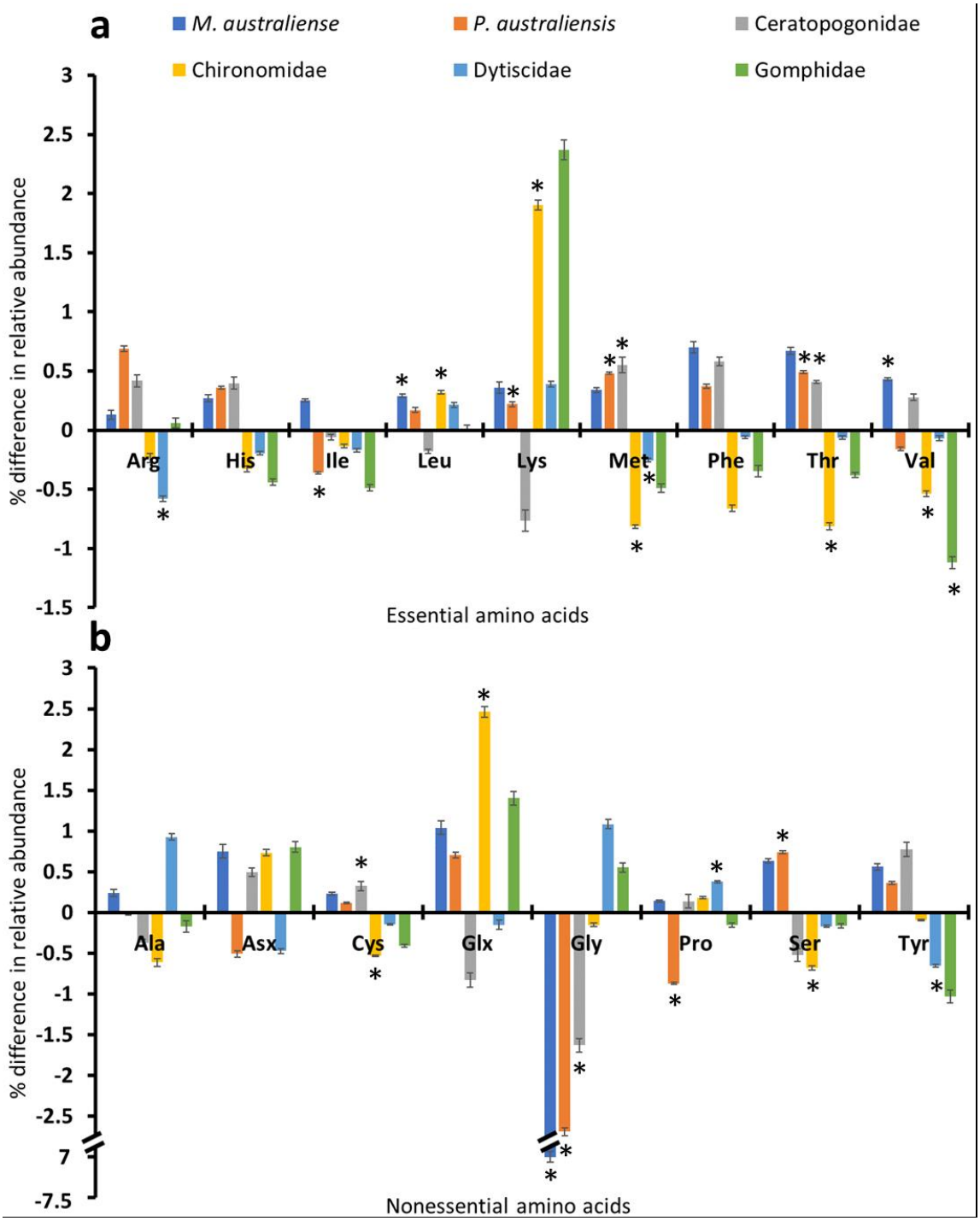


Xxxxxx 6.2. Xxxxxxxxx xxxxxxxxx xxxxxxxx (XXX) xxxxx xx xxx xxxxx xxxx (XX) xxxxxxxx (xxx%) xx xxx xxxxx xxxxxxxxxxxxxxxx xxxx. *Xxxxxxxxxxxx xxxxxxxxxxx*, *Xxxxxxx xxxxxxxxxxxx*, *Xxxxx* xx. xxx *Xxxxxxxxxxx* xx. xxxx xxxxxxxxx xxxx xxxxx xxxxx xxxxx xxx Xxxxxx Xxxxx xxxxxx xxxxxx xxx xxxxxx xx 2015 – 2016; Xxxxxxxxxxx, Xxxxxxxxxxxxxx, Xxxxxxxxxx xxx Xxxxxxxxx xxxx xxxxxxxxx xxxx xxxxx xxxxx xxxxx x xxxxxxxxxxxxx xxxxxxxx xx xxxx xxxx xxxxxxxx xx xxx Xxx Xxxxx; Xxxxxx xxxx xxxxxxxxx xxxx xxxx 95% xxxxxxxxxx xxxxxxxx xxxxx xx xxx xxxxxxx.


a
M. australiense
P. australiensis
Ceratopogonidae
Chironomidae
Dytiscidae
Gomphidae
% difference in relative abundance
Arg
His
Ile
Leu
Lys
Met
Phe
Thr
Val
Essential amino acids
b
% difference in relative abundance
Ala
Asx
Cys
Glx
Gly
Pro
Ser
Tyr
Nonessential amino acids

Xxxxxx 6.3. Xxxxxxx xx xxx xxxxxxxx xxxxxxxxx xx xxxxx xxxxx (XX) xx xxxxxxxxxxxxxxxxxx xxx xx xxxxxxx (xxxxxx) xxx xxxxxxxxxxxxx (XXX) xxxxxx. **x**. Xxxxxxx xx xxxxxxxxx XXx; **x**. Xxxxxxx xx xxx-xxxxxxxxx XXx. *Xxxxxxxxxxxx xxxxxxxxxxx* xxx *Xxxxxx xxxxxxxxxxxx* xxxx xxxxxxxxx xxxxxx xxxxxx xxx xxxxxx xx 2015 – 2016 xxxx xxx Xxxxxx Xxxxx. Xxxx xxx *X. xxxxxxxxxxx* xxx *X. xxxxxxxxxxxx* xxxxxxxxx xxx xxxxxxxxxx xx xxx xxxx xxxxxxxx xxxxxxxxx xx xxxxxxxxxx XX xx xxxxxx xx xxxxxx. Xxxxxxxxxxx, Xxxxxxxxxxxxx, Xxxxxxxxx xxx Xxxxxxxx xxxx xxxxxxxxx xxxx xxx xxxxxxxx xxxxx xxx xxxxx xxxxxxxx xxxxx xxxxx xx xxxx xxxx xxxxxxxx xxxxxxxxxxxxx xxxxxxxx xxxxxx xxx Xxx Xxxxx. Xxxx xxx xxxxx xxxx xxxxxxxxx xxxxxxx xx xxxxxxxx xxxxxxxxx (xxx%) xx xxxxxxxxxx XX xx xxxxxxxxxxx xxxxx xx xxxxxxxx xxxxx. XXx xxx xxxx xxxxx xxxx xxxxxxx xxxxxxxxxxxx xxxxxxx xxxxxx-xxxxxx xx xxxxxxxx-xxxxxxxx xxxxx xx xxxxxxx xx * xx xxx xx xxxx xxx.

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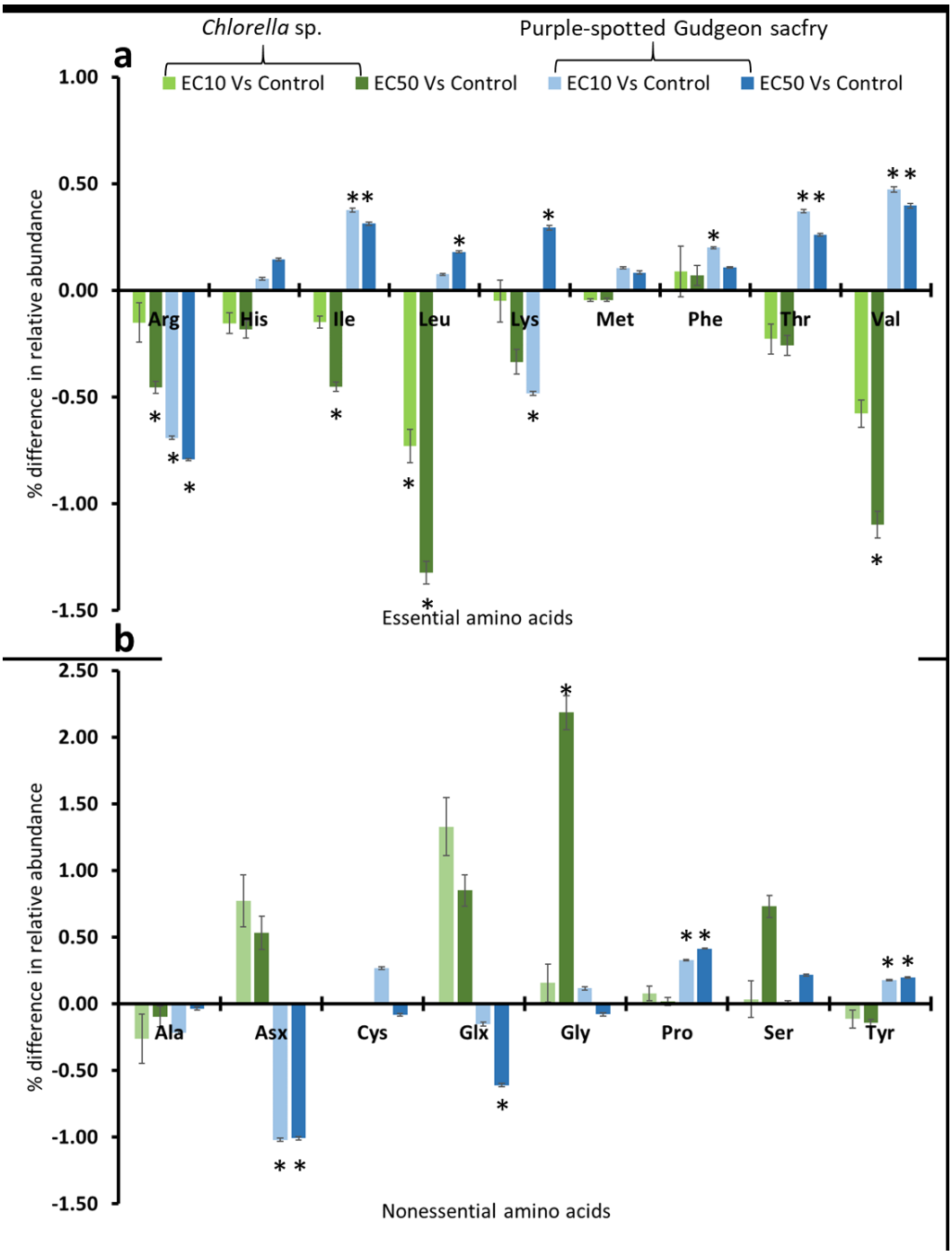
a
Chlorella sp.
Purple-spotted Gudgeon sacfry
EC10 Vs Control
EC50 Vs Control
EC10 Vs Control
EC50 Vs Control
1.00
0.50
0.00
-0.50
-1.00
-1.50
% difference in relative abundance
Arg
His
Ile
Leu
Lys
Met
Phe
Thr
Val
Essential amino acids
b
2.50
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0.50
0.00
-0.50
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-1.50
% difference in relative abundance
Ala
Asx
Cys
Glx
Gly
Pro
Ser
Tyr
Nonessential amino acids

Xxxxxx 6.4. Xxxxxxx xx xxx xxxxxxxx xxxxxxxxx xx xxxxx xxxxx (XX) xxxxxxxxx xx xxx% xx *Xxxxxxxxx* xx. (xxxxx xxxx) xxx Xxxxxx-xxxxxxx Xxxxxxx (*Xxxxxxxx xxxxxxxx)* xxxxxx (xxxx xxxx). Xxxx xxx xxxxxxxxxx xxx xxxxxxxxxx xx xxxxxxxx xxxxxxxxx xx xx xxxxx xxxx xx x xxxxxx (Xx) xxxxxxxxx xxxxxxxx xxxx xxxxxxxx **x.** Xxxxxxx xx xxxxxxxxx XXx; **x.** Xxxxxxx xx xxx-xxxxxxxxx XXx.

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xxxxx://xxx.xxx/10.1016/x.xxxxxxxxxxx.2020.736277

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