Assessing the sub-lethal effects of copper, cadmium, pentachlorophenol and 3,4-dichloroaniline on Freshwater rotifers *Brachionus calyciflorus* using the energy budget biomarkers

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ABSTRACT

Chemical pollutants can impact aquatic organisms at different levels of biological organisation, so an understanding of effects at these different levels is important in the assessment of impacts. At the cellular level, the Cellular Energy Allocation (CEA) approach is well established to evaluate energy budget in response to chemical stress. The hypothesis is that toxicants elicit metabolic changes in stressed organisms, depleting their energy reserves and, subsequently, causing adverse impacts on reproduction and growth. However, the CEA is currently limited to very few model aquatic species. Rotifers are potentially suitable models. Having one of the fastest reproductive rates within the metazoans, they are crucial components of aquatic food webs, connecting lower with higher trophic levels. This thesis applied the CEA approach to assess the toxicity of Cu, Cd, PCP, and 3,4-DCA to the freshwater rotifer *Brachionus calyciflorus*. Firstly, acute and chronic (population density/growth) toxicity tests were conducted to determine LC_{50}, LOEC, and NOEC and define concentration ranges for CEA assessment. Cu and 3,4-DCA (LC_{50} 0.012 and 123.8; NOEC 0.0024 and 6.5 mg/L), respectively, showed the highest and lowest toxicity. Analytical methods were then developed to measure cellular energy reserve components (Ea: carbohydrate, protein, and lipid) in *B. calyciflorus*. For the gradient of sub-lethal concentrations assessed relative to the control, Cu and Cd had no effect, but PCP and 3,4-DCA significantly reduced Ea, especially under the highest exposure concentrations. Given time and logistical constraints, consumed energy (Ec) was interpolated from regression models of Ec against Ea (both as % of control) based on published values across different marine taxa (daphnids, enchytraeids, and microalgae). The relevant CEA was then calculated for each toxicant, decreasing under both heavy metal and organic exposures relative to concentrations. Overall, this thesis validates the suitability and sensitivity of CEA for the assessment of chemical stress in rotifers

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ABBREVIATIONS

3,4DCA	**3,4-dichloroaniline**
AEC	Adenylate energy charge
ANOVA	Analysis of Variance
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CBBG	Coomassie brilliant blue G-250 dye
Cd	Cadmium
CEA	Cellular energy allocation
Cu	Copper
DEB	Dynamic energy budget theory
DFERA	Department for Environment Food and Rural Affair
DMSO	Dimethyl sulphoxide
DO	Dissolved Oxygen
Ea	Available energy
EA	Environment agency
Ec	Consumed energy

EC	European Commission
EQS	Environmental quality standards
ETS	Electron transport system
h	Hours
LC_{50}	The concentration of metal lethal to 50% of test population
LOEC	The lowest observed effect concentration
LT50	The median lethal time for a test population exposed to a defined concentration of metal
NOEC	No observed effect concentrations
NR	Nile Red
Pb	Lead
PBST	Phosphate-buffered saline with Tween 20
PC	Phosphatidylcholine
PCP	Pentachlorophenol
PD	The population density

PPCPs	Personal care products and pharmaceuticals
r	The intrinsic growth rate
SFG	Scope for growth
SPSS	Sigma Stat software (SPSS)
SPV	The Sulfo-Phospho-Vanillin (SPV)
TN	Total nitrogen
TP	Total phosphorus
UPW	Ultrapure water
USEPA	United States Environmental Protection Agency
WFD	Water Framework Directive
Zn	Zinc

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CHAPTER ONE

Chapter 1: Introduction

1.1 Overview

The negative impact of industrialisation in the last century, with the global use of oil, coal, and sewage systems, is clear (Moiseenko, 2008; Nikinmaa, 2014). High amounts of chemical pollutants were introduced into the aquatic environment at different national and international levels (Schwarzenbach et al., 2006). As a result, the conservation of natural resources became an urgent necessity, and it was important to understand anthropogenic substances, their path of movement and form of transportation, the way they interact within different natural ecosystems, as well as their effects on living organisms at different levels of biological organisation (Moiseenko, 2008). To face this challenge, a new branch of science was introduced through the integration of several science disciplines: ecotoxicology.

Ecotoxicology combines two different branches of science – ecology and toxicology – in an integrative way (Chapman, 1995; Baird et al., 1996), with the collaboration of different science disciplines. Chemistry, biology, geochemistry, medicine, and pharmacology all play a part in evaluating the impacts of toxic stress on living organisms(Moiseenko, 2008). Ecotoxicology focuses on the impact of environmental chemicals on living organisms through the study of the different responses at different levels of biological organisation, from molecules to populations and ecosystems (Fent, 2001).

By leaving their target sites or transforming into an unsafe derivative, anthropogenic substances become environmental toxicants (Anderson, 1995). Surface water works as natural reservoirs for the effluents of toxic substances and their distribution flows (Moiseenko, 2008). Although this discharge of chemicals is mainly from terrestrial sources, there are also several aquatic sources (Tornero and Hanke, 2016). Through the use of different qualitative and quantitative analyses, aquatic ecotoxicology studies the effects of toxicants on aquatic organisms, as well

as the fate of these chemicals, including their transformation into sediments and through the food chain (Rand and Petrocelli, 1985).

This thesis is concerned with the impacts of two important classes of chemical toxicants in aquatic systems: heavy metals and pesticides. Effects are assessed on the freshwater rotifer *Brachonus calyciflorus* as a model study organism, using energy budget biomarkers as endpoints. In this chapter, the concept of ecotoxicological biomarkers is introduced, with special emphasis on energy budget and bioenergetics markers as applied throughout this thesis. Furthermore, the use of rotifers in ecotoxicological assays is justified. An overview of the particular toxicants used is provided, and their modes of action are compared. The chapter concludes with an overview of the full thesis, detailing the aims and objectives to be addressed.

1.2 Ecotoxicological biomarkers

A range of tools are available for ecotoxicological assessments, selected based on factors such as the objective of the analysis, type of toxicant, time of exposure, and organisms studied (Connon et al., 2012). These tools used for the prediction of ecotoxicological impacts of chemicals on the aquatic environment and provided information for studies taking place under controlled experimental conditions or in the field. Examples of these tools include the risk assessment test of lethal and sublethal effects of acute and chronic exposures, biomarkers, as well as some new approaches, such as transcriptomics, proteomics, metabolomics, and epigenetics (Connon et al., 2012).

Biomarkers are highly sensitive indicators for toxicants and their use in ecotoxicology is very promising as an alternative or complement to traditional assessment tools. The necessity for more sensitive early-warning signals in ecotoxicological studies led to their development as ecotoxicological assessment tools (Martinez-Haro et al., 2015). According to Depledge (1994), a biomarker can be defined as a "biochemical, cellular, physiological or behavioural variation

that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants". Due to their high sensitivity, biomarkers have been widely used in ecotoxicological and risk-assessment studies, and this use has been widely reviewed (Hagger et al., 2006; Picado et al., 2007; Schettino et al., 2012; Connon et al., 2012; Martinez-Haro et al., 2015; Schuijt et al., 2021).

1.3 Energy budget and bioenergetic markers

Metabolic responses are increasingly used as markers for chemical stress. Energy metabolism is a key factor for survival, function, adaptation to stress, and resistance to changes in ambient conditions (Sokolova et al., 2012). Therefore, extreme disturbances in an organism's habitat, such as pollution, increase the energy expenditure of stressed animals (Moiseenko, 2008). This additional energy expenditure is the direct cost of recovery and maintenance of homeostasis by stressed individuals, affecting their energy gain, transformation, and storage systems (Sokolova et al., 2012). The availability of energy and its acquisition, conversion, and reservation capacities are limited for any organism. Therefore, the organism's fitness is mainly dependent on its ability to regulate energy expenditure (Sokolova et al., 2012).

Several methodologies have been used to assess the effects of pollution stress on energy metabolism and energy reserves in aquatic organisms, such as adenylate energy charge (AEC), physiological energetic responses translated to scope for growth (SFG), dynamic energy budget theory (DEB), and cellular energy allocations (CEA). These strategies and methodologies are based on energy homeostasis concepts and bioenergetic approaches (Sokolova, 2013) and are discussed in more detail in the following headings.

1.3.1 Adenylate energy charge (AEC)

Although acute toxicity tests can evaluate the effects of pollutants on test organisms in the laboratory, they do not take into account the delayed effects of chronic exposure to pollutants,

which impacts organisms with less resistance and can result in mortality. It was necessary, therefore, to find more sensitive tools to evaluate the effects of pollutants on living organisms.

The use of the adenylate energy charge (AEC) method is an example of this approach (Sylvestre and Le Gal, 1987). The AEC method showed good sensitivity to stress and reliability in short-term bioassay tests; however, it was argued that the field monitoring of AECs in invertebrates was not valuable as a pollution-assessment tool, because only pollution-resistant species would be found (Verschraegen et al., 1985). As explained by Sokolova (2013), ATP stores only get depleted when organisms are severely stressed, while the animals tend to accelerate ATP production for biological maintenance and adaptation under moderate stress (Sokolova et al., 2012). In short-term bioenergetic availability tests, the organism is exposed to sudden stress, so that the changes in AECs become more sensitive and representative. Although the AEC method and the adenylate nucleotide ratios have been used as biomarkers for evaluating the stress responses in different organisms, there are doubts about their physiological implications for metabolism at the cellular level (Isani et al., 1997; Thébault et al., 2000; Hardie and Hawley, 2001).

1.3.2 Scope for growth (SFG)

Analysis of physiological responses that control energy budgets to determine the available energy for growth has been reported as a useful assessment tool for the biological impacts of pollution (Widdows, 1983; Widdows et al., 1987). The measurement of the available energy for growth, termed scope for growth (SFG), demonstrates the integration of various types of cellular responses resulting from ecological consequences at higher levels of biological organisation (Widdows and Johnson, 1988).

Over the past two decades, several studies have used the SFG approach to evaluate the effects of chemical pollution on aquatic organisms (Widdows et al., 1995; Sobral and Widdows, 1997;

Verslycke et al., 2004a; Munari and Mistri, 2007), which have shown a direct relationship between SFG reduction and exposure to pollutants. More recently, the SFG approach has been used in studies to assess the effects of different environmental stressors (e.g., temperature, salinity, hypoxia, and ocean acidification) (Wang et al., 2011; Guzmán-Agüero et al., 2013; Sanders et al., 2014; Pedersen et al., 2014; Zhang et al., 2015, 2016). Despite the success of the SFG approach in evaluating the effects of environmental stress in these studies, the research was mostly limited to molluscs,bivalves in particular, and trying to apply the same parameters to a complex, wide range of invertebrate organisms could prove problematic.

1.3.3 Dynamic energy budget theory (DEB)

In 2010, Kooijman proposed quantitative bioenergetic approaches, called dynamic energy budget (DEB) models. These models were applied to link the bioenergetics of individuals to the population growth in different animals by integrating a variety of environmental causes of stress, such as toxic pollution. Although these models have proved to be useful in estimating the consequences of bioenergetic shift at higher ecological levels, they are also very complex, and both time and effort-consuming, due to the high degrees of parameterization (Sokolova et al., 2012). As a result, they do not seem to have been used very much, possibly because "none of the state variables can be measured directly" (Sousa et al., 2010).

1.3.4 Cellular energy allocation

Changes in energy reserves only – mainly carbohydrates and lipids – have been used to evaluate the effects of chemical pollution stress on aquatic organisms (Donker, 1992; Leung and Furness, 2001; Durou et al., 2007; Villarroel et al., 2009), where the changes in energy reserves were linked to the stress status. Feeding and respiration rates (and sometimes other components of the energy budget) can also be measured to determine the net available energy by calculating the gained (stored) and consumed energy in the form of direct oxygen consumption (RO_2).

The theory of cellular energy allocation (CEA), expounded by De Coen and Janssen (1997, 2003) and De Coen et al. (2001), was introduced as a biological equivalent or biochemical alternative approach to the scope for growth, to assess the toxic stress of pollutants on aquatic organisms. This approach quantified the available stored energy and consumed energy, where the storage of carbohydrate, protein, and lipid is compared with respiration rate, as shown by the electron transport system (ETS) activity at the cellular level (De Coen and Janssen, 2003). The cellular energy budget, expressed as CEA, is calculated as the difference between stored energy and consumed energy by integrating the changes in energy budgets over exposure time (De Coen and Janssen, 1997).

Sokolova (2013) suggested that the reduction of energy reserves is related to moderate stress while its depletion is a mark of severe stress. Several studies have used the CEA to assess the impacts of sublethal concentrations of toxicants (moderate stress). Some studies have validated the high *in situ* sensitivity of CEA to toxic responses compared with SFG (Smolders et al., 2004; Verslycke et al., 2004a, b), which have been followed by successful application of CEA approach in studies of pollutant impacts on invertebrates (Moolman et al., 2007; Erk et al., 2011; Novais et al., 2013; Gomes et al., 2015a, b). Most of these studies confirmed the high sensitivity of CEA to exposure to pollutants and its ability to be linked with higher levels of biological organization. Because food supply is essential for energy uptake by organisms, most CEA studies provided food during exposure times (De Coen and Janssen, 1997, 2003; Soetaert et al., 2007; Novais and Amorim, 2013; Novais et al., 2013), although a few did not feed the tested organisms (Verslycke and Janssen, 2002; Beaumelle et al., 2014). Recently, Gomes et al. (2016) did not recommend food and no-food comparison studies but recommended stopping feeding during exposure (if possible) to improve the sensitivity of chemical effects studies.

1.3.4.1 Scopes of oxygen consumption

Sokolova (2013) and Sokolova et al. (2012) emphasised the role of respiration scopes in identifying the tolerance limits of stress and metabolic adaptation mechanisms in aquatic organisms. In optimal conditions, with no environmental stressors, the animal demonstrates maximum energy allocation and high aerobic scope, providing more energy for growth, reproduction, and deposition. In the case of environmental stress, there are changes in the metabolic processes as an adaptation for survival, thus the aerobic scope decreases, or even stops, and affects the organism's fitness by delineating the energy flux allocated to production and activity. Along with the SFG and DEB approaches, which integrate the measurement of oxygen consumption with other energy budget components into one model as an indicator of environmental stress, oxygen consumption (VO_2) alone has been the target of several studies (Dietz, 1974; Feder, 1981; Zanders and Rodríguez, 1992; Pillai and Diwan, 2002; Vutukuru, 2005; Vijayavel and Balasubramanian, 2006; Melatunan et al., 2011), connecting the sensitivity of oxygen consumption to ecological stress or disturbance.

Although the SFG and DEB models have been successful in measuring the scope of aerobic respiration by calculating many elements of the energy budget related to production, growth, excretion, and gained and consumed energy, these models do not apply to all organisms due to limitations in the measured parameters. Alternatively, the monitoring of the reduction or depletion of energy reserves is considered an indirect way to assess the aerobic scope (Sokolova, 2013). The CEA approach overcomes the problems inherent in the SFG and DEB models by including the quantification of energy consumption (respiratory activity) as ETS activity, which can be measured at the cellular level, for the evaluation of toxic effects on a wide range of organisms.

1.3.4.2 Electron transport system (ETS)

In 1948, Lehninger and Kennedy discovered that oxidative phosphorylation takes place in the mitochondria (Nelson and Cox, 2008). Since then, many studies have been developed, and many discoveries made, to describe the electron flow during this process, originally called the electron transport chain (Keilin, 1966). In the 1970s, it was determined that because it was more complex than a chain, it should be considered the electron transport system. This system is present in the mitochondrial membrane of eukaryotes and the cell membrane of prokaryotes, sharing, in general terms, the mechanism of the flow of electrons to produce ATP (Nelson and Cox, 2008).

The ETS consists of a series of transporters of electrons that generate a flow of protons, which produces an electrochemical potential across the inner mitochondrial membrane, positive in the internal part and negative in the mitochondrial matrix, which then will boost the synthesis of ATP as the ultimate purpose of this system. This method can be used to measure and evaluate various parameters, such as primary production, fluxes of carbon in the ocean, nutrient retention efficiency, and heterotrophic energy production (Packard et al., 1988; Packard and Gomez, 2013; Osma et al., 2014; Packard et al., 2015), and the impact of toxic pollutants in the CEA approach as mentioned above.

Most methods used to determine the respiratory oxygen consumption rate (RO_2) in incubated organisms are complex and time-consuming. It is difficult to apply the same natural conditions, and errors of manipulation cannot be avoided (Herrera et al., 2011; Osma et al. 2016a). To measure respiration as oxygen consumption, organisms are incubated in a sealed container. After some time of incubation, the oxygen concentration will have decreased due to respiration.

The most common method used to determine this decrease in oxygen for incubated organisms is the Winkler titration, as modified by Strickland and Parsons (1968). It involves determining

the oxygen concentration at the beginning and end of the incubation, and the difference between them is interpreted as the amount of oxygen consumed by the organism in the time interval. Other techniques and instruments were developed to measure oxygen concentration. Oxygen electrodes, with the Clark-type electrode (Clark, 1956) the most common, calculate the oxygen concentration depending on an electrochemical reaction. The measurement of oxygen concentration with optodes is based on the ability of oxygen to alter the fluorescence emission of certain dyes (oxygen-quenchable fluorophores), so optodes calculate oxygen concentration via fluorescence quenching (Lubbers and Opitz, 1985; Opitz, 1986).

Indirect methods were developed to overcome the problems of incubation. These methods were based on the enzymology behind respiration, such as the ETS method that was developed in the early 1970s (Packard et al, 1971) and applied in marine zooplankton by Owens and King (1975). It led to a series of improvements in the ETS method and its application in many studies, especially in establishing the relationship between ETS and oxygen consumption (King and Packard, 1975; Finlay et al., 1983; Arístegui and Montero, 1995).

The method commonly used today (Packard et al., 2004; Gomez et al., 1996) was designed as an index of respiration by all organisms living in a given volume of seawater (Packard et al., 1971) and to reduce the dependency on time-consuming methods for the estimation of the respiratory activity. This method uses basic precepts of enzymology: enzyme activity is measured at the maximum level (V_{max}) and pyridine nucleotide substrates (NADH and NADPH, and sometimes succinate) are required at saturation to provide the necessary sensitivity to the Complex I and Complex II of the ETS and stimulate the ETS activity to full capacity. As a result, the potential respiration (ϕ) can be calculated (Maldonado et al., 2012).

1.4 Rotifers in ecotoxicology

Aquatic systems support a vast diversity of invertebrates and planktonic species, which can be impacted by metal and organic pollution. Amongst them, rotifers occur in high densities relative to other zooplanktons, mainly because of their rapid reproduction rate (Herzig, 1987). Rotifers prey on primary producers, such as algae and cyanobacteria (Rico-martínez et al., 2016; Dodds and Whiles, 2020; Gilbert, 2020). They, in turn, serve as prey for several organisms at higher trophic levels, including insects, copepods, and larval fish (Wallace and Snell, 2001; Kostopoulou et al., 2012; Dodds and Whiles, 2020). As a result of these food web linkages, rotifers play an important role in the flow of energy between different trophic levels in aquatic ecosystems (Wallace et al., 2006) and are, therefore, important study organisms.

Snell and Persoone (1989) described some of the persistent problems relevant to using standard invertebrate test animals in ecotoxicological bioassays, such as variations in water and food quality, difficulty in standardisation of physiological conditions, high cost, long periods of acclimation to culture conditions, and genetic variation during stock cultures. To overcome these problems, Snell and Persoone proposed the use of invertebrates that form dormant eggs (cysts) as an alternative to the livestock culture of invertebrates. They outlined a protocol for producing neonates from *B. plicatilis* cysts and used them in toxicant bioassays of the marine environment. At that time, rotifer cysts had only just started to be used for toxicity testing (Snell and Hoff, 1986). Ecotoxicological studies have continued with the use of rotifers, ever since.

Rotifers offer several advantages as a model organism for ecotoxicological assessments. They have a rapid rate of reproduction, short life with multiple life stages and they can be grown in a lab setting and don't come under the same stringent regulations as fish and, as such, have been recommended as appropriate test organisms in experimental studies (Snell and Janssen, 1995; Snell and Joaquim-Justo, 2007; Dahms et al., 2011). These advantages have enhanced the use of rotifers as a model organism for ecotoxicological studies (Kaneko et al., 2005;

Dahms et al., 2011; Moreira et al., 2016; Won et al., 2017). Also, the characteristic feature of rotifers in producing dormant cysts has facilitated the production of commercial toxicity kits to be used for acute and chronic toxicity tests: Rotoxkits (http://www.microbiotests.be) (Moreira et al., 2016). Rotifer cysts have the added advantage of being easy to collect, stock, and ship and so are readily distributed globally. The advantages are further detailed in the following headings.

1.4.1 Rotifer sensitivity to chemicals

Rotifers are considered sensitive primary consumers, having shown more sensitivity to some types of chemical toxicants than other common model organisms such as daphnids (Snell and Joaquim-Justo, 2007; Dahms et al., 2011; Rico-Martínez et al., 2016). Despite the existence of several studies on the impacts of organic compounds and heavy metal pollutants on rotifers, which make up a large baseline database (Dahms et al., 2011), compared with other model animals, such as daphnids, this database of toxicant screening studies are relatively few (Moreira et al., 2016). Rico- Martínez et al. (2013, 2016) screened some of these studies and illustrated the use of various rotifer species in different ecotoxicological tests, presenting their sensitivity to different organic compounds and metals using various endpoints within sublethal concentration ranges. In addition, Won et al. (2017) discussed the promising prospects of rotifers for ecotoxicological studies and summarised some of the individual and molecular endpoints. For their advantageous features in ecotoxicological studies, rotifers have been regarded as having equal or more importance than other invertebrate model organisms, including cladocerans (Dobsikova, 2004).

1.4.2 Rotifers and bioconcentration of pollutants

Rotifers are efficient organisms to bioaccumulate and biomagnify environmental pollutants (Dahms et al., 2011). As they are generalist feeders, they use their cilia to move water and trap

various food items in their natural habitats (Barnes et al., 2001), maximising the uptake of harmful toxicants from the water column during feeding (Won et al., 2017).

Being also subject to predation, rotifers are key organisms in transferring energy and recirculating nutrients between different trophic levels in aquatic food webs (Barnes et al., 2001). The transfer of toxicants from water and sediments to rotifers has been reported in some studies (Cargouet et al., 2004; García-García et al., 2007). Because rotifers can take up chemicals from both water and sediments and deliver them to higher trophic levels, they can be used to determine the flow patterns of several pollutants from one trophic level to another (Dahms et al., 2011).

However, there exist some limitations to the use of rotifers in ecotoxicological studies. Calculation of rotifer bioconcentration factors for different toxicants is not well investigated and studies on this are limited (Rico-Martínez et al., 2013). In addition, studies concerning the effects of sublethal exposure to toxic pollutants on life-history parameters are still limited (Dahms et al., 2011), even though they have large population sizes and contribute significantly to secondary production in aquatic ecosystems (Snell and Janssen, 1995; Wallace et al., 2006).

1.5 Rotifers and energy budget biomarkers

In general, there is a scarcity of studies that apply any of the energy biomarkers in rotifers. However, there are some studies relevant to changes in energy allocations that have been concerned with oxidative stress biomarkers, as reviewed by Won et al. (2017), who reported that under stress conditions , the energy required for activating antioxidant mechanisms, the balance of redox conditions, and repair DNA is compensated by energy allocated for reproduction and growth. Moreover, the content of protein, lipid, and carbohydrate was measured in some rotifer species by several studies (Ben-Amotz et al., 1987; Frolov et al., 1991; Frolov and Pankov., 1992; Carić et al., 1993), All these studies on the biochemical

composition of rotifers were conducted in normal conditions using a different diet with different concentrations to investigate the effects of different food and their concentrations, especially on the content of protein, lipid, and carbohydrate. In these studies, however, the amount of each fraction was expressed as a percent of dry mass without transformation to energetic equivalents using the enthalpy of combustion.

In terms of measuring oxygen consumption in rotifers, several studies were carried out using various methods (such as cartesian divers, polarography, and closed bottles) as reviewed by Galkovskaya (1995). The marine rotifer *Brachionus plicatilis* was the most used species, and most of these studies (about 80%) were conducted using the closed bottle method.

1.6 Toxicants examined in the current study

Aquatic organisms can be impacted by several anthropogenic stressors (Reid et al., 2020). Indeed, according to the Chemical Abstracts Service (CAS), there are about 133 million unique compounds that can be used and released into the environment (Dodds and While, 2020). Given the severity of potential impacts, pollution as a result of nutrients, pharmaceuticals, heavy metals, and pesticides have been prioritized by regulators, especially the EU Water Framework Directive (DEFRA, 2014).

Nutrients leach mainly from farmlands and result in eutrophication and potentially toxic phytoplankton blooms (Bowen and Valiela, 2001; Howarth and Marino, 2006; MPCA, 2008; Kiedrzyńska et al., 2014). Personal care products and pharmaceuticals are typically released from sewage treatment facilities and can disrupt the endocrine system of aquatic organisms (Ebele et al., 2017). Some metals such as Zn, Cu, Fe, Mn, Co, Ni, Mo, and V play an essential role as micronutrients for biological functioning in several organisms (Duffus, 2002). They are common in trace amounts in natural waters (Herawati, 2000), but can be toxic to organisms at higher concentrations (Prashanth et al., 2015). Conversely, non-essential metals such as Cd,

As, Hg, and Pb are toxic even at relatively low concentrations, as they are not useful for metabolic activities (Herawati, 2000; Fakhri et al., 2018). Pesticides, like nutrients, are also mainly leached from agricultural run-off during high rainfall. They can be acutely toxic or can impair reproductive and embryonic development, increase oxidative stress, and/or the inhibit growth and enzymatic activities (Harmon, 2009; Harmon and Wiley, 2010; Harmon and Wiley, 2012; Amoatey and Baawain, 2019).

In the current study, representative toxicants were selected from the aforementioned groups. Cu and Cd were selected as commonly occurring inorganic compounds as well as to compare the effects of an essential metal to a non-essential metal. Additionally, pentachlorophenol (PCP) and 3,4-dichloroaniline (3,4-DCA) are pesticides (organic compounds) with different chemical modes of action and were selected to compare the effects of organic chemicals with metals. Several studies have indicated that these four chemicals are toxic to zooplankton species at relatively low concentrations (Table 1.1). In the following subheadings, the toxicants are discussed in more detail and their modes of action are compared.

1.6.1 Copper (Cu)

Copper is an essential metal. As a catalytic co-factor for several enzymes and protein groups, it is required in trace amounts metabolic activities (Padrilah et al., 2018). However, high concentrations (more than 20 µg Cu/g) can be toxic to organisms (Solomon, 2009). The concentration of copper in water bodies can be greater than the background levels (natural levels) due to the large inputs of copper from terrestrial sources. Examples include the use of copper as an ingredient in pesticides for agricultural purposes, agricultural runoff, the use of copper pipes in water treatment plants, and emissions from vehicles on roadways (Lahman, 2015). In addition, copper sulphate is usually applied to kill snails or slugs and control algae in irrigation systems (Padrilah et al., 2018).

Table 1.1: Acute toxicity range in terms of 48-hour mortality or Immobilisation effects of copper, cadmium, Pentachlorophenol, and 3,4-Dichloroaniline on different groups of zooplankton.

Compounds	Zooplankton Group	Scientific name	Endpoint*	Concentration mg/L	References
Cu	*Cladocera*	*Daphnia magna*	EC_{50}/LC_{50}	0.017-0.054	(Mount and Norberg,1984; Elnabarawy et al.,1986; Versteeg et al.,1997)
	Copepods	*Notodiaptomus conifer*	EC_{50}	0.029-2.119	(Lalande and Pinel-Alloul,1986; Soto et al.,2003; Gutierrez et al., 2010)
	Rotifers	*Brachionus calyciflorus*	EC_{50}/LC_{50}	0.026-0.33	(Snell and Moffat,1992; P´erez-Legaspi and Rico-Mart´ınez,2001)
Cd	*Cladocera*	*Ceriodaphnia dabia*	EC_{50}/LC_{50}	0.066-0.424	(Mount and Norberg,1984; Elnabarawy et al.,1986; Bodar et al.,1990)
	Copepods	*Tropocyclops prasinus*	EC_{50}/LC_{50}	0.149 - 2.23	(Lalande and Pinel-Alloul,1986; Baliarsingh et al.,2010)
	Rotifers	*Brachionus calyciflorus*	EC_{50}/LC_{50}	0.070-0.350	(Snell and Moffat,1992; Ṕerez-Legaspi and Rico-Mart'ınez,2001)
PCP	Cladocera	*Daphnia magna*	EC_{50}/LC_{50}	0.143-1.23	(Mount and Norberg,1984; Elnabarawy et al.,1986; Hickey and Vickers,1992; Liber and Solomon,1994)
	Copepod	*Eucyclops neumani*	EC_{50}/LC_{50}	0.033-0.173	(Willis,1999; Soto et al.,2003)
	Rotifers	*Brachionus calyciflorus*	EC_{50}	0.27	(Snell and Moffat,1992)
3,4-DCA	*Cladocera*	*Daphnia magna*	EC_{50}	0.054 - 13.0	(Crossland and Hillaby,1985; Pedersen et al.,1998; ECETOC,2003)
	Copepods	no data	no data	no data	
	Rotifers	*Brachionus calyciflorus*	LT_{50}	2.5	(Janssen et al.,1994)
	Rotifers	*Brachionus calyciflorus*	LT_{50}	5	(Janssen et al.,1994)

*EC_{50} -median effective concentration- is a substance concentration that is expected to cause a specified toxic effect in 50% of the test population. LC_{50} -median lethal concentration-; is a substance concentration that is expected to cause 50% mortality in the test population. LT_{50}- median lethal time; is the time in which a specified concentration of a test substance is lethal to 50% of the test population.

Copper can accumulate in aquatic organisms as it is not biodegradable, resulting in toxic effects such as mortality, inhibition of reproduction, behavioural changes, and an overall change in the diversity of organisms in an ecosystem (Snell and Janssen, 1995; Gama-Flores et al., 2007a, b; Padrilah et al., 2018). These negative effects can be attributed to the high ability of copper to bind to proteins in the cells, potentially damaging regular cellular functions and structures. Furthermore, copper can produce free radicals by precipitating in the Fenton reaction (Padrilah et al., 2018).

1.6.2 Cadmium (Cd)

The average concentration of cadmium in the earth's crust is about 0.1 mg Cd/kg (Stoeppler, 1991). According to Amer (2014), the natural sources of cadmium include volcanic activities, sea spray, and biogenic materials, but most of the cadmium in the ecosystem is present due to anthropogenic activity. Cadmium is present in numerous products such as paints, coating, plastics, nickel-cadmium batteries, pesticides, and fertilizers (Amer, 2014). In non-polluted waters, cadmium concentrations are estimated to be lower than 0.1 µg/L (Stoeppler, 1991). However, cadmium in the forms of chemicals, pesticides, and other manufacturing products and by-products can reach and pollute aquatic systems through wastewater, discharge water, floods, and storms (Amer, 2014).

Cadmium has no known role in metabolism, so it is considered a non-essential element for organisms (Chapman et al., 2003). The metal can bioaccumulate in aquatic organisms, (Blackmore, 1998) even at relatively low concentrations (Amer, 2014). The uptake of cadmium in the organisms can increase levels of free radicals, as reactive oxygen species in the cells, diminishing antioxidant defences and resulting in negative effects, such as lipid peroxidation, protein degradation, and DNA damage (Amer, 2014). Cadmium can also interfere with zinc and calcium metabolism by easily binding to receptors, therefore decreasing the uptake of these essential metals (Treuner, 2002).

1.6.3 Pentachlorophenol (PCP)

Pentachlorophenol (C_6CI_5OH) is an organochlorine pesticide used in agriculture, industries, and disease vector control (Gad and Pham, 2014; Fraser, 2017). The main source of pentachlorophenol in aquatic ecosystems comes from the usage of this chemical to control molluscs and algae populations. Also, as it is used extensively in agriculture, it easily leaches through surface runoff to nearby waterbodies (Gad and Pham, 2014).

PCP has low solubility in water (8 mg in 100 ml) but relatively higher solubility in fat, therefore, it can be classed as a persistent organic pollutant (POP) (Jones and de Voogt, 1999). Consequently, it rapidly accumulates in aquatic organisms from the food chain and directly from water (Gad and Pham, 2014), which can cause toxicity to aquatic organisms and humans (Jones and de Voogt, 1999). The uptake of pentachlorophenol affects organisms by damaging the oxidative phosphorylation enzymatic system, binding with the protein of the mitochondria, and preventing ATPase activity. This inhibits the synthesis of ADP from ATP and, therefore, prevents the release of energy. This mechanism can also increases the cellular oxygen demand by inhibiting oxidative phosphorylation, which increases the respiration rate in exposed individuals (Gad and Pham, 2014).

1.6.4 3,4-dichloroaniline (3,4-DCA)

3,4-dichloroaniline ($C_6\ H_5\ Cl_2\ N$) is a chemical intermediate material used to generate 3,4-dichlorophenylisocyanate and some herbicides, such as propanil, linuron, diuron, and neburon (European Chemicals Bureau, 2006). Therefore, 3,4-dichloroaniline can be found in natural environments as a degradation compound of diuron, linuron, and propanil (Mercurio, 2016).

3,4-dichloroaniline is released through wastewater into aquatic environments in two common ways: during industrial chemical production and the use of plant protection products (European Chemicals Bureau, 2006; Sorokin et al, 2012). Products such as linuron, diuron, and propanil

could leach into water bodies and the biotransformation of these herbicides can produce 3,4-dichloroaniline, which bioaccumulates in aquatic organisms and is more toxic than the original herbicides (Giacomazzi and Cochet, 2004; European Chemicals Bureau,2006; Kiss and Virág., 2009).

The toxicity of 3,4-DCA to aquatic organisms can be related to its polar narcosis (Sorokin et al, 2012). 3,4-dichloroaniline can attach to the quinone protein in Photosystem II, inhibiting photosynthesis in algae, plants (Mercurio, 2016), and photosynthetic micro-organisms by inhibiting both the process of oxygen production and the electron transfer (Giacomazzi and Cochet, 2004; de Lima et al., 2018).

1.7 Research aims and objectives

The freshwater rotifer *Brachionus calyciflorus* can be useful as a model organism to evaluate the effects of sublethal concentrations of some organo-choro compounds and heavy metals on the total energy budgets and energy homeostasis in stressed individuals. Linking the effects of toxic stress on the energy budget status of rotifers and the effects on reproduction, using the population growth rate as the endpoint, will be very useful in understanding the cellular findings of energy reserve changes at higher levels of biological organization. The model will be better able to predict the toxic impacts at both population and ecosystem levels. To achieve these aims, the project will investigate the objectives listed in Table 1.2.

1.8 Thesis structure

This thesis is divided into seven chapters, inclusive of the current introductory chapter. Chapter Two assessed the population growth of rotifers under the stress of heavy metals and pesticides. Measurements of stored energy are described in Chapters Three and Four, while Chapter Five evaluated the measurements of energy consumption. Chapter Six discussed energy budget

calculations. Finally, the conclusions, recommendations for future work, and limitations of the research are presented in Chapter Seven.

Table 1.2: Thesis objectives and structure

Chapters	Objectives
2	To determine the lethal concentrations of four toxicants, copper (Cu), cadmium (Cd), pentachlorophenol (PCP), and 3,4-dichloroaniline (DCA), on the freshwater rotifer *Brachionus calyciflorus*.
2	To examine the influence of the sub-lethal concentrations on the population density of rotifers.
2	To examine the influence of sub-lethal concentrations on an intrinsic rate increase of population growth of rotifers
2	To determine the sub-lethal concentrations of these toxicants to establish appropriate concentrations to use in future experiments
3	To develop analytical methods for the determination of protein, lipid, and carbohydrate concentrations in rotifers.
3	To assess the utility of these methods for measuring the relevant compounds.
3	To establish appropriate dilution factors to ensure samples are within the linear range of the calibration curves
3	To establish the optimal assay times to achieve robust assessment outcomes.
4	To measure the total lipid, protein, and carbohydrate content in rotifers exposed to sublethal concentrations of toxicants
4	To evaluate the relative influence of the four toxicants on lipid, protein, and carbohydrates content
5	To complete energy consumption measurements using existing data from the literature.
6	To investigate how the presence of toxicants affects the energy budget (including energy available and consumed) of rotifers.
6	To investigate which of toxicant with its relevant concentration is most toxic to rotifers
7	To highlight the importance of the cellular energy allocation (CEA) methodology to assays of the effects of chemical stress on the energy metabolism
7	To conclude the optimization of the CEA procedure for the freshwater rotifer *Brachionus calyciflorus*
7	To validate whether freshwater rotifers can be used as a model organism for assessment of toxicity.
7	To make recommendations about the use of the four selected toxicants in the aquatic environment.

CHAPTER TWO

Chapter 2: Maintaining cultures of the freshwater rotifer (*Brachionus calyciflorus*) and establishing the lethal and sub-lethal effects of four toxicants: copper (Cu), cadmium (Cd), pentachlorophenol (PCP), and 3,4-dichloroaniline (DCA)

2.1 Introduction

In ecotoxicology, various assessment tools are used in assessing the impacts of chemical contaminants on aquatic ecosystems. The most currently used tools have been reviewed by Schuijt et al. (2021). These tools measure responses at diverse levels of biological organization. At the sub-organismal level (cells), two methods are common: biomarkers (such as cellular energy metabolism, immunotoxicity, histopathology, and gross indices) and *in vitro* bioassays (such as genotoxicity and oxidative stress). At whole-organismal (individuals), population, and community levels, endpoints such as reproduction, mortality, growth, and development are applied in bioassays (Schuijt et al., 2021).

Many toxicity thresholds are used to summarise the results of toxicity tests in quantifying the lethal and sub-lethal toxicity of various chemicals. Examples of these thresholds include LC_{50}, LOEC, and NOEC. The LC_{50} (median lethal concentration for 50% of the population) is useful for quantifying acute lethal impacts; whereas the LOEC (lowest-observed-effect concentration) and NOEC (no-observed effect concentration) are more protective thresholds useful in expressing sub-lethal impacts. Specifically, the LC50 is the level of concentration causing 50% mortality effects; the LOEC is the lowest concentration causing detectable adverse effects; and the NOEC is the highest concentration lacking detectable adverse effects of chemicals on tested organisms. The combined use of these thresholds improves understanding of potential risks posed by various pollutants (Connon et al., 2012). Overall, toxicity tests should be sensitive enough to detect the effects of different chemical contaminants in various organisms, reproducible, useful in risk evaluation, cost-effective, and accurate (Rico-Martínez et al.,

2016). A good test organism should be easy to culture in the laboratory, have a short life cycle with multiple life stages, be small, be ecologically and economically relevant, and be sensitive to the required assessment (Brown, 2001).

Rotifers are considered useful test organisms for toxicity studies due to several biological traits (see full discussion in Chapter One). The use of freshwater rotifers and marine species in toxicity studies was first reported by Snell and Persoone (1989a, b). Since then, several rotifer species have been used in toxicity tests (Janssen et al., 1994; Azuara-Garcia et al., 2006; Gama-Flores et al., 2007*;* Juárez-Franco et al., 2007; Nandini et al., 2007; Sarma et al., 2007; Park et al., 2018; Hernández-Flores et al., 2020). These species vary in sensitivity for different chemical contaminants (Rico-Martínez et al., 2016). In particular, the freshwater rotifer *Brachionus calyciflorus* has been widely used to investigate the impact of several pollutants, including hydrocarbons (Snell et al., 1991a; Ferrando and Andreu-Moliner, 1992), pharmaceuticals (Parrella et al., 2014), organic compounds (Radix et al., 2002; Mihaich et al., 2009; Zhang et al., 2013), multi-metal mixtures (Xu et al., 2014; 2015), single metals (Snell et al., 1991a,b; Janssen et al., 1994; Radix et al., 2000; Sarma et al., 2006; Gama-Flores et al., 2007; Santos-Medrano and Rico-Martínez, 2013; Hernández -Ruiz et al., 2016), and pesticides (Ferrando and Andreu-Moliner, 1991; Snell et al., 1991a, b; Fernández-Casalderry et al., 1992; Ferrando et al., 1993a, b; Janssen et al., 1994; Radix et al., 2000; Xi et al., 2007; Ke et al., 2009; Zhang et al., 2016; Gharaei et al., 2020).

Many toxicity thresholds have been reported for rotifers in toxicity tests. For example, the LC_{50} in short-term (24 – 48h) exposures to measure mortality as a direct impact of toxicants with or without feeding of the test organisms (Snell et al., 1991a, b; Fernández-Casalderry et al., 1992; Milam et al., 2004; Azuara-Garcia et al., 2006; Nandini et al., 2007; Arias-Almeida and Rico-Martínez, 2011; Pérez-Legaspi et al., 2012; Zhang et al., 2016; Lee et al., 2018). On the other hand, chronic toxicity tests can assess sub-lethal parameters such as changes in the reproductive

cycle, behaviour, *in vivo* enzymatic activity, rate of ingestion, rate of filtration, and swimming ability (Ferrando et al., 1993a, b; Burbank and Snell, 1994; Juchelka and Snell, 1994; Snell and Janssen, 1995; Milam et al., 2004; Wallace et al., 2006; Perez-Leqaspi et al., 2012; Chen et al., 2014; Rico-Martinez et al., 2016). Another common chronic endpoint is the intrinsic growth rate (r), which is seen as the most sensitive. The intrinsic rate of population growth, often known as the r-value or intrinsic growth rate, is the highest potential growth rate of a population under optimal conditions. (Cortés,2016). These sub-lethal parameters are generally determined under conditions of chemical stress over a period of hours, days or weeks upon which NOEC and LOEC thresholds are reported (Snell and Moffat, 1992; Janssen et al.,1994; Radix et al., 2002; Sarma et al., 2006; Gama-Flores et al.,2007; Mihaich et al., 2009; Pérez-Legaspi et al., 2012; Zhang et al., 2013; Rico-Martínez et al., 2016).

The overall aim of this chapter is to describe the conditions required to maintain rotifer cultures in the laboratory and assess the lethal and sub-lethal impacts of four toxicants on the freshwater rotifer *B. calyciflorus*, determining the threshold concentrations. This aim was achieved by:

(a) Starting a culture of freshwater rotifers *B. calyciflorus* from cysts and maintaining it under controlled conditions in the laboratory to generate test organisms for toxicity assessment.

(b) Conducting acute toxicity tests to assess the lethal effects (as 24h LC_{50}) of copper (Cu), cadmium (Cd), pentachlorophenol (PCP), and 3,4-dichloroaniline (3,4-DCA) on neonates of the freshwater rotifer *B. calyciflorus*.

(c) Evaluating the influence of sub-lethal concentrations of the four toxicants on the population density and intrinsic growth rate on populations of *B. calyciflorus,* quantifying LOEC and NOEC after 24, 48, and 72h of exposure with food provided.

(d) Determining the range of sub-lethal concentrations to use for the energy budget experiment in a subsequent study. This included chronic toxicity tests using the population density and the

intrinsic growth rate of the population of rotifers as parameters after 24, 48, and 72h of exposure without food provided.

Experimental outcomes, here, helped to inform further work in Chapter 4, which needed to be undertaken below the lethal thresholds for the toxicants of interest.

2.2 Methods and materials

2.2.1 Starting and maintaining a culture of freshwater rotifers

The monogonont rotifer, *B. calyciflorus*, was used as the test organism in this study. The culture of rotifers was started from cysts (Product Code: fwrest5k) cultured by Florida Aqua Farms Inc., USA (purchased from Zebrafish Management Ltd, UK). In this study, the rotifer cysts were hatched in mass cultures in the laboratory using moderately hard synthetic freshwater (EPA) as a medium (Table 2.1). The EPA medium was prepared (in 10L glass media bottle) according to USEPA (2002), using deionised adjusted to pH 7.5 with 1 M potassium hydroxide (KOH) or hydrochloric acid (HCl). The freshwater medium (EPA) was autoclaved at 126 ºC and >15 psi for 10 minutes (the total autoclave time from start to finish was 80 minutes) and allowed for 24h to cool to 25˚C. Thereafter, the medium was poured into sterile 20L plastic containers and stored in an incubator until use.

A batch culture of freshwater microalgae, *Chlorella vulgaris*, was used as food source for the rotifer culture. The *C. vulgaris* cysts were purchased from cultured by the Scottish Association for Marine Science (SAMS; Product Code: CCAP 211/11B) and supplied in 15 ml glass tubes containing Bold Basal Medium (BBM). The cysts were cultured using autoclaved BBM (see Table 2.2 for composition) which had been allowed for 24h to cool to 20˚C in an incubator. To set up the algal culture, one tube of cysts was added to 1L of BBM in 2 L conical flask, sealed with aluminium foil, and incubated at 20 ± 1˚C, cool white, fluorescent light, intensity of 4000 Lux (Biospherical meter, Model: QSL-2101) and under a light: dark cycle of 16:8 h/day.

Table 2.1: Composition of the freshwater medium (EPA)

Chemical name (Formula)	Weight (g)	Deionised water (L)	Concentration (g/L)
Magnesium Sulphate ($MgSO_4.7H_2O$)	0.6	10	0.06
Calcium sulphate ($CaSO_4.2H_2O$)	0.6	10	0.06
Potassium Chloride (KCl)	0.04	10	0.004
Sodium Bicarbonate ($NaHCO_3$)	0.96	10	0.096

Table 2.2: Composition of the Bold Basal Medium (Borowitzka and Borowitzka, 1988).

Chemical name (Formula)	Weight (g)	Deionised water (L)	Stock solutions concentration (g/L)	Volume required (mL)
Sodium Chloride (NaCl)	0.62	0.25	2.48	10
Calcium Chloride ($CaCl_2 \cdot 2H_2O$)	0.62	0.25	2.48	10
Sodium Nitrate ($NaNO_3$)	6.25	0.25	25.00	10
Potassium Phosphate, Dibasic (K_2HPO_4)	1.87	0.25	7.48	10
Potassium Phosphate, Monobasic (H_2PO_4)	4.37	0.25	17.48	10
Magnesium Sulphate ($MgSO_4.7H_2O$)	1.87	0.25	7.48	10
Disodium EDTA	5.00	0.1	50.00	1.0
Potassium Hydroxide (KOH)	3.10	0.1	31.00	1.0
Boric acid (H_3BO_3)	1.14	0.1	11.4	1.0
Ferrous Sulphate ($FeSO_4 \cdot 7H_2O$)	0.49	0.1	4.90	1.0
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.048	0.025	1.92	0.1
Cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$)	0.020	0.025	0.80	0.1
Zinc Sulphate ($ZnSO_4 \cdot 7H_2O$)	0.353	0.025	14.12	0.1
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	0.058	0.025	2.32	0.1
Cupric Sulphate ($CuSO_4 \cdot 5H_2O$)	0.063	0.025	2.52	0.1
Deionised water (diH_2O)	-	-	-	935.5

Growth phases for *C. vulgaris* were not determined in the current study. However, the exponential growth phase of *C. vulgaris* cells (log growth) has been observed to be between 5 and 12 days. The *Chlorella* cells were, therefore, harvested during this period by decanting an aliquot of the culture into 15 mL tubes, which were then centrifuged at 3000 rpm (low speed to minimize cell damage) for 5 min. Thereafter, the supernatant was removed, and algae cells were rinsed in deionised water and re-suspended in an EPA medium using a vortex mixer to reach a volume of 10 mL. The suspension of the live *Chlorella* cells was stored in a refrigerator

at 4 ˚C and used within 3 days to minimize the risk of bacterial contamination. The viability of the microalgae cells harvested through this method was demonstrated by visible cell growth after incubation in a 500 ml flask containing EPA medium at conditions earlier described.

Throughout this project, the sole source of nourishment for the rotifer cultures was the live microalgae, except for certain instances, where rotifer cultures were supplemented with Roti-Rich, a blend of essential components such as mineral mixture, vitamin mixture, yeast, and phytoplankton, produced by Florida Aqua Farms and supplied by Zebrafish Management.

The rotifer culture was established following the protocol described by Rico-Martinez and Dodson (1992), with some modifications. Hatching of eggs was initiated by adding the content of cyst vials (approximately 80,000 eggs) into a 200 mm Petri dish filled with freshwater medium (EPA), ensuring that all the eggs were submerged. Thereafter, the culture was covered and incubated at 25 ± 1˚C under continuous illumination with cool, white, fluorescent light with an intensity of 4,000 Lux. At this stage, the use of continuous light conditions is essential for hatching resting eggs by triggering the release of diapause (Minkoff et al.,1983).

Once hatching began, after approximately 16 - 24h, food was supplied to the rotifers by adding just enough microalgae (*Chlorella vulgaris*) to change the colouration of the media to pale green. One day later, the culture was transferred into a 0.5 L container, adding fresh medium and microalga. The culture was then maintained at 25 ± 1 ˚C and cool, white, fluorescent light with intensity from 2,000 to 4,000 Lux under a light: dark cycle of 16:8 h/day. At this stage, the presence of light remains a crucial factor for the growth of microalgae, which serve as food source for rotifers, but it is not required for the growth of rotifers due to the absence of photosynthesis. Afterwards, rotifers were harvested by sieving the medium (41µm aperture) and then transferred into 2, 5, and 10 L containers after 4, 6, and 8 days, respectively, with new medium and algal food. When the rotifers were transferred to a larger container (10 L), they

were fed twice daily with live harvested algal cells. The density of *Chlorella* cells in the rotifer culture was maintained at 2.0×10^6 cells/ml. At each feeding step, a sample from the culture was taken, and the density of the algae cells was estimated by comparison with a reference sample containing a level of 2.0×10^6 cells/ml (established using an automated cell counter).

For oxygen supply, an aquarium pump **(**M-102 Mouse Air Pump) was used to aerate the rotifer culture by bubbling air. Too much oxygen flow can be stressful for the rotifers; therefore, a modest bubble (2-3 bubbles a second) was produced by adjusting the aquarium pump.

On Day 10, all rotifers were harvested and transferred to a 25 L container with 20 L of new freshwater medium and algal food. The culture was maintained for another 8 to 10 days to allow for population growth. Between Days 18 and 20, the density of rotifers reached about 10 individuals per 100 µL (Figure 2.1). At this level, the culture was harvested, and rotifers were transferred to two 25 L containers filled with 20 L of new freshwater medium and algal food. Cultures were then maintained in the incubators as described earlier.

Several processes were followed during the setup of the culture. These include culture examination, medium exchange, container cleaning, and rotifer harvesting. The cultures were checked every day by observing a 20 mL subsample under a dissecting microscope (at a magnification of 20-50X). Several parameters were checked, such as the medium quality (clean or dirty), presence of microorganisms, and rotifer swimming speed, which was useful evidence of contamination or starvation. If any of these indicators were unsatisfactory, culture medium exchange and container cleaning were done urgently. However, if satisfactory, then the culture medium exchange and the container cleaning were done twice a week.

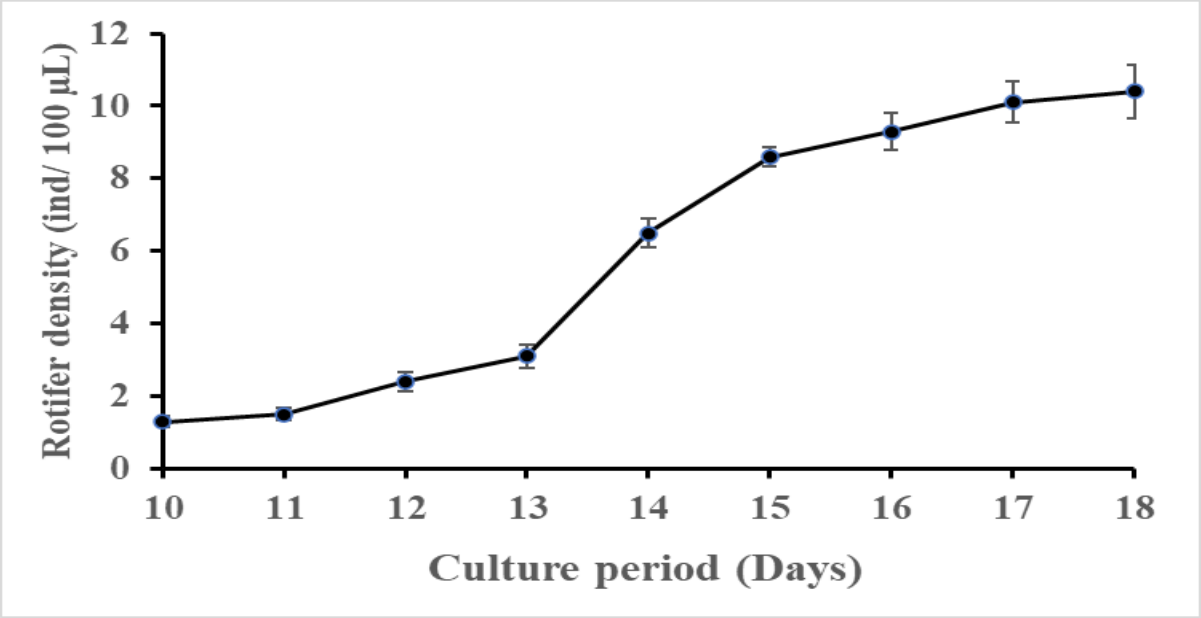

Fig. 2.1: Density of freshwater rotifers (*Brachionus calyciflorus)* in a 20 L culture. Each plot is the mean ± SEM (N=10).

Culture medium exchange was carried out by sieving half of the existing medium and then replenishing it with fresh medium. Rotifers on the sieve were gently washed back into the container. When required, the culture container was cleaned by stopping the aeration pump for 15 min to settle suspended debris, which was then easily siphoned off the container bottom.

Determination of rotifer growth phases was not performed in the current study. However, rotifer density was estimated daily in the 20 L cultures. This was performed using ten homogenous 1 mL subsamples, retrieved from the culture using a plastic pipette and gently mixed in 1.5 mL Eppendorf tubes. Thereafter, a 100 µL subsample was fixed in 30 mm Petri dishes by adding 100 µL of 5% Lugol's iodine solution and counted under a dissecting microscope at a magnification of 10 – 12X. This staining step was necessary to make the rotifers more visible during the counting process, especially at relatively high density (>7 rotifers/100 µL) and given the rapid movement. Figure 2.1 shows the average number of rotifers per 100 µL of the culture. This was multiplied by 10 to obtain the average number of

rotifers in 1 mL, which was then averaged across the ten subsamples and multiplied by the culture volume to obtain the average number of rotifers in the culture (rotifers/mL).

Rotifer harvesting was done, when the density exceeded 100 rotifers/mL in order to achieve a balance between population growth and food availability. This measure prevented overcrowding, which can lead to food shortage, decrease in dissolved oxygen, and an increase in excretory products. To achieve this, the rotifers were harvested using a 41 μm sieve, rinsed with a clean freshwater medium, and washed into a 2000 mL conical flask. A flashlight was shone at the neck of the conical flask to attract the rotifers, which concentrated near the edge at the top of the flask. The rotifers were then easily pipetted into a clean container with freshwater medium (EPA) and live microalga.

2.2.2 Chemicals and test solutions

All laboratory wares used throughout these experiments were washed using Decon90 and distilled water, soaked in 15% (v/v) HNO_3 for 24 - 48h, and thereafter rinsed several times (until neutral pH) using ultrapure water (UPW; Elga Purelab Ultra, 18.2 MΩ cm). Afterwards, washed wares were dried in a laminar flow cabinet and, thereafter, enclosed in clean plastic boxes to be used when needed.

Copper (Cu), cadmium (Cd) pentachlorophenol (PCP), and 3,4-dichloroaniline (3,4-DCA) were used as toxicants in this study. 100 mg of $CuSO_4$ (anhydrous -analytical reagent grade - purchased from BDH Chemicals Limited, UK), 100 mg of $CdCl_2$ (hemi pentahydrate**,** analytical reagent grade -purchased from Fisher Scientific, UK), 10 mg of PCP (purchased from Sigma-Aldrich) and 1000 mg of 3,4-DCA (purchased from Acros Organics, UK). These amounts of different toxicants were used to prepare the stock solutions in an EPA medium (Table 2.3). These high-concentration stock solutions were adjusted to pH 7.5 by adding some droplets of 1M potassium hydroxide (KOH) or hydrochloric acid (HCl), where applicable.

Table 2.3: Concentrations of stock solutions and the corresponding molarities of the four chemicals used in the current study

Toxicants	Molecular weight g/mol	Concentrations mg/L	Molarities µM
Cu	63.55	39.80*	626
Cd	112.40	49.20*	438
PCP	266.34	10.00	37
3,4-DCA	162.02	1000	6172

*The concentrations of Cu and Cd were adjusted for the percentage composition in the relative molecular weight of $CuSO_4$ (159.61 g/mol) and $CdCl_2$ (228.36 g/mol).

2.2.3 Acute toxicity experiments

Range-finding tests for each toxicant were conducted for a duration of 24h. These tests investigated the acute effects (lethality) of used toxicants on the rotifer *B. calyciflorus.* The generated LC_{50} were then used to determine appropriate toxicant concentrations for chronic exposure experiments.

2.2.3.1 Selection of toxicant concentrations

The toxicant concentrations used for these tests were selected based on the median lethal concentration data available in the literature for freshwater rotifers. According to published data, the lethal concentration (LC_{50}) ranges for copper (Cu), cadmium (Cd), pentachlorophenol (PCP), and 3,4-dichloroaniline (3,4-DCA) were found to be 0.01-0.07, 0.18-1.3, 0.21-2.16, and 61.4-62 mg/L, respectively (Ferrando and Andreu-Moliner, 1991; Snell et al., 1991a; Ferrando et al., 1992; Snell and Moffat, 1992; Preston et al., 1999; Sarma et al., 2006). From these datasets, the mean values of LC_{50} for each chemical were recalculated, yielding results of 0.04, 0.93, 1.20, and 61.70 mg/L, respectively. Finally, four concentrations for each chemical were chosen in the present study to allow us to investigate the potential effects on rotifers at

concentrations within this range (Table 2.4). Test solutions with different concentrations were prepared by diluting the stock solutions in a freshwater medium (EPA).

Table 2.4: Concentrations of the four toxicants used in acute toxicity tests on freshwater rotifers

Toxicants	The mean value of LC_{50} concentrations mg/L*	Concentrations used in the current trial mg/L			
Cu	0.04	0.01	0.02	0.03	0.04
Cd	0.93	0.33	0.65	1.30	2.60
PCP	1.20	0.80	1.60	2.40	3.20
3,4-DCA	61.70	24.80	49.60	74.40	99.20

*The mean value of LC_{50} concentrations calculated from the previous studies

2.2.3.2 Experimental setup

A culture of 500 mL for rotifers was established by using a small portion of resting eggs to yield the adequate number of rotifers needed for this test. These eggs were cultured as earlier described, with the culture maintained only for three days. Thereafter, some rotifers were harvested from the culture and placed into a Petri dish (100 mm) containing a new medium. They were fed live food (2×10^6 cells of *Chlorella vulgaris* per ml) for 2h. The acute toxicity tests of the different toxicants were performed in 24-multiwell plates to determine the LC_{50}. One ml of the toxicant solution, at appropriate concentrations, was pipetted into each well using a micropipette. Each toxicant concentration was filled into one column of wells, which consisted of four wells. Under a dissecting microscope at a magnification of 10-12 X, six neonate rotifers were then transferred from the Petri dish into each well using a micropipette. Each plate consisted of a control with no toxicant (only EPA medium) and four toxicant concentrations with four replicate wells for each treatment. The plates were incubated under

standard experimental conditions for acute toxicity bioassays (temperature 25 ˚C and in darkness), as described by Snell and Persoone (1989a).

Due to the short duration of the test (24h), the rotifers were not fed, and the medium was not renewed during the test period. The number of dead and live animals for each toxicant treatment was counted under a dissecting microscope after 24h to calculate the percentage mortality for each toxicant concentration.

2.2.4 Chronic toxicity and population growth experiments using high-range concentrations

For the chronic toxicity tests, the population density (number of individuals) and the intrinsic rate of population increase (r) parameters were measured as endpoints to identify LOEC (the lowest observed effect) and NOEC (the no observed effect concentrations). Four high-range sub-lethal concentrations of each toxicant were chosen based on the LC_{50} values obtained in the range-finding experiments. These concentrations were selected by dividing the LC_{50} value of each toxicant by 5,10,15 and 20 (Table 2.5). Test solutions with different concentrations were prepared by diluting the stock solutions in a freshwater medium (EPA).

Table 2.5: High-range concentrations of the four toxicants used for the first chronic toxicity test.

Toxicants	LC_{50} mg/L	High range concentrations mg/L			
Cu	0.012	0.0006	0.0008	0.0012	0.0024
Cd	1.460	0.07	0.09	0.14	0.29
PCP	1.950	0.09	0.13	0.19	0.39
3,4-DCA	123.800	6.15	8.2	12.3	24.6

2.2.4.1 Experimental setup

One ml of the toxicant solution, at appropriate concentrations, was pipetted into each well of a 24-multiwell plate using a micropipette. Each toxicant concentration was filled only into one column of wells, which consisted of four wells. Some rotifers were harvested from the previously described culture (500 ml) and, thereafter, placed into a Petri dish (100 mm) containing a new medium. Under a dissecting microscope at a magnification of 10-12 X, three neonate rotifers were randomly selected from the Petri dish and placed into each well as described above. Each plate consisted of a control with no toxicant (only EPA medium) and four toxicant concentrations with four replicate wells for each treatment. In the case of 3,4-DCA, which was dissolved with a minimal amount of acetone, a second control with acetone treatment was included.

Food was supplied in this experiment, and the medium, which contained 2×10^6 cells per ml of *Chlorella vulgaris*, was not renewed during the 72h exposure period. The plates were placed in an incubator under standard experimental conditions (temperature 25 ˚C and in darkness), as described by Snell and Moffat (1992). The number of dead and live organisms for each group of treatments was recorded after 24, 48 and 72h to determine the changes in population density. The intrinsic rate of population increase (r) was determined according to the following formula:

$$r = (\ln Nt - \ln N_0)/ T$$

where: ln Nt is the natural logarithm of the number of rotifers after 1, 2 and 3 days; ln N_0 is the natural logarithm of the initial number of rotifers (equal to 3); and T is 1, 2 or 3 days. Depending on the values of the intrinsic rate of population increase (r) and/or the population density, the statistical differences were established to determine the LOEC and NOEC.

2.2.5 Chronic toxicity and population growth experiments using low-range concentrations

A second chronic toxicity experiment was conducted to determine the low range of sub-lethal concentrations of the toxicants. The concentrations used for these tests were chosen based on the values of no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) which were obtained from the first chronic experiment. Therefore, five concentrations were selected in decreasing order of toxicity dependent on the LOEC (or NOEC when no LOEC was detected) to be used for each toxicant.

In the earlier experiment, the LOEC values for Cd and 3,4-DCA were determined to be 0.070 mg/L and 8.200 mg/L, respectively. As a result, to keep test concentrations below the respective LOEC values for Cd and 3,4-DCA, concentrations of 0.007 mg/L and 0.800 mg/L were the maximum used for these compounds. In contrast, the NOEC values for Cu and PCP were found to be 0.002 mg/L and 0.390 mg/L, respectively. Therefore, these concentrations were used as the maximum concentrations (Table 2.6). Test solutions were prepared by diluting stock solutions in freshwater medium (EPA). The rotifers were exposed for 24, 48, and 72h to determine the effect of these low-range sub-lethal concentrations on population growth parameters. This information was then used to define both toxicant concentration ranges and exposure time, which was later applied in the energy budget experiments (Chapter 4).

Table 2.6: Low-range sub-lethal concentrations used for the second chronic toxicity test based on NOEC or LOEC values

Toxicants	NOEC/LOEC mg/L	Low range concentrations mg/L				
Cu	NOEC (0.002)	0.0000002	0.000002	0.00002	0.0002	0.002
Cd	LOEC (0.070)	0.0000007	0.000007	0.00007	0.0007	0.007
PCP	NOEC (0.390)	0.000039	0.00039	0.0039	0.039	0.390
3,4-DCA	LOEC (8.200)	0.00008	0.0008	0.008	0.08	0.800

2.2.5.1 Experimental setup

An appropriate volume of each toxicant solution was pipetted into each well of a 24-multiwell plate to get the desired concentration of each toxicant. As described in the previous experiments, each column consisted of four wells which were filled only with the same toxicant concentration. Some rotifers were harvested from the previously described culture (500 ml) and, thereafter, placed into a Petri dish (100 mm) containing new medium and food (2×10^6 cells of *C. vulgaris* per ml). After 2h, five neonate rotifers were randomly selected from the Petri dish and placed into each well. This was accomplished using a micropipette under a dissection microscope at a magnification of 10-12X. Each plate consisted of a control with no toxicant (only EPA medium) and five toxicant concentrations with four replicate wells for each treatment. In the case of 3,4-DCA, which was dissolved with a minimal amount of acetone, one control and another control + acetone treatments were used. Food was not supplied during the experiment and the medium was not renewed during the 72h exposure period.

The plates were maintained in darkness at a temperature of 25 ˚C. The number of dead organisms for each toxicant treatment was recorded after 24, 48 and 72h. The changes in population density and the intrinsic rate of population increase (r) were also determined as earlier described.

2.2.6 Determination of the effect of the absence of food

To evaluate the effect of food absence on the responses of rotifers under different toxicants, the values of the population density (PD) were converted to a percentage of the initial number of individuals for each toxicant concentration in the first chronic (with food) and the second chronic (without food) exposure experiments, while the values of intrinsic growth rate were used without changes. For each toxicant under every experiment, the values of PD and r under all concentrations were treated as one treatment, which was compared to control values under

the two experiments and the values of the other experiment. Thus, in total, four values were compared for each toxicant: control food, control no food, toxicant food, and toxicant no food.

2.2.7 Relationship between population density and the intrinsic rate of population increase (r)

The results of population growth rate were plotted against population density to investigate the relationship between population growth rate and population density under both feeding and no-feeding conditions.

2.2.8 Data analysis

To determine the LC_{50} values for each toxicant the probit analysis model was used. It is a statistical method commonly used to analysis the mortality data of organisms in response to toxic concentrations (Postelnicu, 2011). It was used to calculate the percentage of dead rotifers at each concentration and Pearson's chi-square test was performed to examine the goodness of fit of the model used ($p \leq 0.05$). Furthermore, a one-way analysis of variance (ANOVA) was performed on the results of the first chronic exposure experiment. The results of the second chronic experiment were analysed by one-way ANOVA to determine the significant differences in response between the different sub-lethal concentrations of each toxicant. In addition, a two-way ANOVA test was used to determine the effect of duration of exposure on population density and growth rate for both chronic experiments, where the time of exposure and concentration of toxicant were used as factors to assess the effect of time on each concentration of each toxicant. Furthermore, one-way ANOVA was used to test whether there was a significant effect of the absence of food. All these analyses were done using Sigma Stat software (SPSS) (v.25). Differences were considered significant at the $\alpha = 0.05$ level.

2.3 Results

2.3.1 Acute toxicity test

Probit statistical analysis was used to calculate the LC_{50} concentrations for each toxicant. This was accomplished by converting the toxicant concentrations to their natural logarithms while the mortality response was converted to Probit (regression analysis). The number of organisms exposed in each assay was also considered. The Probit values were then plotted (on the y-axis) against the logarithm of concentration (on the x-axis) to determine the LC_{50} for each toxicant. The results revealed that Cu was the most toxic, having the lowest LC_{50} (0.012 mg/L) in comparison with the other toxicants tested. On the contrary, 3,4-DCA had the lowest toxic effect, with an LC_{50} value of 123.800 mg/L. PCP and Cd had intermediate LC_{50} values of 1.950 mg/l and 1.460 mg/L, respectively (Table 2.7).

2.3.2 Chronic toxicity effects of high and low-range sub-lethal concentrations of used toxicants on the population density (PD) and the intrinsic growth rate (r)

2.3.2.1 The chronic toxicity of copper (Cu)

The exposure to different high-range concentrations of Cu (0.0006, 0.0008, 0.0012 and 0.0024 mg/L) did not have a significant effect on the population density (PD) of *B. calyciflorus*. One-way ANOVA showed that after 24, 48, and 72h of exposure, there was no effect of Cu on PD ($F_{4,15}$, $p = 0.52$; $F_{4,15}$, $p = 0.51$; and $F_{4,15}$, $p = 0.082$ respectively). On the contrary, there was a significant effect of duration of exposure (F2, 45, $p = 0.001$). PD in the control group increased with increasing duration of exposure after 48 and 72h. However, under all Cu concentrations, there was a significant increase only after 72h compared to 24 and 48h (Figure 2.2 A).

Table 2.7: The median lethal concentration values (LC_{50}) for *B. calyciflorus* compared to previous studies

Toxicants	LC_{50} mg/L	Source
Cu	***0.012***	***This work***
Cu	0.026	(Snell et al., 1991a)
Cu	0.026	(Snell and Moffat, 1992)
Cu	0.076	(Ferrando et al., 1992)
Cd	***1.460***	***This work***
Cd	1.300	(Snell et al., 1991a)
Cd	0.180	(Sarma et al., 2006)
Cd	1.300	(Snell and Moffat, 1992)
PCP	***1.950***	***This work***
PCP	0.210	(Preston et al., 1999)
PCP	2.160	(Ferrando et al., 1992)
PCP	1.200	(Snell and Moffat, 1992)
PCP	1.200	(Snell et al, 1991a)
3,4-DCA	***123.800***	***This work***
3,4-DCA	62.000	(Snell et al,1991a)
3,4-DCA	61.400	(Ferrando and Andreu-Moliner, 1991)
3,4-DCA	61.500	(Ferrando et al., 1992)

In terms of the intrinsic rate of natural increase (r), Cu did not induce significant effects at any exposure concentration. However, the duration of exposure had a significant effect on r ($F_{2,45,}$ $p = 0.004$). This effect of duration of exposure was driven by the results at 0.0006 mg Cu/L, which had a significantly higher r-value after an exposure of 72h compared with its value after 24 and 48h ($p = 0.015$ and 0.044 respectively) but did not differ from other concentrations at any time (Figure 2.2 B). These results indicate that there was no detected LOEC for Cu across the concentration range tested. All concentrations did not show any significant effect on PD and r, including the maximum concentration (0.0024 mg/L) which was considered the NOEC in this study. This NOEC was noted to be similar to those in previous studies (Table 2.8).

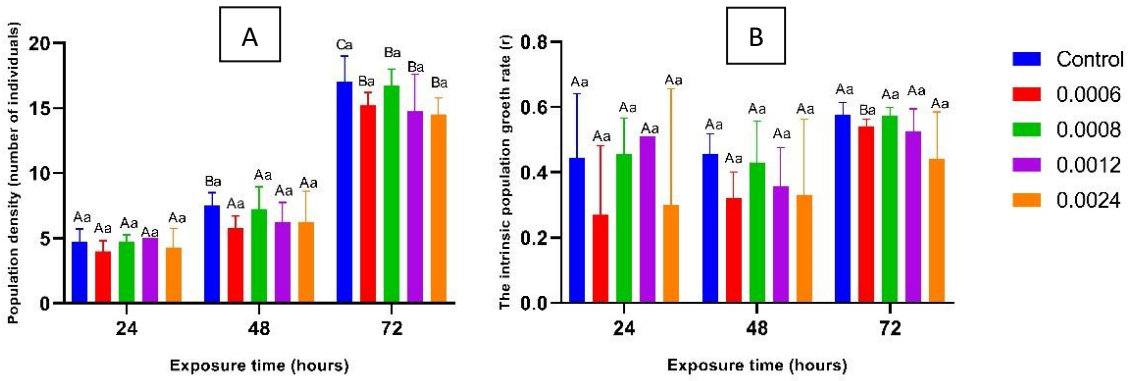

Fig. 2.2 The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (*high range*) concentrations of Cu (mg/L), for different durations of exposure (hours). Different lower-case letters indicate significant differences between Cu concentrations at a given exposure time while upper-case letters indicate significant differences of the same concentrations after different durations of exposure. Differences were considered significant at $p \leq 0.05$. Errors are $\pm$ standard deviation (N = 4).

Table 2.8: NOEC and LOEC values for *B. calyciflorus* in this study compared to previous studies

Toxicant	NOEC mg/L	LOEC mg/L	Source
Cu	***0.0024***	***-***	***This work***
Cu	0.0025	0.005	(Janssen et al., 1994)
Cu	0.020	0.030	(Snell and Moffat,1992)
Cu	-	0.030	(Gama-Flores et al., 2007)
Cd	***<0.070***	***0.070***	***This work***
Cd	-	0.020	(Gama-Flores et al., 2007)
Cd	-	0.005	(Sarma et al., 2006)
Cd	0.040	0.080	(Snell and Moffat,1992)
PCP	***0.390***	***-***	***This work***
PCP	0.400	0.800	(Janssen et al., 1994)
PCP	0.110	0.190	(Snell and Moffat,1992)
3,4-DCA	***6.150***	***8.200***	***This work***
3,4-DCA	2.500	5.000	(Ferrando et al.,1993a)
3,4-DCA	5.000	10.000	(Janssen et al., 1994)

Accordingly, we started with the NOEC value to obtain the five low-range concentrations that were used in the second sub-lethal exposure assessment (0.0000002, 0.000002, 0.00002, 0.0002 and 0.002 mg/L). These concentrations of Cu did not show any significant effect on PD at any exposure duration ($F_{4,15}$, P = 0.062; $F_{4,15}$, P = 0.069; and $F_{4,15}$, P = 0.229 for 24, 48 and 72h of exposure respectively). The duration of exposure had a significant effect on PD values. At all treatments, the values of PD significantly decreased with increasing duration of exposure ($F_{2,37}$, P = 0.001; Figure 2.3 A). Similar to PD, the Cu concentrations did not affect the r after any duration of exposure ($F_{5,18}$, P = 0.052; $F_{5,18}$, P = 0.125; and $F_{5,18}$, P = 0.459 for 24, 48, 72h respectively). The effect of duration of exposure was significant ($F_{2,36}$, p = 0.001). There was a significant reduction in r for all the treatments with prolonged exposure (Figure 2.3 B).

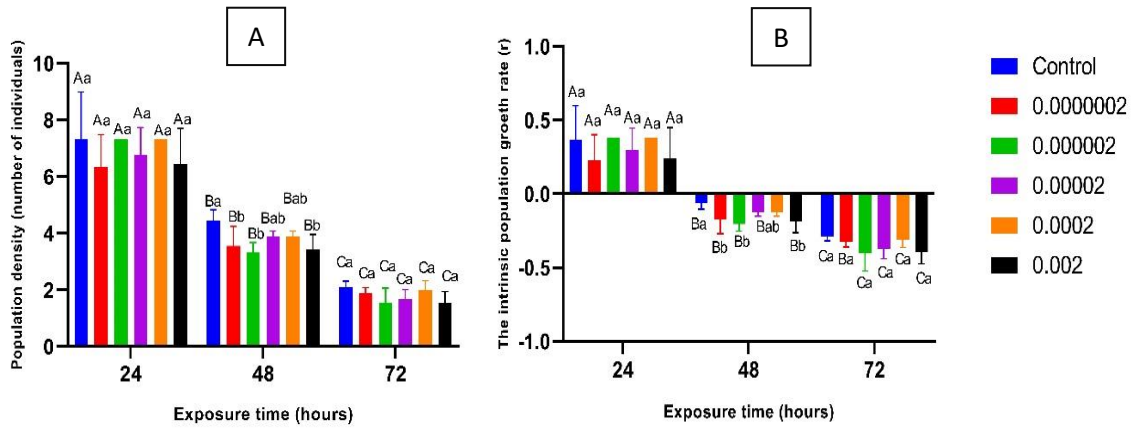

Fig. 2.3: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to low-range sub-lethal concentrations of Cu (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between Cu concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at p ≤ 0.05. Errors are ± standard deviation (N = 4).

2.3.2.2 The chronic toxicity of Cadmium (Cd)

The exposure to Cd high-range concentrations (0.07, 0.09, 0.14 and 0.29 mg/L) significantly affected the PD. However, these effects were observed only after 48 and 72h of exposure ($F_{4,16}$, p = 0.001 for both durations) but not after 24h ($F_{4,16}$, p = 0.203). After 48h, the PD was

significantly reduced under the highest three concentrations. Whereas after 72h, all the Cd concentrations had significantly lower PD in comparison with the control, and the PD steadily decreased with increasing Cu concentration. Furthermore, the duration of exposure had a significant effect on PD ($F_{2,45}$, p = 0.001). The PD increased steadily under control conditions, with an increase in the duration of the experiment. For Cd, the highest concentration (0.29 mg/L) had the lowest PD value after 48 and 72h compared with 24h of exposure, while other concentrations only had significantly lower values after 72h of exposure (Figure 2.4A).

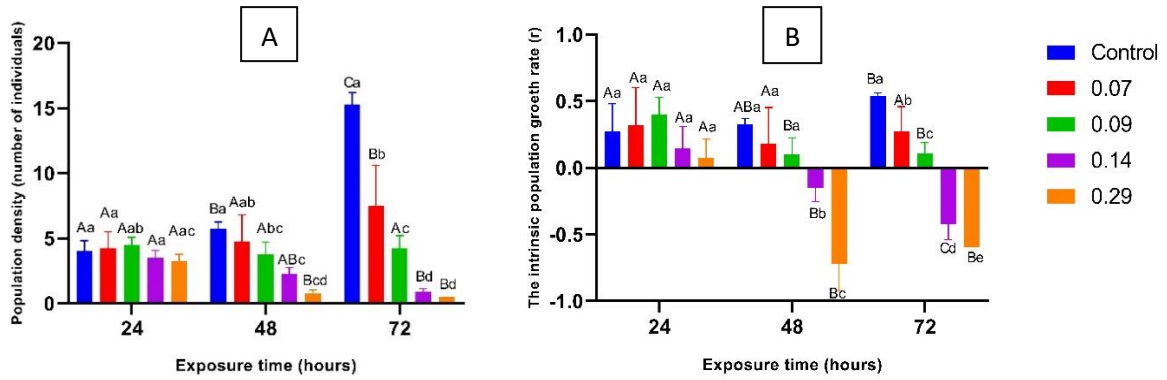

Fig. 2.4: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to high-range concentrations of Cd (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between Cd concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at p ≤ 0.05. Errors are ± standard deviation (N = 4).

The effect of these Cd concentrations on r was similar to the case of PD. Cd concentrations significantly reduced the r-value after 48 and 72h of exposure ($F_{4,16}$, p = 0.001 for both durations) but not after 24h ($F_{4,16}$, p = 0.391). After 48h of exposure, the two highest concentrations (0.14 and 0.29 mg/L) significantly decreased r in comparison with the control, and there was also a significant difference in r between the pair. After 72h, all Cd concentrations significantly decreased r and differed from each other and the control group,

including the lowest concentration (0.07 mg/L). The increase in exposure duration significantly reduced the r ($F_{2, 45}$, p = 0.001) after 72h for the control and after 48 and 72h for the highest three concentrations (Figure 2.4 B). As a result, the LOEC value for Cd in the present study was 0.07 mg/L, which was the lowest Cd concentration used. Thus, it was concluded that the NOEC value was lower than 0.07 mg/L (Table 2.8).

Accordingly, depending on the LOEC value, five low-range concentrations were chosen to be used in the second sub-lethal exposure assessment (0.0000007, 0.000007, 0.00007, 0.0007 and 0.007 mg/L). The exposure of rotifers to low-range sub-lethal concentrations of Cd significantly reduced the PD only after 48h of exposure ($F_{5,18}$, p = 0.021) but not after 24h ($F_{5,18}$, p = 0.079) or 72h of exposure ($F_{5,18}$, p = 0.418). The increase in the duration of exposure significantly affected the PD ($F_{2,}$ 36, p = 0.001), as PD decreased significantly with increasing time of exposure under all treatments (Figure 2.5 A).

The effect of the sub-lethal Cd concentrations on r was similar to that of PD. There was a significant reduction in r only after 48h of exposure ($F_{5,18}$, p = 0.001), and all the concentrations reduced the r-value compared to the control except the lowest concentration. Meanwhile, after 24h ($F_{5,18}$, p = 0.073) and 72h ($F_{5,18}$, p = 0.367) of exposure, there were no differences observed in the r values between all treatments and the control group. The increase in exposure duration had an identical effect as in the case of PD (Figure 2.5 B).

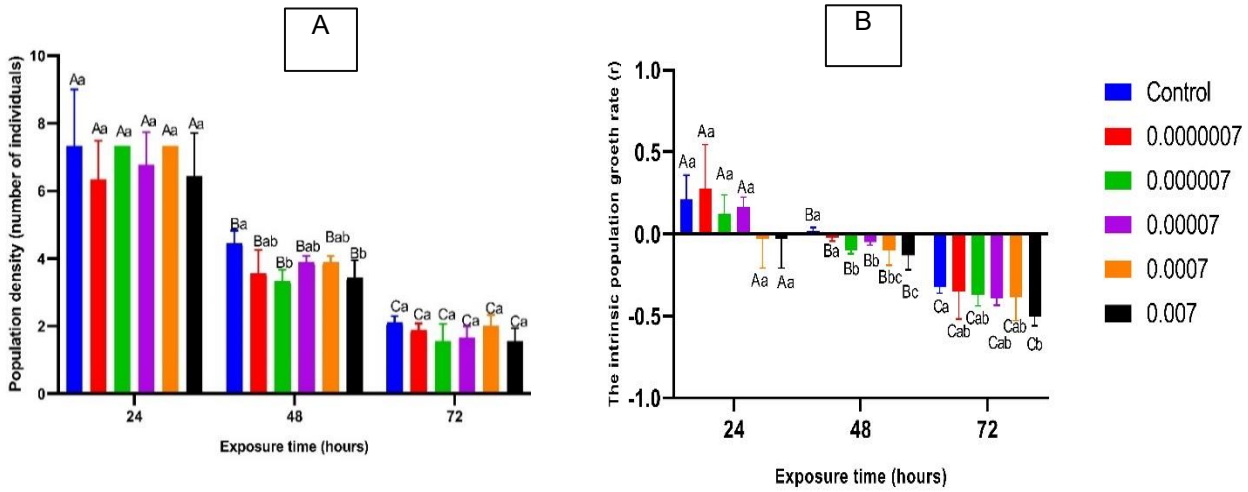

Fig. 2.5: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (low range) sub-lethal concentrations of Cd (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between Cd concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at $p \leq 0.05$. Errors are ± standard deviation (N = 4).

2.3.2.3 The chronic toxicity of Pentachlorophenol (PCP)

The exposure of rotifers to PCP high-range concentrations (0.09, 0.13, 0.19 and 0.39 mg/L) did not have any significant effect on the PD after any duration of exposure ($F_{4,15}$, p = 0.64; $F_{4,15}$, p = 0.96; and $F_{4,15}$, p = 0.092, for 24, 48, and 72h of exposure respectively). The increase in the duration of exposure resulted in steady and significant reductions of PD at every PCP concentration and the control ($F_{2,45}$, p = 0.001). The PD values significantly decreased from 24 h to 48h and from 48h to 72h of exposure for every treatment (Figure 2.6 A).

The exposure of rotifers to PCP did not have a significant effect on r for any exposure duration ($F_{4,15}$, P = 0.70; $F_{4,15}$, P = 0.960; and $F_{4,15}$, P = 0.055 for 24, 48, 72h respectively). However, the duration of exposure had a significant influence on r ($F_{2,45}$, p = 0.002). For the control, r was significantly higher after 72h of exposure than after 24 and 48h, while there was no change in r with increasing time of exposure to PCP (Figure 2.6B). Hence, similar to Cu, it was not possible to determine the LOEC for PCP because the test concentrations did not show a

significant difference from the control. The highest used concentration (0.39 mg/L) was, thus, considered the NOEC value. (Table 2.8).

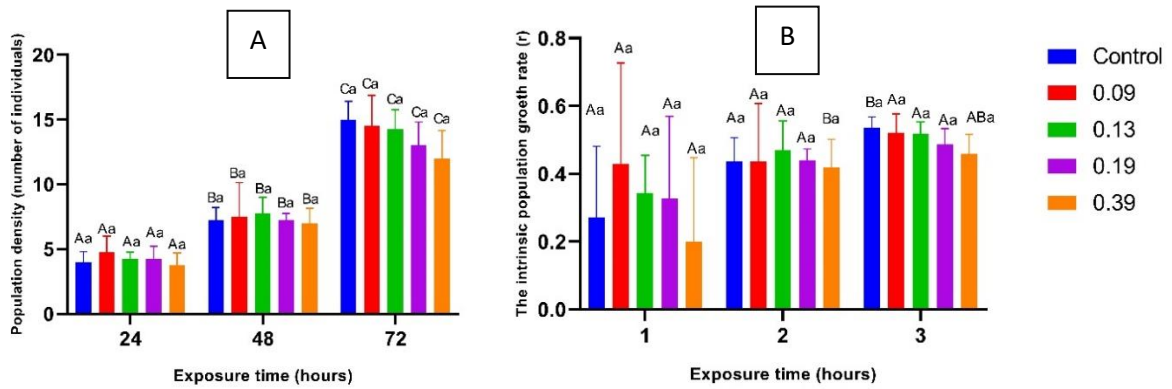

Fig. 2.6: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (high range) concentrations of PCP (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between PCP concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at $p \leq 0.05$. Errors are $\pm$ standard deviation (N = 4).

Accordingly, we started with the highest PCP concentration (0.39 mg/L) to obtain the five low-range concentrations used in the second sub-lethal exposure assessment (0.000039, 0.00039, 0.0039, 0.039 and 0.39 mg/L). There was no significant effect of these PCP concentrations on either the PD ($F_{5,12}$, $p = 0.634$; $F_{5,12}$, $p = 0.231$; and $F_{5,12}$, $p = 0.764$ for 24, 48, 72h of exposure respectively) or r ($F_{5,12}$, $p = 0.628$, $F_{5,12}$; $p = 0.258$, and $F_{5,12}$; $p = 0.619$ for 24, 48, 72h of exposure respectively) after any duration of exposure. Furthermore, the effect of increasing the duration of exposure was similar for both the PD ($F_{2,36}$, $p = 0.001$) and r ($F_{2,36}$, $p = 0.001$). In all cases, these were reduced after 24, 48, and 72h of exposure (Figure 2.7 A, B).

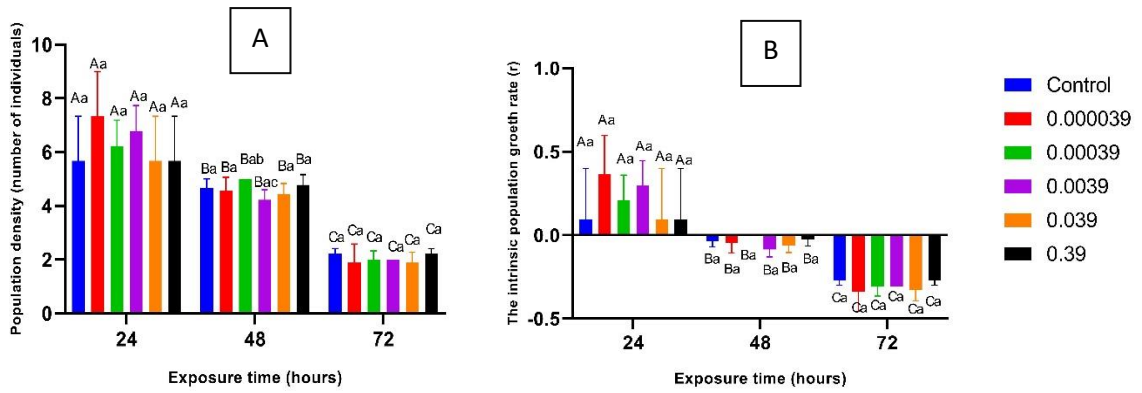

Fig. 2.7: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (low range) sub-lethal concentrations of PCP (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between PCP concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at $p \leq 0.05$. Errors are ± standard deviation (N = 4).

2.3.2.4 The chronic toxicity of 3,4-dichloroaniline (3,4-DCA)

The exposure of rotifers to 3,4-DCA high-range concentrations (6.15, 8.2, 12.3 and 24.6 mg/L) had a significant effect on PD, which was observed at every duration of exposure ($F_{5,18}$, p = 0.016, $F_{5,18}$; p = 0.022; and $F_{5,18}$, p = 0.001 for 24, 48, and 72h of exposure respectively). There were no significant differences in PD between the control group and the "control + acetone" group. The most significant reductions in PD were observed after 72h of exposure, where the highest three concentrations had lower PD values compared to the two controls. Furthermore, the duration of exposure had a significant effect on PD only in the two controls ($F_{5,54}$, p = 0.003). Both controls had the highest PD values after 72h of exposure. Duration of exposure did not affect PD at any of the 3,4-DCA concentrations (Figure 2.8 A).

The effect of 3,4-DCA exposure on r ($F_{5,54}$, p = 0.001) was similar to PD, where the significant reduction in r was observed at every exposure duration for the highest three concentrations ($F_{5,18}$, p =0.011; $F_{5,18}$, p = 0.033; and $F_{5,18}$, p = 0.001 for 24, 48, and 72h of exposure respectively). Moreover, there were no significant differences in r between the control group and the "control + acetone" group. All the exposure concentrations had significantly lower r

compared to the control treatments after 72h of exposure, except the lowest concentration (6.15 mg/L). And there was no significant effect of the duration of exposure on r ($F_{2,36}$, p=0.39) (Figure 2.8 B). From this result, the LOEC for 3,4-DCA was estimated at 8.2 mg/L, while the NOEC value was estimated at 6.15 mg/L. These values were in agreement with previously reported results for rotifers (Table 2.8).

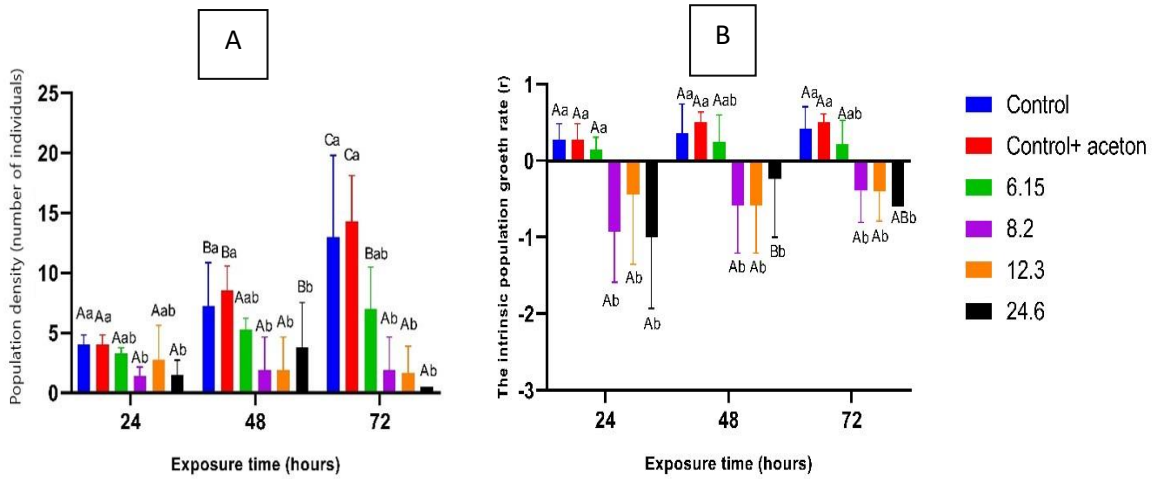

Fig. 2.8: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (high-range) concentrations of 3,4-DCA (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between 3,4-DCA concentrations at the same duration of exposure while upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at p≤0.05. Errors are ± standard deviation (N = 4).

Accordingly, given the LOEC value, five low-range sub-lethal concentrations were chosen to be used in the second sub-lethal exposure assessment (0.00008, 0.0008, 0.008, 0.08 and 0.8 mg/L). There was no significant effect of these 3,4-DCA concentrations on either the PD ($F_{5,12}$, p = 0.221; $F_{5,12}$, p = 0.285; and $F_{5,12}$, p = 0.081 for 24, 48, 72h of exposure respectively) or the r-value at any duration of exposure ($F_{5,12}$, p = 0.605; $F_{5,12}$, p = 0.245; and $F_{5,12}$, p = 0.123 for 24, 48, 72h of exposure respectively). Furthermore, increasing the duration of exposure decreased PD, and this was significant after 72h of exposure ($F_{2,36}$, p = 0.001). The values of r,

however, decreased significantly ($F_{2,36}$, p = 0.001) with an increase in the duration of exposure under all treatments after 48, and 72h of exposure (Figure 2.9 A, B).

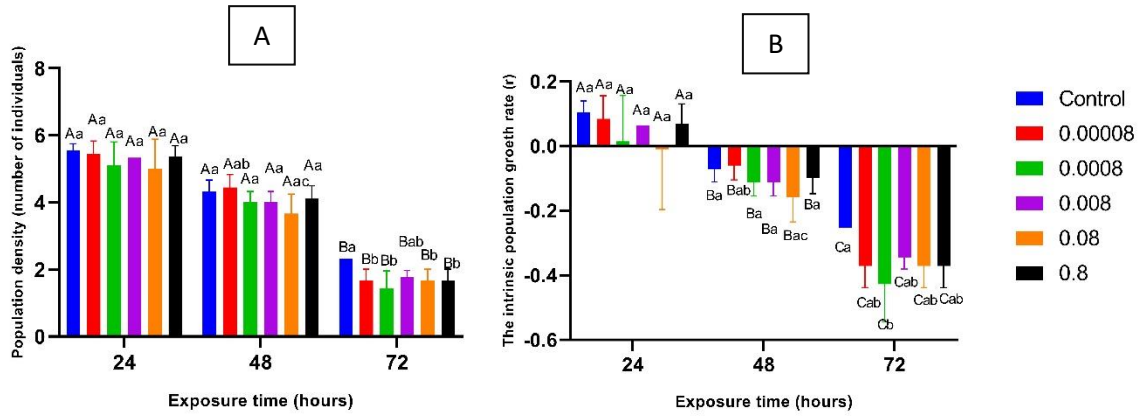

Fig. 2.9: The mean values of population density (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to different (low-range) sub-lethal concentrations of 3,4-DCA (mg/L), for different durations of exposure. Different lower-case letters indicate significant differences between 3,4-DCA concentrations at the same duration of exposure while different upper-case letters indicate significant differences of the same concentrations under different durations of exposure. Differences were considered significant at p ≤ 0.05. Errors are ± standard deviation (N = 4).

2.3.3 Effect of food absence on the PD and r under different toxicants

2.3.3.1 Under copper chronic exposures

There was no effect of food absence on the PD and r of *B. calyciflorus* after 24h of exposure, as there were no differences between both controls and Cu exposure conditions ($F_{1,31}$, p = 0.073 and $F_{1,31}$, p = 0.113 for PD and r, respectively). However, after 48 and 72h of exposure, there were significant differences between the values of PD and r between controls and the Cu exposure conditions, which was significantly higher when the rotifers were fed (p = 0.001 for all comparisons). For "food" conditions, PD was observed to increase with increasing exposure time, while the r remained constant through the whole period under both control and Cu exposure. When not fed, the PD and r values of the rotifers steadily decreased with increasing exposure time (Figure 2.10 A and B).

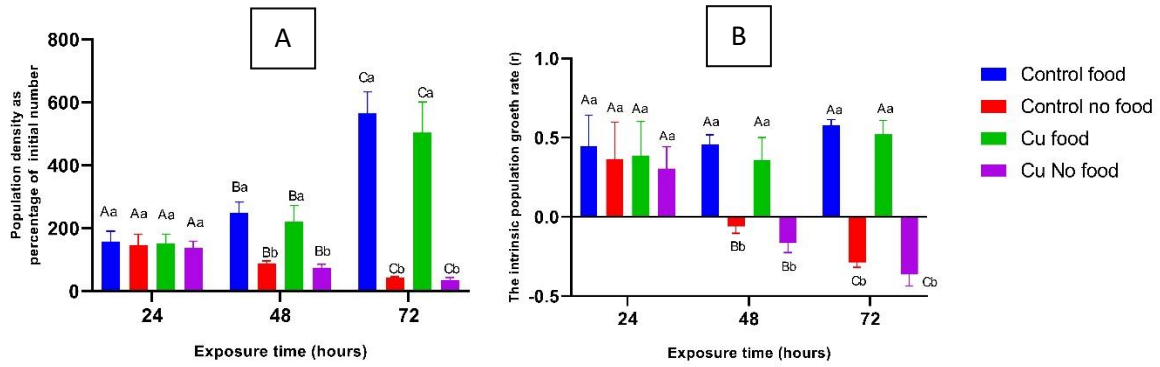

Fig. 2.10: The mean values of the population density as a percentage of the initial number of individuals (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to chronic toxicity of Cu under food and no food conditions for different durations of exposure. Different lower-case letters indicate significant differences between treatments at the same duration of exposure while upper-case letters indicate significant differences in the same treatment under different durations of exposure. Differences were considered significant at $p \leq 0.05$. Errors are ± standard deviation (N = 4).

2.3.3.2 Under cadmium chronic exposures

There was no effect of food absence on the PD and r for *B. calyciflorus* after 24h of exposure to Cd, as there were no differences between values of both controls and Cd exposure conditions ($F_{1, 31}$, $p = 0.078$ and $F_{1, 31}$, $p= 0.080$ for PD and r, respectively). However, after 48 and 72h of exposure, there were significant differences in the values of PD and r between controls and the Cd exposure conditions. Under "control food" values, these were significantly higher when compared with "no food" and "Cd food" conditions ($p \leq 0.002$ for all comparisons). For "food" conditions, PD increased with increasing exposure time after 72h of exposure, while r increased after 72h for the control and decreased after 48 and 72h for Cd exposure. Under "no food" conditions, PD and r values decreased with increasing exposure time (Figure 2.11 A and B).

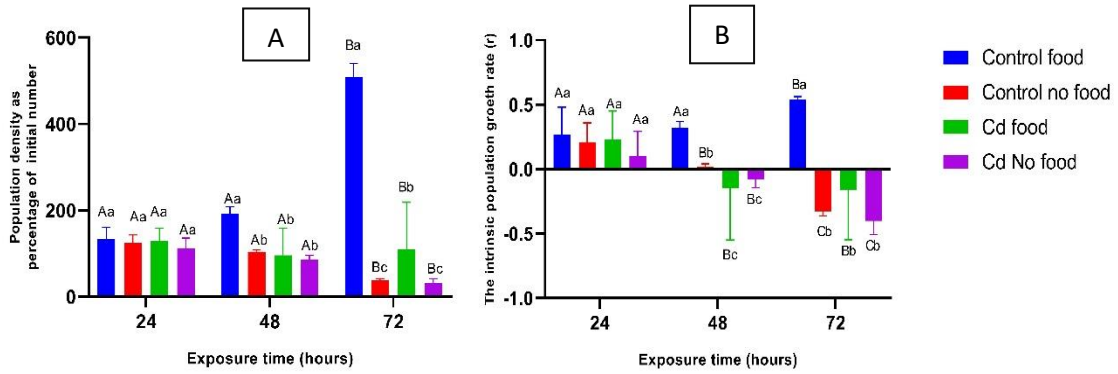

Fig. 2.11: The mean values of the population density as a percentage of the initial number of individuals (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to chronic toxicity of Cd under food and no food conditions for different durations of exposure. Different lower-case letters indicate significant differences between treatments at the same duration of exposure while different upper-case letters indicate significant differences in the same treatment under different durations of exposure. Differences were considered significant at $p \leq 0.05$.

2.3.3.3 Under Pentachlorophenol chronic exposures

There was no effect of food absence on the PD and r for *B. calyciflorus* after 24h of exposure to PCP. Furthermore, there were no differences between values of both control and PCP exposure conditions ($F_{1, 31}$, $p = 0.107$ and $F_{1, 31}$, $p = 0.302$ for PD and r, respectively). However, after 48 and 72h of exposure, there were significant differences between the values of PD and r for the controls and the PCP exposure conditions. Under "control food" values were significantly higher when compared with "no food" and "PCP food" conditions ($p \leq 0.001$ for all comparisons). For "food" conditions, PD increased with increasing exposure time, while the r remained constant during the whole duration of exposure for control and PCP exposure. Under "no food," conditions, the PD and r values decreased with increasing exposure time (Figure 2.12 A and B).

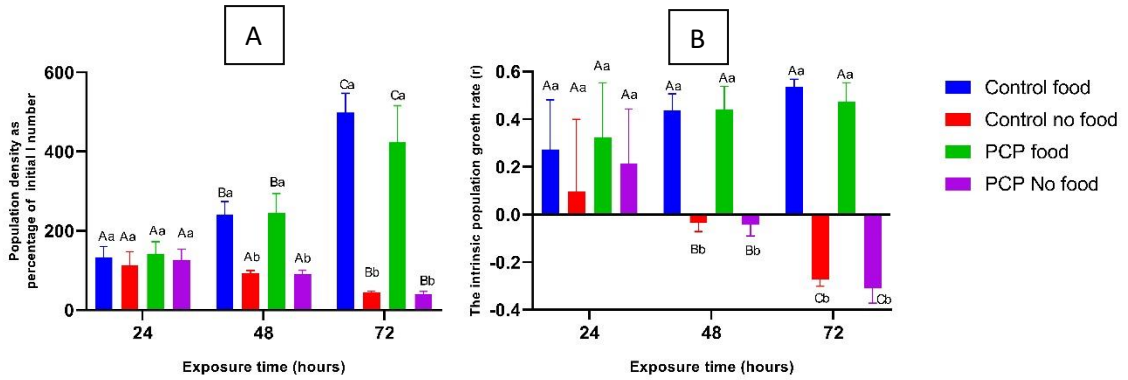

Fig. 2.12: The mean values of the population density as a percentage of the initial number of individuals (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to chronic toxicity of PCP under food and no food conditions for different durations of exposure. Different lower-case letters indicate significant differences between treatments at the same duration of exposure while upper-case letters indicate significant differences in the same treatment under different durations of exposure. Differences were considered significant at $p \leq 0.05$.

2.3.3.4 Under 3,4-dichloroaniline chronic exposures

There was a significant effect of food absence on the PD and r for *B. calyciflorus* after 48 and 72h of exposure to 3,4-DCA. After 24h, the PD and r values of the "no food" control and 3,4-DCA exposure conditions were similar to the "food" control, but all the "no food" conditions were significantly higher than the 3,4-DCA condition when fed ($F_{1, 31}$, p= 0.045 and $F_{1, 31}$, p= 0.004 for PD and r, respectively). After 48 and 72h of exposure, the food control condition had significantly higher PD and r values compared to the two "no food" conditions and the "food" 3,4-DCA condition ($p = 0.001$ for all comparisons). For "food" conditions, the PD increased with increasing exposure time for both control and 3,4-DCA conditions, while the r remained constant during the whole duration of exposure for control and decreased for the "food" 3,4-DCA exposure condition. Under "no food" conditions, the PD and r values significantly decreased after 72h of exposure (Figure 2.13 A and B).

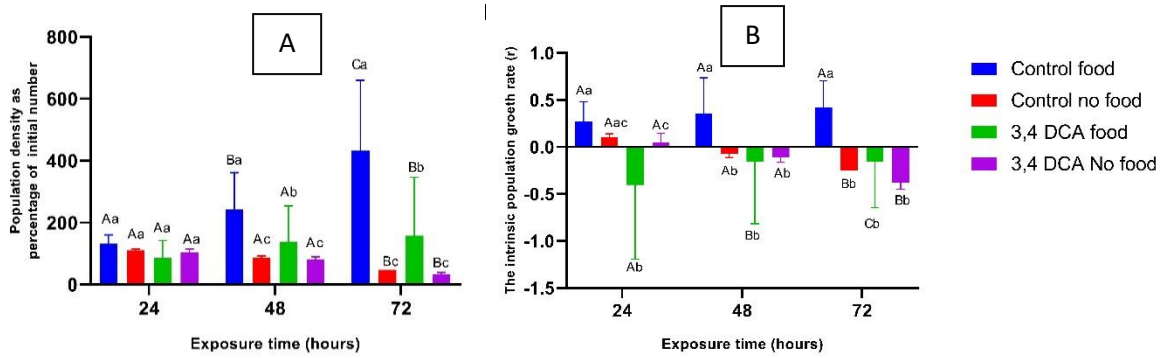

Fig. 2.13: The mean values of the population density as a percentage of the initial number of individuals (A) and the intrinsic growth rate (B) of *B. calyciflorus* exposed to chronic toxicity of 3,4-DCA under food and no food conditions for different durations of exposure. Different lower-case letters indicate significant differences between treatments at the same duration of exposure while upper-case letters indicate significant differences in the same treatment under different durations of exposure. Differences were considered significant at $p \leq 0.05$.

2.3.4 The relationship between PD and r under chronic toxicity with and without food

The relationship between the PD and the r was positive for all toxicants under both "food" and "no food" conditions; i.e., population density increased with an increase in the growth rate. However, the slope of increase in r when the PD increased was higher for all toxicants under "no food" conditions compared to the "food" conditions. The slope of the "no food" condition ranged from 0.119 for 3,4-DCA to 0.139 for Cu, while it ranged from 0.020 for Cu to 0.106 for 3,4-DCA under the "food" condition (Figures 2.14 A and D respectively). Meanwhile, Cd (Figure 2.14 B) and PCP (Figure 2.14 C) had intermediate values for "food" and "no food" conditions. Furthermore, the goodness of fit of the model under "no food" conditions was higher than in the "food" conditions which had lower R^2 values.

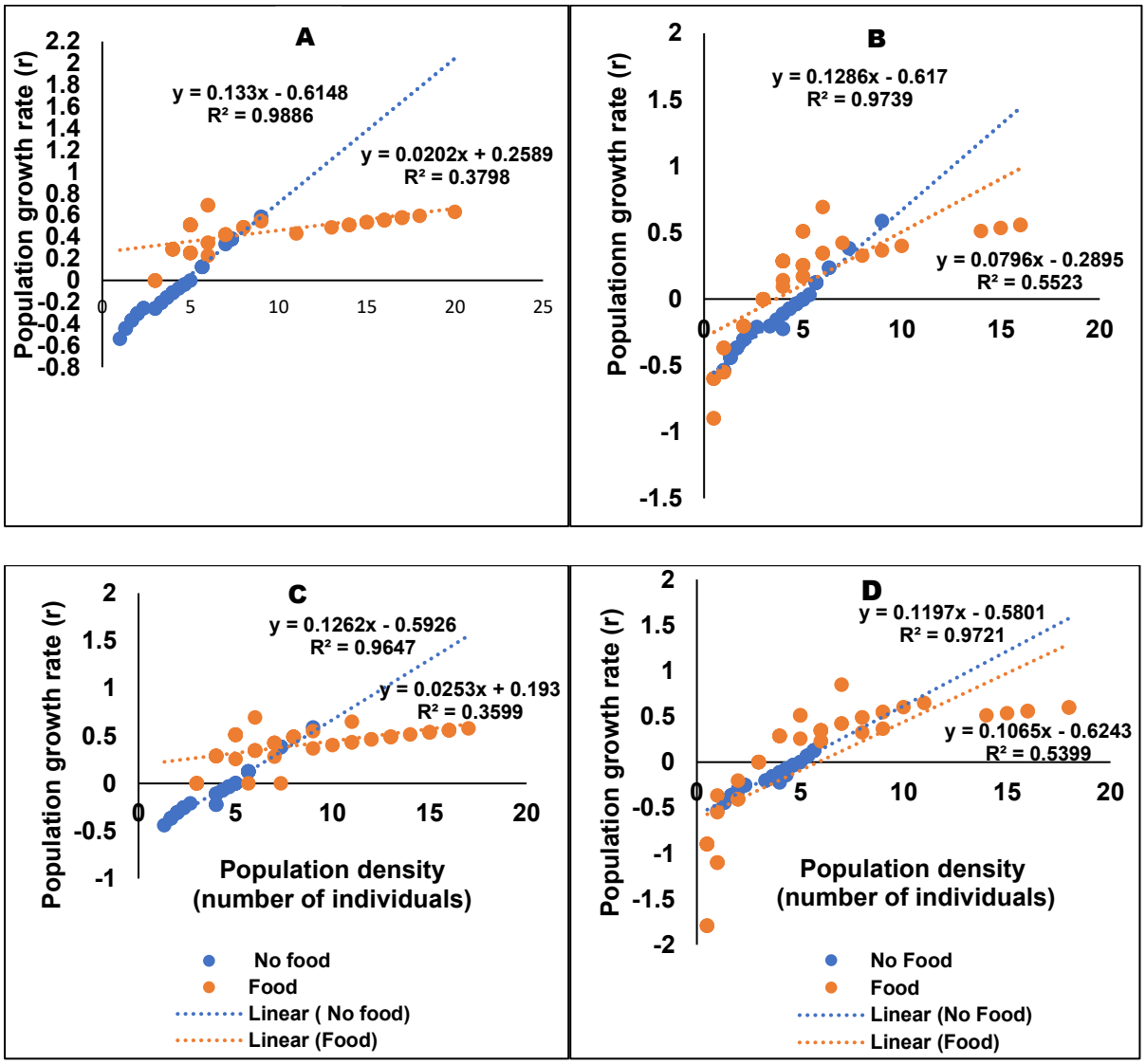

Fig. 2.14: Relationship between the population density and the intrinsic growth rate for *B. calyciflorus* exposed to Cu (A), Cd (B), PCP (C), and 3,4-DCA (D) chronic exposure under food (orange) and no food (blue) conditions.

2.4 Discussion

2.4.1 The median lethal concentration (LC_{50}) of the different toxicants

Modern concentration-response tests use a variety of thresholds to compare the varying toxicities of substances. The LC_{50} is a particularly important and widely used measure, among these. This study showed that the median lethal concentration (LC_{50}) of all toxicants fell within the range of concentrations assessed, except for 3,4-DCA which had a higher LC_{50} than the assessed range. Cu, with the lowest LC_{50} (0.012 mg/L) was the most toxic. This value for Cu is less than half of the LC_{50} reported by Snell et al. (1991a) for *B. calyciflorus* and much lower than that reported for *B. calyciflorus* (Snell and Moffat, 1992) (Table 2.9).

Contrary to Cu, 3,4-DCA was the least toxic, with the highest LC_{50} (124 mg/L). This concentration was double the value reported by Snell et al. (1991a), Ferrando and Andreu-Moliner (1991) and Ferrando et al., (1992) for *B. calyciflorus*.

PCP and Cd had intermediate LC_{50} values of 1.950 mg/l and 1.460 mg/l, respectively (Table 2.9). The LC_{50} value of PCP for this study was similar to values reported by Ferrando et al. (1992) and Snell and Moffat (1992) for *B. calyciflorus.* However, it was higher than those reported by Preston et al. (1999) and Snell et al. (1991a). For Cd, LC_{50} values in this study were similar to the results reported by Snell et al. (1991a) but higher than the results reported by Sarma et al. (2006).

Although acute toxicity tests in the present study were performed for 24h and in similar conditions to that of previously reported studies, differences between the results may be due to differences in rotifers species or strains. Rotifers, as a species complex, include various closely related species having similar characteristics but which may vary in their responses to environmental stressors (Granada et al.,2023). Moreover, toxic effects might also vary with age of the rotifers. Although the neonates were used for the current and in previously reported

studies, it cannot be confirmed that they were at the same age in terms of number of hours. Rotifer neonates can enter the juvenile stage within a few hours (and reach the adult stage within 21h) after hatching. Therefore, these seemingly small differences between populations could affect test sensitivity. Neonates, for example, might be more sensitive than juvenile and adult rotifers due to their lower ability to metabolise toxicants.

Table 2.9: Concentrations of the LC_{50}, NOEC, LOEC, and the upper concentrations used in the cellular energy allocation (CEA) experiment of the four test toxicants

Toxicants	LC50	NOEC	LOEC	Upper concentrations used in the CEA experiment
	mg/L	mg/L	mg/L	mg/L
Cu	0.012	0.0024	>0.0024	0.006
Cd	1.459	<0.070	0.070	0.006
PCP	1.957	0.390	> 0.390	0.390
3,4-DCA	123.800	6.150	8.200	0.800

2.4.2 Chronic toxicity of high-range sub-lethal toxicant concentrations on the population density (PD) and the intrinsic growth rate(r)

The two heavy metals used in this study differently influenced the PD and r of rotifers at high-range exposure concentrations. Cu had a similar effect as the control: the PD increased with an increase in time of exposure, but r remained similar throughout the experiment. Cd was found to be more toxic, eliciting a significant reduction in PD and r with increasing concentration and exposure time.

This difference in Cu and Cd toxicity may be due to using higher concentration ranges for Cd compared with Cu; however, the severity of the impact of Cd has been previously reported to be greater than that of Cu (Gama-Flores et al., 2007). The increase in PD can occur in low concentrations of some essential metals, such as Cu, which can stimulate reproduction. This phenomenon is called hormesis, which has been observed in several zooplankton species

(Calabrese and Baldwin, 2003; Gama-Flores et al., 2007). However, essential trace elements have been found to be more toxic in some cases (Santos-Medrano and Rico-Martınez, 2013).

Although the reproduction rate can be negatively influenced by most toxicants, for example Cd in this study, stimulatory effects have also been observed under low concentrations of some metals (Gentile et al., 1982). The present study found that the NOEC of Cu was 0.0024 mg/L after 72h (Table 2.9), which agrees with results from Janssen et al. (1994) who reported no effects on r for *B. calyciflorus* at 0.0025 mg Cu/L. However, our results were lower than those reported by Snell and Moffat (1992), who reported a NOEC of 0.020 mg Cu/L after 48h of exposure. Furthermore, they reported the LOEC for Cu to be 0.030 mg/L, in agreement with Gama-Flores et al. (2007). Their findings may explain why a LOEC was not determined in this study, as it was out of the concentration ranges that were used.

For Cd, the reduction in PD after 48 and 72h exposure period might be explained by the poor rate of food ingestion (Charoy, 1995), as energy intake by rotifers is only sufficient for maintenance, leading to the reduction of the population growth rate (Nandini and Rao, 1998). The LOEC value of Cd (0.070 mg/L) found in the current study (Table 2.9) agrees with that reported by Snell and Moffat (1992), but it is higher than those reported by Sarma et al. (2006) and Gama-Flores et al. (2007), all for *B.calyciflorus*. As the LOEC for Cd in the present study was 0.070 mg/L, it may be concluded that the NOEC is lower than 0.070 mg/L, which was in accordance with Snell and Moffat (1992), who determined it to be 0.040 mg/L.

The two organic toxicants used in this study influenced the PD and r of rotifers differently. The range of concentrations of PCP had no effect on PD and r, similar to control exposures. In contrast, 3,4-DCA significantly decreased PD and r under three out of four of the used concentrations. The negative impact of the 3,4-DCA on PD and r may be due to hindering food consumption and assimilation, as the feeding rates of the rotifers can be reduced under the

stress of toxicants (Ferrando and Andreu, 1993). Additionally, the toxicants may affect the number of offspring produced by delaying the age of the first reproduction and, thus, increasing the birth interval (Snell and Moffat, 1992). Furthermore, enzymes of acetylcholine metabolism, such as choline acetyltransferase and acetylcholinesterase, can be disrupted in vertebrates and some invertebrates due to organic toxicants, and this can also occur in rotifers, causing lower growth and reproduction (Ferrando et al., 1993a). The NOEC value for the 3,4-DCA found in the current study (Table 2.9) was higher than previously reported results by Ferrando et al. (1993a) and Janssen et al. (1994), while the LOEC value was within the range of the two values reported by Ferrando et al. (1993a) and Janssen et al. (1994).

In this study, a LOEC value for PCP could not be determined (Table 2.9), as the PCP concentration used, chosen as dilutions of $LC_{50,}$ did not show any negative effect on PD and r. This result was in agreement with Janssen et al. (1994), who reported that PCP concentrations of 0.20 and 0.40 mg/L did not affect the PD of *B. calyciflorus* after 72h of exposure. A significant decrease in PD was, however, found at 0.80 mg/L, which was identified as the LOEC. The results of the current study disagree with those of Snell and Moffat (1992), who determined the LOEC and NOEC value of PCP for *B. calyciflorus* to be 0.19 mg/L and 0.11 mg/L, respectively. Consequently, the actual LOEC value is more likely to be similar to the results of Janssen et al. (1994). Assuming that these rotifer species are the same strains would suggest that the aforementioned differences can be acceptable within the normal range of variation for a single species. However, the rotifers assessed may represent dissimilar strains within a species complex (Granada et al., 2023), which might be responsible for the differences between the conclusions of this study and those of other studies.

It can be concluded that the negative chronic effects of 3,4-DCA were most severe, followed by the effects of Cd, which also resulted in negative r values. On the contrary, PCP and Cu concentrations used in this study were not toxic, with PD and r outcomes being similar to the control. Comparing the acute with the chronic toxicity results, the order of the highest and lowest toxicity appears to be reversed. Various authors (Snell and Moffat, 1992; Marcial et al., 2005) have also reported that chronic exposure tests may be more sensitive to toxicity than acute tests. Also, this study showed that not only the concentration of the toxicant but also the duration of exposure was important for evaluating toxic effects on rotifers.

2.4.3 Chronic toxicity effects of low-range sub-lethal concentrations of used toxicants on the population density (PD) and the intrinsic growth rate(r)

Results of the sub-lethal chronic exposures showed that all concentrations used for each toxicant had similar responses compared to the control after 24h of exposure, and if there was any difference, it was after 48 and/or 72h of exposure. Consequently, the highest concentrations used in this sub-lethal chronic test were used as the highest concentrations in the energy budget experiments (Chapter 4) and diluted to generate a range of concentrations. However, for both Cu and Cd, we used one intermediate concentration to be comparable with the literature, as the CEA requires sub-lethal concentrations that can induce differential energy allocations in natural populations of the studied organism. Therefore, the highest concentration used in the energy budget assessments was 0.006 mg/L for Cu and Cd, but 0.390 and 0.800 mg/L, respectively, for PCP and 3,4-DCA. The selected duration of exposure was 24h (Table 2.9).

2.4.4 Effect of food absence and the relationship between the population density (PD) and the intrinsic growth rate(r)

Except for 3,4- DCA where there was a significant difference between food and no food conditions, the absence of food had no effect on the responses of PD and r across the sub-lethal concentrations used after exposure for the first 24h for the toxicants assessed. However, the

no-food conditions had higher PD and r values that were not different from the control condition with food. These results agree with a previous study (Kirk, 1997) that described the average starvation survival time to range from about 10 to 120 h in several species of rotifers. Accordingly, the duration of exposure for future experiments (i.e., the energy budget experiments) was recommended to be 24h. The food/no-food experiments confirmed a no-food regime to be acceptable.

The relationship between the PD and the r was positive for all toxicants under both food and no-food conditions, with population density increasing with an increase in the growth rate. However, the slope of increase in r when the PD increases was higher for all toxicants under no-food conditions compared with the food conditions. This result may be explained according to the strategy of energy allocation which proposed that energy may be more allocated to reproduction under food-limited stress conditions (Kirk, 1997; Kirk, 1999).

2.5 Conclusion

This chapter provided an extensive understanding of the appropriate circumstances and procedures required for effective rotifer culture maintenance in laboratory conditions. Different fundamental elements of the rotifer cultivation process were discussed, including the culture media, suitable temperature, pH, light, and nutrient content. Furthermore, periodic monitoring of the rotifer cultures was discussed. Examples involved the regular removal of waste materials and monitoring of water quality to ensure clean and favourable environmental conditions that enable the normal growth and reproduction of the rotifers.

Furthermore, the lethal effects of four toxicants, Cu, Cd, PCP, and 3,4- DCA, on neonates of the *B. calyciflorus* were identified after 24h of exposure, quantifying the threshold concentrations as LC_{50}. Afterwards, LOEC and NOEC for chronic high- and low-range sub-

lethal exposures where quantified, using population growth parameters as endpoints after 24, 48, and 72h test periods with food provided.

Thirdly, the influence of low-range sub-lethal concentrations, which were determined based on the NOEC or LOEC values, on population density and the intrinsic growth rate of population parameters of rotifers were evaluated through chronic toxicity after 24, 48, and 72h of exposure without providing food. The results obtained from these latter tests provide the appropriate upper limit concentrations to be used in the CEA experiments in Chapter Four, which need to be undertaken below the lethal thresholds for the toxicants of interest.

CHAPTER THREE

Chapter 3: Method development of assays to determine protein, lipid, and carbohydrate contents in the freshwater rotifers *Brachionus calyciflorus*

3.1 Aims of this chapter

This chapter aims to:

a) Review the literature relating to methods to assess protein, lipid, and carbohydrate in biological samples.

b) Use this information to pilot rotifer-specific methods to assess protein, lipid, and carbohydrate in samples.

c) Fully develop analytical methods for the determination of protein, lipid, and carbohydrate concentrations in rotifer material as well as ensure appropriate dilution factors, calibration range, and optimisation of assay times.

d) Benchmark results obtained with previously published data.

3.2 Introduction

All animals need to consume food which can be broken down by metabolic processes. Proteins, lipids, and carbohydrates are the principal components of food. In one sense, these different compounds represent energy available (Ea; i.e., chemical energy which can also be transformed and used by organisms) for cellular processes such as growth, reproduction, maintenance of structures, and other functions. In another sense, the amounts and proportions of proteins, lipids, and carbohydrates in an organism represent the energy stored. Jointly, with energy consumption, the energy available within an organism is a critical component of achieving an energy budget balance. Thus, a cellular energy balance approach to quantifying energy available [Ea] and energy consumption [Ec] can be used to establish the relative stress an organism is experiencing (De Coen and Janssen, 1997, 2003). The first step in establishing an energy balance is to quantify the available energy, i.e., amounts of protein, lipid, and

carbohydrates in an organism. Many methods have been used to measure the protein, lipid, and carbohydrate content in different biological samples, and these are reviewed below. However, there remains a need to establish methods suitable for rotifer samples. Thus, the purpose of this chapter is to trial and validate analytical approaches specifically for rotifer samples.

3.2.1 General review of methods for protein analysis

The measurement of the absorbance at 280 nm (UV range) is the simplest method that has been used to measure protein content in samples (Olson and Markwell, 2007). Proteins can absorb UV light easily due to their aromatic amino acid content. Amino acids such as tyrosine, tryptophan, and phenylalanine (aromatic) have a high ability to absorb UV light, and this absorbance has been correlated with total protein concentration in a sample (Olson and Markwell, 2007; Aitken and Learmonth, 2009). This method is simple, straightforward, and very accurate when testing pure protein or different solutions for the same protein (Olson and Markwell, 2007) but is not perfect for protein mixtures, as the proportion of aromatic amino acid and non-aromatic amino acid content can differ (Olson and Markwell, 2007). Moreover, some non-protein contents can interfere with results, as these molecules are also able to absorb UV light. Therefore, high errors can result, particularly when protein mixtures or complicated samples are assessed (Olson and Markwell, 2007; Aitken and Learmonth, 2009).

Consequently, several colorimetric methods were designed to overcome drawbacks associated with measuring UV absorbance. These methods are based on the interaction between protein samples and specific reagents to produce a colour change that can be measured by spectrophotometry. The protein content is calibrated according to a standard curve of known concentrations of a specific protein used as a reference. Depending on the kind of chemicals, there are three commonly used assays: the Lowry assay, the Bradford assay, and the Smith assay (Sapan et al., 1999).

The Lowry method was developed by Lowry et al. (1951). It has good sensitivity to different proteins, and it has been used extensively by researchers for decades. The method is based on the Biuret reaction and uses two reactions in sequence. The first is the reaction between a copper ion complex (Cu^{2+}) and the peptide bonds of protein in alkaline solution to produce reduced copper (Cu^{+})-protein complex. The second reaction is between the reduced copper-protein complex and the Folin-Ciocalteureagent (phosphomolybdate and phosphotungstate) to produce reduced Folin reagent (Gornall et al., 1949; Olson and Markwell, 2007; Waterborg, 2009). The amount of reaction product can then be measured with a spectrophotometer in the range of 500 to 750 nm (Lowry et al., 1951; Olson and Markwell, 2007).

However, as is the case with a direct UV-absorption measurement, the Lowry method is dependent on the amino acid content. Thus, the method is a proxy for protein content and not an absolute measure. Nonetheless, this method has some advantages. It is sensitive and, therefore, can be used to measure protein concentrations as low as 10 µg/mL (Waterborg, 2009). Several substances can, however, interfere with this method, such as detergents, EDTA, carbohydrates, glycerol, uric acid, calcium, magnesium, and others (Olson and Markwell, 2007). Also, this method is time-consuming when compared with other methods (Olson and Markwell, 2007).

The Bradford method (Coomassie Blue, CB, G-250 dye-binding) is a colorimetric assay that was introduced by Bradford et al (1976). This method depends on the binding of Coomassie brilliant blue G-250 dye (CBBG) to amino acids such as arginine, tryptophan, tyrosine, histidine, and phenylalanine. The CBBG binds to these molecules, generating an absorbance maximum at 595 nm when examined by spectrophotometry. The Bradford method has some advantages, which include being straightforward, rapid (Kruger, 2009), and cost-effective (Olson and Markwell, 2007). Moreover, it can be conducted at room temperature and is

compatible with substances such as solvents, salts, and buffers (Thermo Fisher Scientific Inc., n.d.).

The Bradford assay is more sensitive – as it can be used to measure protein concentrations as low as 1 µg/mL – and also more accurate – as it is less prone to interference by reagents and non-protein substances – than the Lowry assay (Kruger, 2009). As a result of these factors, it has been used widely and has become the most common approach for protein quantitative analysis in many laboratories.

For all its popularity, the Bradford method has some disadvantages. For example, the main response to CBBG is from arginyl, so this method is used as an essential assay only when the sample is rich in arginyl and using an arginine-rich standard (Olson and Markwell, 2007). Furthermore, CBBG can bind easily to the lysyl residue of the protein (Compton and Jones,1985; Congdon et al., 1993). Therefore, this variation in the response to different proteins can affect the results (Read and Northcote, 1981; Friedenauer and Berlet, 1989; Stoscheck,1990). Also, it is customary to lyse samples in a particular surfactant solution such as BugBuster (Novagen), and this may contain other substances that are not compatible with the Bradford assay. It has been reported that detergent or surfactant can interfere with results; thus, the cleaning approaches used must be sensitive to this limitation (Olson and Markwell, 2007).

Two basic formats of the Bradford method were developed depending on the detection range. The micro-assay format has been designed for protein masses between 1 and 20 µg. On the other hand, the macro-assay format has been designed for protein masses in the range of 20 to 100 µg. It is more practical to use the micro-assay format in the microwell plate, as it only requires a small volume of the sample and reagents, generating limited waste. A further

advantage is that one concentrated sample can be used for a large number of assays (Olson and Markwell, 2007).

The Smith assay (Bicinchoninic Acid [BCA]) assay was also developed by Smith et al. (1985) for determining protein content. There are similarities between the Lowry and BCA assays. For example, they have similar sensitivity, but the BCA is faster. This is because the BCA is steady under alkaline conditions and, therefore, can be conducted in one rather than the two steps required in the Lowry method (Wiechelman et al., 1988; Gates, 1991; Walker, 2009). Both assays are based on the Biuret reaction, which relies on the transformation of Cu^{2+} to Cu^{+} in alkaline conditions. But in the case of the BCA method, the Cu^{+} will be generated by reaction with BCA, cysteine, cystine, tryptophan, and tyrosine residues in the sample (Olson and Markwell, 2007).

The result of this reaction can be the production of a purple coloration, which needs to be incubated at high temperatures to increase the sensitivity and reduce the variation in the response of protein compositions. The BCA assay can be incubated for 15 min at 60 ˚C to end the reaction (Olson and Markwell, 2007). Therefore, when these samples are incubated at room temperature to cool, the absorbance does not increase significantly. Alternatively, samples can be incubated at 37 ˚C for 30 min. (Olson and Markwell, 2007). Walker et al. (2009) indicated that, after the incubation time, the colour is steady for at least one hour. The absorbance can be determined at 562 nm by a spectrophotometer. Another benefit of the BCA assay is that it is compatible with most substances that interfere with the Lowry assay, such as several detergents and denaturing agents (Olson and Markwell, 2007).

The BCA method has some disadvantages. The first is that the response to the BCA assay depends on the amino acid composition of the protein tested. Therefore, the BCA assay can only determine the absolute concentration of protein when the results are compared to similar

known samples on the standard curve (Walker, 2009). Furthermore, the presence of reducing sugars (e.g., glucose) can make the BCA assay less accurate (Gates, 1991; Walker, 2009). A high concentration of reducing sugars can cause intense colour development when the BCA assay is incubated at 60 ˚C which can maximize the sensitivity and therefore it is preferable, for the BCA assay, to be incubated at 37 ˚C as the interference of reducing sugars can be substantially reduced (Gates,1991).

The BCA assay was adapted for high-throughput analysis by using a 96-microwells format. This assay can be used when many unknown samples are being analysed. A major benefit of this format is that many points can be generated for the calibration curve. Consequently, the accuracy of the assay can be increased (Olson and Markwell, 2007).

3.2.2 General review of methods for lipid analysis

The most widespread methodology of lipid analysis is the gravimetric technique (Liu, 2014). This method requires lipid extraction from biological samples using solvent; thereafter, the extract needs to be dried and the mass of lipid measured (Han et al., 2011). Such gravimetric approaches are the main traditional methods recommended for evaluating the content of lipids in many samples (Greenspan and Fowler, 1985; Greenspan et al., 1985; Olsen and Henderson., 1989; Smedes and Askland, 1999). Gravimetric lipid analysis was first introduced in the late 1950s by Bligh and Dyer (1959). In their research, they succeeded to measure the lipid content in fish tissue by using a mixture of chloroform, methanol, and water as a solvent to homogenize the sample, and then measuring extracted lipids by the gravimetric quantitative approach. The method was also applied for lipid measurements in several samples, such as in fish (Linko et al., 1985; Payne et al., 1999), milk (Arnould et al.,1995), microalgae (Cooksey et al., 1987; Feng et al., 2013; Liu, 2014) and invertebrates (Smolders et al., 2004; Erk et al., 2011)

Although the gravimetric quantification technique is straightforward and used extensively, it has some drawbacks. For example, a large volume (580 mL) of homogenized sample is needed (Bligh and Dyer 1959); it is labour-intensive and time-consuming (Chen et al., 2009); and the precision depends upon the efficiency of the lipid extraction step (Liu, 2014).

Another traditional method for evaluating the content of lipids in samples is high-performance liquid chromatography (HPLC) (Olsen and Henderson, 1989). HPLC can be used extensively to perform several biochemical and analytical tasks such as the identification, purification, separation, and quantification of the specific element from mixtures (Han et al., 2011). Specifically, it can be used to analyse lipid classes in crude lipid samples (Jones et al., 2012).

HPLC is a useful methodology. It can be automated and can provide accurate results in a short time (Han et al., 2011; Liu, 2014). On the other hand, HPLC is expensive (Han et al., 2011), has low sensitivity to certain substances, and requires specialist skills (Liu, 2014).

The Sulfo-Phospho-Vanillin (SPV) method is another method for analysing lipids. It was established by Chabrol and Charonnat (1937). SPV is a colorimetric test completely different from traditional methods due to its simplicity and fast response (Cheng et al., 2011; Han et al., 2011). Cheng et al. (2011) employed this assay to test the lipid content in different types of microalgae samples. These samples were treated with a suitable solvent (e.g., chloroform, methanol, or a mixture of methanol and chloroform) to extract the lipid. Thereafter, the extracted lipid was loaded into microplates and incubated for 10 min at 90 ˚C to evaporate the solvent. Sulfuric acid was then added, and the microplate was again incubated for 20 min at 90 ˚C. After a short time to cool down to 25 ˚C, the background absorbance was read at 540 nm. Thereafter, the vanillin-phosphoric-acid reagent was added to produce colour, and the absorbance was read at 540 nm.

Sulfo-Phospho-Vanillin (SPV) assay is considered a relevant and appropriate method, as it can be applied to the quantification of total lipids for a wide variety of samples (Johnson et al., 1977; Nakamatsu and Tanaka, 2004; Haskins et al., 2010; Cheng et al., 2011). The method is inexpensive and requires only 100µL of homogenized samples. Thus, the method can be adapted for 96 well plates which considerably increases sample throughput (Cheng et al., 2011; Pinger et al., 2022). Furthermore, the SPV assay requires less time (<2 hours) and less labour (Han et al., 2011; Pinger et al., 2022).

However, some problems can occur when the SPV assay is applied for measuring the lipid content in a different source of oil. For example, it was found that the results of the mean lipid/absorbance ratio were 0.0055 and 0.0075 Au/µg for cod liver oil and canola oil, respectively (Cheng et al., 2011). These results suggest that there was a relationship between the assay response and the fatty acid composition of lipids and therefore an appropriate standard must be selected (depending on the source of oil) for analysing lipid content in various types of samples (Cheng et al., 2011). In addition to reagent response, the solvent used to extract lipid is important, with some solvent regimes being more efficient than others as indicated by Sheng et al. (2011) and Cheng et al. (2011). Therefore, the selection of solvent for lipid extraction is an essential part of lipid measurement. Because the extraction process is influenced by the polarity of the solvents used, it is common for a mixture of solvents to be used to ensure the extraction of a range of lipids with contrasting polarity (Sheng et al., 2011).

The selection of solvent needs to consider the lipid form in the sample matrix. For example, in animal, plant, and microbial cells, the lipid content is surrounded by the cytoplasmic membrane system, which is double-layered and made up mostly of phospholipids, consisting of a polar head and two nonpolar tails (fatty acid residuals). Therefore, polar organic solvents are generally utilized together with non-polar solvents to extract lipids. Polar solvents are needed to penetrate the lipid bilayer of the cytoplasmic membrane system, but these cannot be used

alone as they have low levels of solubility and select for neutral lipids. For this reason, nonpolar solvents must be applied to increase the breadth of lipid extraction (Sheng et al., 2011; Liu, 2014).

The Nile red fluorescence staining method is an alternative assay that can be used for lipid quantification. It is worth mentioning that the most important advantage of the Nile red fluorescence method, when compared with other methods, is that there is no need to extract the lipid, as the measurements can be performed directly on the sample. However, the use of solvents in this method is necessary for two reasons: 1) to dissolve the dye and 2) to carry the dye into the sample. Nile red is a benzophenoxazone dye; its molecular formula is $C_{20}H_{18}N_2O_2$ and its molecular weight is 318 g/mol. The solubility of Nile red in water is very limited (<1 μg/mL) but can increase to 1 mg/mL in some organic solvents such as acetone (Liu, 2014).

The fluorescent Nile red dye was initially applied in mammalian tissues as a selective dye for the detection of lipid droplets (Greenspan et al., 1985; Fowler and Greenspan, 1985). Thereafter, it was often employed to assess the lipid content of microalgae (Elsey et al., 2007; Chen et al, 2011; Liu, 2014), yeast species (Kimura et al., 2004; Sitepu et al., 2012), mammalian oocytes (Genicot et al.,2005), and invertebrates (Cole et al., 1990; Castell and Mann, 1994; Alonzo and Mayzaud, 1999). This assay has been under modification in the last thirty years. For example, Cooksey et al. (1987) used this assay to study the lipid content of algal cells, adding Nile red acetone solution to a suspension of cells. After shaking on a vortex, the samples were analysed using a spectrofluorometer, with the excitation and emission wavelengths at 488-525nm and 570-600nm, respectively. Their results demonstrated that the lipid contents resulting from Nile red fluorescence staining as well as gravimetric and chromatographic methods were linearly correlated.

Another experiment on the green alga *Botryococcus braunii* showed that the Nile red staining method can be as accurate as the gravimetric method (Lee et al., 1998). These authors reported a linear relationship between the lipid content determined using the gravimetric method and *in vivo* fluorescence of cells stained with Nile red.

Additionally, the Nile red fluorescence assay was used to decide the fluorescence intensity value (FIV) corresponding to the concentration of intracellular lipids (μg/mL) at the maximum of the corrected spectrum. The results showed that there is a linear relationship between the FIV and lipid contents determined by the gravimetric method in some yeasts and oleaginous fungi (Kimura et al., 2004).

Chen et al. (2009) also made improvements to the Nile red method. For a high throughput approach and rapid determination of neutral lipid in *Chlorella vulgaris*, they used a 96-well plate-based Nile red assay. 25% dimethyl sulfoxide (DMSO) was employed in their study as a solvent and stain carrier. According to Chen et al. (2009), neutral lipid content can be quantified, sensitively, and thoroughly analysed using a spectrophotometer with excitation and emission wavelengths of 530 and 575 nm, respectively.

Furthermore, Chen et al. (2011) developed the Nile red fluorescence method using a two-step microwave-assisted staining method. They used this technique for *in vivo* quantification of neutral lipids in microalgae. Samples were prepared in DMSO and incubated at 1200 W in the microwave oven for 1 min ahead of the staining step. Their method produced accurate results when compared with gravimetric analysis.

Moreover, the lipid content in *Chlamydomonas reinhardtii* has been evaluated using the Nile red fluorescence technique (Kou et al., 2013). In this research, a 1 μg/mL Nile red solution in 5% DMSO was used. After a 5 – 15 min incubation, the fluorescence was read by a spectrophotometer at 528 nm and 576 nm for excitation and emission, respectively. They

reported that the range of 1-8 $\times 10^6$ cells/mL was optimal for quantifying lipid content. Liu et al. (2013) suggested that to improve the quantification of lipid content analysis the green algal samples can be homogenized in an ultrasonic bath before staining with Nile red.

3.2.3 General review of methods for carbohydrate analysis

There are various methods to measure the total carbohydrate content in biological samples. These methods can be chemical (such as the phenol–sulfuric acid method and the anthrone–sulfuric acid method) or enzymatic methods. While the latter technique has some advantages and can be applied easily, it is limited due to its specific targeting of particular carbohydrates in the sample (Brummer and Cui, 2005). The chemical methods have limitations as they rely on sugar molecules in the sample reacting with the reagents, and different sugars have different reactivities. Thus, these assessments of the total carbohydrate are influenced by the reactivity of the sugars in the sample. These methods are, therefore, applied when an approximate value of total carbohydrate is desired. While the specific enzymatic method is recommended when the accurate value of particular sugars is required (Brummer and Cui, 2005).

Phenol-Sulfuric Acid assay was developed by Dubois et al. (1951). In their account, 5 mL of sulphuric acid was added to 2 mL of the mixture of sugar solution and phenol to produce an orange-yellow coloration. Thereafter, absorbance at 490 nm was measured and compared with a standard curve to obtain the content of the sugar in the sample. This method is widely used due to its advantages of being simple and relatively inexpensive (Dubois et al.,1956; Brummer and Cui, 2005). Furthermore, the phenol–sulfuric acid assay has been reported to be the most straightforward, dependable, and convenient colorimetric method to measure the total sugar in many samples when compared with other methods (Dubois et al.,1956; Masuko et al., 2005).

According to Dubois et al. (1956), the Phenol-Sulfuric Acid assay involves three essential steps. Firstly, concentrated sulfuric acid and 5% phenol are added to the sample solution and

shaken thoroughly on a vortex mixer. Following this, the samples are incubated at 25 to 30 ˚C in a water bath for the colour to develop. Finally, the absorbance of samples at 490 nm is obtained. In this technique, the hot acidic solution is responsible for dehydrating and transforming the carbohydrates into various furan derivatives which can react with phenolic reagents (BeMiller, 2017; Nielsen, 2017). As a result of these reactions, a dark-coloured product appears. The absorbance of this product can then be measured at 490 nm using a spectrophotometer (Dubois et al., 1956; Brummer and Cui, 2005; BeMiller, 2017; Nielsen, 2017). The phenol–sulfuric acid assay has a high success rate when applied to samples containing single and mixtures of carbohydrates. It is common practice to use glucose as a standard to generate calibration curves. Consequently, the results are conventionally expressed as glucose equivalents (Brummer and Cui, 2005). The accuracy of this technique has been reported to be ±2 % (Nielsen, 2017).

A modification to the Phenol-Sulfuric Acid method was attempted by Rao and Pattabiraman (1989) to streamline the technique. Under this modified method phenol was added directly after adding the sulfuric acid to the samples. However, colour intensity (absorbance at 490nm) was noted to decrease when like materials were compared. It was concluded that the phenol was subjected to sulfonation *in situ* and this led to inaccurate results. It was recommended that the addition of phenol should be after the dehydration of carbohydrates by sulfuric acid and after cooling the mixture of sulfuric acid.

A significant modification was, however, achieved by Masuko et al. (2005) to miniaturise the assay. This modification optimized this approach using 96-well microplates to increase sensitivity, use less reagents, and obtain a greater linear range of 1–150 nmol for D-mannose (Man). Additionally, this modified method did not need shaking, and rapid throughput was achieved in no more than 15 min. Masuko et al. (2005) concluded that due to these several

advantages, their modification can be the best method to assess large numbers of samples in a straightforward process.

Common to all of these approaches is the hazard associated with the concentrated sulfuric acid (corrosive/harmful) and phenol (carcinogen) solutions. The small amount of reagent needed for the multi-well assay helps to mitigate chemical risks, and this is a significant benefit. The Phenol-Sulfuric Acid method has been used to measure the content of carbohydrates in many samples such as urine, blood, starch, and plant gums (Dubois et al., 1956). It has also been used to measure carbohydrates in foods, soft drinks, and beer (Nielsen, 2017).

Anthrone–sulfuric acid is another colorimetric analysis used to measure carbohydrates. This method was firstly applied for the detection of carbohydrates (Dextran) in blood and urine samples by Bloom and Willcox (1950). Like the phenol-sulfuric acid assay, furan derivatives can be produced by condensation reactions of carbohydrates in response to strong acids (Brummer and Cui, 2005). Rather than phenol, anthrone (9,10- dihydro-9-ozoanthracene) is used as the reagent to generate coloured compounds. The absorbance of the blue-green coloured product can be read spectrophotometrically at 625 nm (Brummer and Cui, 2005).

Anthrone–sulfuric acid colorimetric assay has been under modification by several authors such as Zipf and Waldo (1952), Scott and Melvin (1953), Roe (1954), and Roe (1955). The most important modification to the anthrone–sulfuric acid method was presented by Laurentin and Edwards (2003). They succeed to develop the anthrone-sulfuric acid microassay using 96-well microplates. In their study, samples were loaded into a microplate and incubated for 15 min at 4 ˚C. Anthrone solution was then added, and the microplate was vortex-mixed thoroughly and again incubated for 3 min at 92 ˚C. Thereafter, to stop the reaction, the microplate was incubated for 5 min at 25 ˚C. Finally, the absorbance was read at 630 nm. Laurentin and Edwards (2003) reported that the developed assay is appropriate for routine analysis of

carbohydrates as it decreases the amount of reagent and assay time by 800% and 40%, respectively. Therefore, the hazards and the cost of the assay can be reduced.

Anthrone–sulfuric acid method needs a standard curve to determine the carbohydrate content (Brummer and Cui, 2005). This method is more relevant to assess samples that have only one kind of sugar, because the different kinds of sugars in the sample can produce different rates and quantities of colour development (Brummer and Cui, 2005).

3.2.4 Selecting an appropriate assay

In the interest of conserving reagents, limiting waste, and working with small quantities of rotifer masses and homogenized samples, multi-well approaches were developed to assess protein, lipid, and carbohydrate contents in rotifer material. It was desirable to streamline all three assays in a way that they would mesh with the generation of a unifying rotifer extract. This means that a single rotifer extraction can be prepared and used for all three analyses (protein, lipid, and carbohydrate content). This would be useful in ensuring consistency across analyses, making findings more comparable and accurate. Given the review of the literature, the following methods were prioritised for further rotifer-specific development (Table 3.1).

3.3 Materials and Methods

3.3.1 Test animals

The monogonont rotifer, *B. calyciflorus*, was used as a test organism in this study. The cultures of rotifers were established, produced, and maintained as described in Chapter 2.

Table 3.1: Summary of multi-well approaches prioritised for development with justification

Target	Method	Justifications
Protein	Bicinchoninic Acid (BCA)	1) Test not inhibited by low to moderate amounts of detergent. 2) The range of detectable protein concentrations is ≤ 20 µg/mL 3) Availability of plate reader to measure the colour produced (absorbance). 4) A small sample is needed (25 µL). 5) Speed and convenience for assessing 96 samples in 35 mins
Lipid	Nile Red Fluorescence Staining	1) Small amounts of samples are required (25 µL). 2) It does not require a complex process to extract the lipid from samples. 3) Availability of a plate reader to measure the fluorescence intensity. 4) 96 samples can be tested in 15 mins 5) It was successfully used to measure lipid content in some microorganisms such as microalgae and yeast cells, so it was useful to apply on rotifers.
Carbohydrate	Phenol–Sulfuric Acid	1) A small amount of sample is needed (30 µL). 2) Has a longer linear range – 1-150 nmol for Man. 3) Only 15 min is required to complete the test. 4) 96 samples can be processed in a simple procedure.

3.3.2 Harvesting of the rotifers

When the rotifer density reached the number of about 100 rotifers/mL in both cultures, they were harvested as described in Chapter 2 and washed several times using deionised water to remove any food residues. Afterward, rotifers were resuspended by putting the sieve upside down into a plastic funnel over a 2000 mL conical flask and washed using deionised water into the conical flask. Thereafter, a flashlight was shone at the neck of the conical flask to attract the rotifers and allow them about 5 min to get their direction to the light and concentrate on the top layer and edges of the flask. Then they were easily collected using a micropipette and transferred to 15 mL centrifuge tubes. Rotifers were frozen, freeze-dried (Scott and Baynes, 1978) and stored at -80 ˚C. The utilisation of freeze drying, also known as lyophilization, was necessary to obtain dried rotifers that are closely comparable resemble their fresh counterparts

and maintaining a prominent level of samples quality. The stability of dried samples produced by this process has been observed to persist for an extended period of up to 24 months, without experiencing notable damage or loss of functionality (Oetjen, 2000; Santiago and Moreira, 2020).

3.3.3 Sample homogenization

The Biomasher disposable homogenizer (model I, Omni International) was used to homogenize the dry powdered sample. This model consists of 1.5 mL centrifuge tube with a filter tube and a pestle.

For sample homogenization, 20 mg of the sample (~1×10^5 freeze dried rotifers) was added into the filter. Next, 100 µL of ice-cold phosphate-buffered saline with Tween 20 (0.1M; pH 8.0) was added to the filter tube, and the sample was homogenized, by pushing and turning the pestle by hand, on ice for 2 min. Thereafter, 900 µL of PBST buffer was added to the filter tube. Then, the homogenised sample was centrifuged at 20,000 *g* for 20 min, maintaining the temperature at 4 ˚C (Srivastava et al., 2006). After that, the filter tubes were discarded and the extractions in the recovery tubes were vortexed. These extractions were sampled as full-strength extract and denoted "X". To prepare the dilution, 100 µL of X was added to 900 µL of PBST buffer. This sample was denoted "X/10" (i.e., diluting 10-fold). Five replicates were prepared and stored at -80 ˚C prior to testing.

3.3.4 Analytical method for total protein content in microwells format

The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Incorporated Company) was used to determine total protein content in homogenized samples. This assay is based on bicinchoninic acid (BCA), which combines with cuprous ions, resulting in a purple-coloured product. The product has high absorbance at 562 mm, which is a nearly linear increase in

protein concentration ranging between 20 and 2000 µg/mL (Thermo Fisher Scientific Incorporated., 2017)

The kit includes two reagents required for the BCA assay. First is BCA Reagent A, which contains sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide. The second is BCA Reagent B, which contains 4% cupric sulfate. To prepare the BCA working reagent (WR), BCA Reagent A was mixed with BCA Reagent B at 50:1 Reagent A: B.

Bovine serum albumin (BSA) was used as a standard. The concentration of the stock solution of BSA was 2 mg/mL, and it was used to generate 1, 0.5, 0.25, 0.125, and 0.063 mg/mL in deionised water.

The analysis was conducted following the protocol described by Thermo Fisher Scientific Incorporated (2017), 25 µL of unknown samples or the known BSA concentrations (standards) were then pipetted into a 96-well microplate, followed by 200 µL of the WR, before being mixed thoroughly on a plate shaker for 30 sec. Thereafter, the plate was covered and incubated at 37 ˚C for 30 min and then cooled to room temperature. Finally, the absorbance was measured at 562 nm using a plate reader. The readings were taken every 10 min up to 60 min in order to examine the effect of the incubation time on the measurements. The calibration line then was created using the known concentrations and their corresponding absorbance values. Concentrations of unknown samples were then calculated according to the calibration line by inserting the absorbance value given by the plate reader.

3.3.5 Analytical method for total lipid content in microwells format

The Nile red fluorescent method was followed to determine neutral lipid content. This method required 25% of DMSO (dimethyl sulfoxide) with 4.20 mg/mL of Nile red and a 10 min incubation time at 37 ˚C. Fluorescence was determined at 530 mm of excitation and 580 mm

of emission, as described by Chen et al. (2009). Phosphatidylcholine (PC) was used as a standard. The concentration of the stock solution of PC was 5 mg/mL, and it was used to generate 2.5, 1.25, 0.63, 0.31, and 0.156 mg/mL in DMSO.

To complete the analysis, 25 µL of unknown samples or the known PC concentrations (standards) were pipetted into a 96-well plate. Then, 32 µL of Nile red solution (Nile red reagent in DMSO 100%) was added to each well. Next, 75 µL of deionised water was pipetted into each well. The plate was incubated (37 ˚C) for 10 min in darkness, followed by vigorous vortexing for 30 sec. The fluorescence was then read at excitation and emission wavelengths of 530 and 580 mm, respectively, using a plate reader. Readings were made every 10 min up to 60 min in order to examine the effect of the incubation time on the measurements. The calibration line then was created using the known concentrations and their corresponding fluorescence values. Finally, concentrations of unknown samples were then calculated according to the calibration line by inserting the fluorescence values given by the plate reader.

3.3.6 Analytical method for total carbohydrate content in microwells format

The Phenol–Sulfuric Acid method was used to determine the carbohydrate content of the samples. This assay used concentrated sulfuric acid (98%) to hydrolyze carbohydrates to monomers. These monomers can then be formed into furfural or its derivatives which react with the 5% developer (phenol). This product was then quantified using a plate reader (Le Parc et al., 2014). To achieve this, a Total Carbohydrate Assay Kit (Abcam Company) was used. The materials included are 3 mL of 5% developer (phenol) and 2 mL of standard stock solution (glucose, 2 mg/mL). This standard stock solution was used to generate, 1, 0.5, and 0.25 mg/mL in deionised water.

The assay was conducted according to the method described by Le Parc et al. (2014), 30 µL of unknown samples or the known glucose concentrations (standards) were added to a 96 well

plate. Then, 150 µL of 98% concentrated sulfuric acid was added to each well. The plate was placed on a shaker and the samples were mixed for 1 min. Thereafter, the plate was incubated at 85-90 ˚C for 15 min. Following incubation, the developer (30 µL) solution was pipetted into each well. The plate was placed on the shaker to mix the samples for 5 min. The absorbance was read at 490 mm. The readings were taken every 10 min up to 60 min using a plate reader in order to examine the effect of the incubation time on the measurements. The calibration line then was created using the known concentrations and their corresponding absorbance values. Concentrations of unknown samples were then calculated according to the calibration line by inserting the absorbance value given by the plate reader.

3.3.7 Validation of the protein, lipid, and carbohydrate methods

Having established the methods and optimised dilution factors and assay read times (Table 3.2), the methods were applied to a "blind" biological sample of unknown concentration. This sample was extracted and the assays of the bicinchoninic acid (BCA) and the Nile red fluorescent method were applied, as described above, to measure protein and lipid contents.

3.3.8 Data analysis

The analysis of variance (ANOVA) was used to test for significant differences between readings obtained after increasing assay times. An independent samples t-test analysis was also used to identify any significant differences between dilution "x" and "x/10" at different time points. Tests were deemed significant at a p-value ≤ 0.05. All procedures were conducted using the SPSS software package (Version 25).

Table 3.2: Validation of the protein, lipid, and carbohydrate methods

Methods (Targets)	Reagents	Reagent's volume (µL)	Sample volume (µL)	Incubation time(min) (°C)	Tests time (Min)
Bicinchoninic Acid (Protein)	BCA	200	25	30 (37 ˚C)	0,10,20,30,40,50, and 60
Nile red fluorescence (Lipid)	4.20 mg/mL Nile Red (NR)	32 NR in DMSO	25	10 (37 ˚C)	0,10,20,30,40,50, and 60
	Deionised water	75			
Phenol–Sulfuric Acid (Carbohydrates)	98% H_2SO_4	150	30	15 (85-90 ˚C)	0,10,20,30,40,50, and 60
	%5 Phenol	30			

3.4 Results

3.4.1 Calibration lines and the use of sample data points

The calibration lines revealed a strong linear relationship between the concentration of standard solutions and the instrumental readings (Figure 3.1). For protein, the calibration line using Bovine Serum Albumin (BSA) standard solutions showed a strong linear relationship between absorbance (nm) and concentrations from 0 mg/mL up to a concentration of 2 mg/mL (R^2 = 0.993). For lipids, the calibration line using phosphatidylcholine (PC) standard solutions also showed a strong linear relationship between fluorescence (nm) and concentrations up to a concentration of 2.5 mg/mL (R^2 = 0.999). In the case of carbohydrates, the calibration line using glucose standard solutions showed a strong relationship between absorbance (nm) and concentrations (mg/mL) up to 2 mg/mL (R^2 = 0.994).

To ensure the accuracy of our results through calibration lines, it is imperative that data points fall within the linear range of the standards for extrapolation. The current study showed that

the concentrations of protein, lipid, and carbohydrate in the unknown samples consistently fell within the linear response range of the calibration lines. Particularly, for protein, the concentration in unknown samples ranged from 0.63 to 1.01 mg/ml for the full-strength (X) samples, and from 0.01 to 0.13 mg/ml for the X/10 samples (where X/10 denotes a tenfold dilution). Correspondingly, for lipid, the concentrations in the unknown samples ranged from 0.15 to 0.57 mg/ml for the full-strength (X) samples and from 0.02 to 0.06 mg/ml for the X/10 samples. Regarding carbohydrate, concentrations ranged from 0.25 to 0.62 mg/ml for the full-strength (X) samples and from 0.02 to 0.06 mg/ml for the X/10 samples. All these unknown sample data were diligently compared to the calibration lines. However, it is important to note that the data for the X/10 samples indicated significant dilution, which may affect the accuracy of their results.

3.4.2 Unknown biological sample

The bicinchoninic acid (BCA) and the Nile red fluorescent assays were not described in the literature to measure the protein and lipid contents in invertebrate samples. Therefore, these assays were applied to measure the protein and the lipid contents of unknown biological samples "blind'' because it was necessary to test the efficiency of these methods in advance. The "blind" sample tested for this purpose was aquarium fish feed. This blind sample contained 46% protein and 6.5% lipid as described by its datasheet. In contrast, the values of 50.3% protein and 7.9% lipid content were observed in this study using the bicinchoninic acid (BCA) and the Nile red fluorescent methods. Hence, the accuracy of the bicinchoninic acid (BCA) and the Nile red fluorescent assays was high as indicated by the convergence of values.

The phenol–sulfuric acid method was used extensively in the literature to measure the carbohydrate content in several invertebrate species (De Coen and Janssen, 1997; De Coen and Janssen, 2003; Verslycke and Janssen, 2002; Verslycke et al., 2003; Gomes et al., 2015; Gomes et al., 2016). Consequently, it was not necessary to test this method on the blind sample.

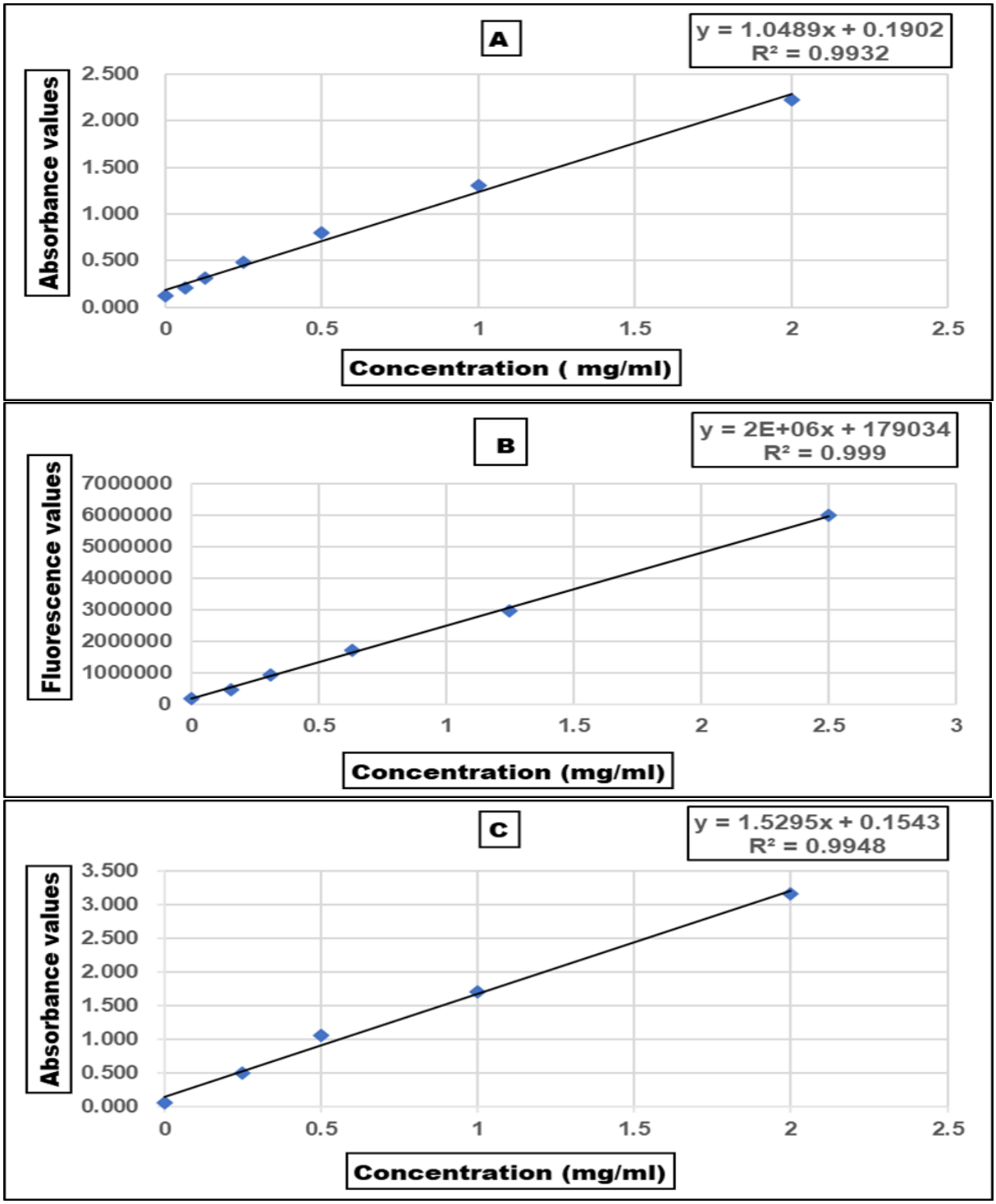

Fig.3.1: A: calibration line of protein test **-** absorbance of bovine serum albumin (BSA) at different concentrations at 562nm**. B:** calibration line of lipid test **-** fluorescence of intensity for phosphatidylcholine (PC) at different concentrations at 530 and 580 nm wavelengths for excitation and emission, respectively**. C:** calibration line of carbohydrate test **-** absorbance of glucose at different concentrations at 490nm.

3.4.3 Protein, lipid, and carbohydrate contents

3.4.3.1 Protein content

The initial value of the protein content in the full-strength extract (X) at the start of the incubation (0 min) was 6.49 ± 0.45%, and there was no variation in readings obtained with increasing assay time (Figure 3.2). There was no significant difference ($P > 0.05$) in readings obtained regardless of the increasing assay time up to 60 min.

The initial value of protein content in the X/10 samples (X/10 has been scaled up by a factor of 10) was 4.46 ± 0.39% at 0 min. Here, protein content slightly increased with increasing assay time, although there was no significant difference ($P > 0.05$) between all the readings.

Where the full strength (X) readings were compared with those of X/10, a significant difference was observed ($P < 0.05$) between sample couplets at all time points. These data suggest that diluting the sample, as in X/10, resulted in low values obtained, although the readings were not affected by protracting the assay time. The readings of protein content seem to be consistent, with only minimal differences across all time points. The values ranged between 4.46 ± 0.39% and 4.71 ± 0.46%. In the full-strength samples, the protein content was observed to be between 6.47 ± 0.44% and 6.54 ± 0.45%. X and X/10 samples were analysed at the same time using the same procedure, reagents, and equipment. The variations between the two samples can therefore be attributed to the limit of quantification. Concentrations of protein included in X/10 samples could be below the quantification limit of the BCA assay which led to measurement inaccuracies.

Therefore, it is recommended that full-strength extracts are used to screen protein concentrations and an assay time of between 0 and 60 mins can be used in future experiments. Moving forward with further assays, protein measurements were made using the full-strength extract X with an assay time of 60 mins.

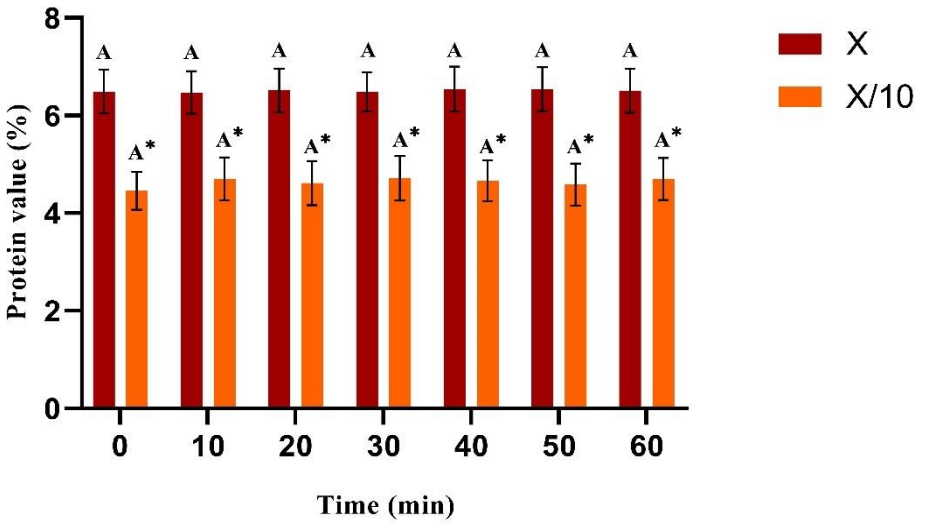

Fig. 3.2: Protein content (%) in full strength sample (X) and diluted sample (X/10 – these values have been scaled by a factor of x10 to allow like-for-like comparison). Significant differences ($P \leq 0.05$) in protein content with increasing assay time are indicated by dissimilar letters (absence of dissimilar letters indicates the absence of any significant difference). Significant differences ($P \leq 0.05$) in protein content where X and X/10 values are compared at a given assay time are indicated with an asterisk**.** Results are expressed as average ± standard error (N=5).

3.4.3.2 Lipid content

The lipid content in the full-strength samples (X) at the start of incubation (0 min) was 1.35 ± 0.1%. With increasing assay time there were differences in results obtained (Figure 3.3), although changes were not statistically significant ($P > 0.05$). The maximum value of lipid content was observed to be 2.58 ± 0.4% after 60 min of incubation. A general trend of increasing value with increasing time was observed, with the lipid value plateauing between 50 and 60 min.

The lipid content in the X/10 samples at 0 min was 2.05 ± 0.2%, differing slightly, but not significantly ($P > 0.05$), with increasing assay time. Lipid content reached a maximum value of 2.84 ± 0.2% after 10 min of incubation and then decreased slightly to 2.70 ± 0.2% after 20

min. From 30 min, lipid content dramatically decreased to a level lower than the initial value (1.80 ± 0.4%). It then subsequently rose to plateau at 40 and 50 min of incubation, with values of 2.52 ± 0.3% and 2.51 ± 0.4%, respectively.

Where the full strength (X) readings were compared with the X/10 readings, a significant difference was observed between sample couplets at 0, 10, and 20 min of incubation (p= 0.03, 0.006, and 0.01 respectively). However, no significant difference was observed between sample couplets from 30 min of incubation.

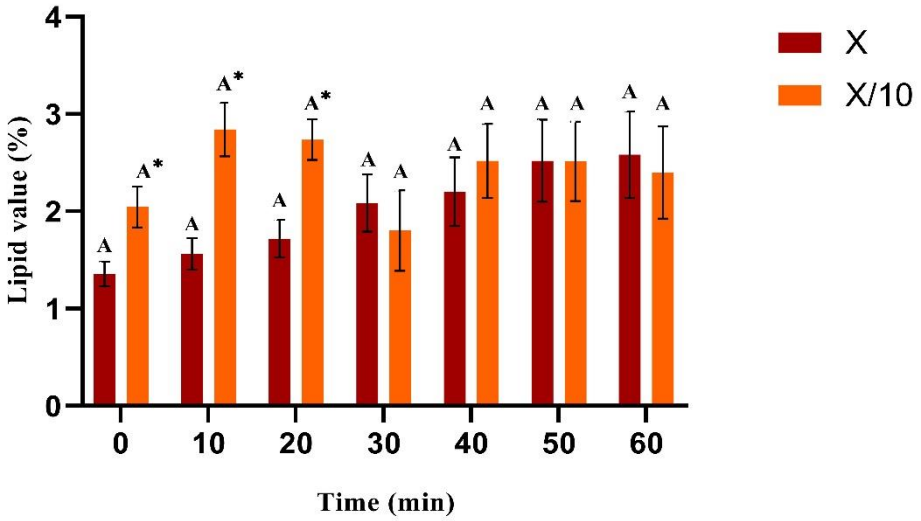

Fig. 3.3: Lipid content (%) in full strength sample (X) and diluted sample (X/10 – these values have been scaled by a factor of x10 to allow like-for-like comparison). Significant differences (P ≤ 0.05) in lipid content with increasing assay time are indicated by dissimilar letters (absence of dissimilar letters indicates the absence of any significant difference). Significant differences (P ≤ 0.05) in lipid content where X and X/10 values are compared at a given assay time are indicated with an asterisk. Results are expressed as average ± standard error (N=5).

Looking across the dataset at all assay times and both dilutions, results were consistent in the 40, 50, and 60 min assay times. These results show no significant difference (P >0.05) between each other. Therefore, it is recommended that full-strength extracts should be used with an assay time of 60 mins to screen lipid concentrations. This recommendation is consistent with the approach suggested for protein (also at full strength and after 60 min assay time).

3.4.3.3 Carbohydrate content

The initial value of carbohydrate content in the full-strength extract (X) at the start of incubation (0 min) was 3.17 ± 0.33% (Figure 3.4). With increasing assay time there was no significant difference ($P > 0.05$) in readings observed. In contrast, the initial value of carbohydrate content in the X/10 samples was 3.56 ± 0.17% at 0 min, and the values decreased with increasing assay time. While the readings were not significantly different ($P > 0.05$) after 0, 10, and 20 mins, a significant decrease ($P<0.05$) in readings was observed from the 30 min assay time.

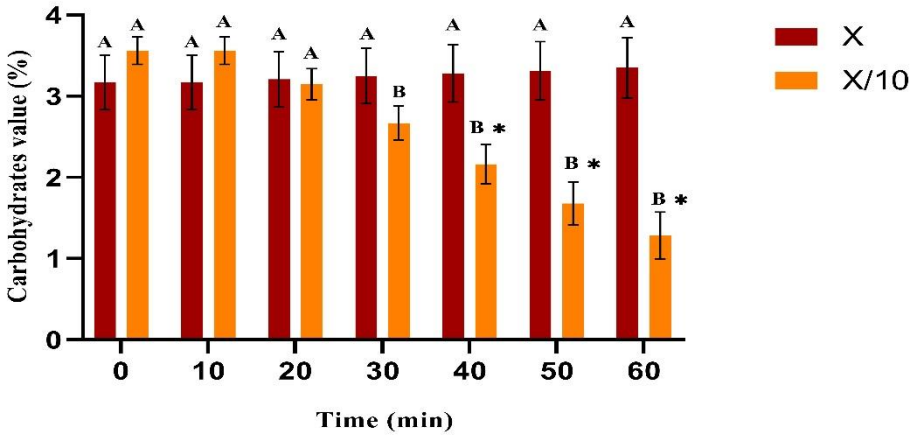

Fig. 3.4: Carbohydrate content (%) in full strength sample (X) and diluted sample (X/10 – these values have been scaled by a factor of x10 to allow like-for-like comparison). Significant differences ($P \leq 0.05$) in carbohydrate content with increasing assay time are indicated by dissimilar letters (absence of dissimilar letters indicates the absence of any significant difference). Significant differences ($P \leq 0.05$) in carbohydrate content where X and X/10 values are compared at a given assay time are indicated with an asterisk. Results are expressed as average ± standard error (N=5).

When the full strength (X) readings were compared with the X/10 readings, no significant difference was observed between sample couplets at 0, 10, 20, and 30 min of incubation. However, readings of couplets were significantly different at 40, 50, and 60 min assay time points ($p = 0.03$, 0.007, and 0.002 respectively). This data suggests that diluting the sample, as in X/10, and protracting the assay time beyond 20 mins resulted in inaccurate results being

obtained. The carbohydrate content is relatively stable at 0, 10 and 20 min with values of 3.56 ± 0.1, 3.56 ± 0.17, and 3.15 ± 0.19%, respectively. Thereafter, carbohydrate content decreases dramatically and reaches a minimum after 60 min (1.23 ± 0.29%).

Based on the reliable data, specifically full-strength samples, which yielded consistent measurements at all time points, the carbohydrate content in the rotifer samples was established to be 3.17 ± 0.33% as a minimum value and 3.35 ± 0.37% as the maximum value (at 0 and 60 min respectively). For future work, it is recommended that full strength (X) extracts are used to screen carbohydrate concentrations and an assay time of 60 mins can be used. Thus, the use of full-strength (X) extracts and an assay time of 60 mins provides a consistent approach to assessing all three components of interest.

3.5 Discussion

3.5.1 Protein content

The documented protein content of saltwater rotifers, across several feed types, ranges between 25.5% and 72% (Watanabe et al., 1978; Ben-Amotz et al., 1987; Frolov et al., 1991; Carić et al., 1993; Lie et al., 1997; Nhu, 2004; Srivastava et al., 2006; Jeeja et al., 2011; Eryalcin, 2018). Scott and Baynes (1978) estimated the protein content in saltwater rotifers to be between 49.1 and 58.8%. Frolov and Pankov (1992) found the quantity of total protein to be ranged between 45.3 to 62.7%. Carić et al. (1993) reported the values of protein to range from 25.5 to 52%. Ben-Amotz et al. (1987) reported a wider range of protein, from 28 to 55%. In the current investigation, the protein content in the freshwater rotifers was shown to be about 6.5%, which represents about 25% of the lowest values found by Ben-Amotz et al. (1987) and Carić et al. (1993).

This relatively low protein content may be related to some factors such as the type of food (here algae, *Chlorella vulgaris*), growth phase, and temperature (Ben-Amotz et al., 1987; Carić et

al.,1993). According to Carić et al. (1993), the phases of the growth cycle can affect the quantity of protein in rotifers. The highest levels of protein were seen in the stationary phase of growth, which can be related to the rapid protein synthesis process in growing rotifers. Protein values were not always significantly influenced by the growth phase. Carić et al. (1993) also reported that protein levels increased during the stationary phase of growth and reached the highest values when rotifers were fed with nanoplankton and *Phaeodactylum tricornutum*. However, lower protein values were found when rotifers in the same phase were fed with baker's yeast *Saccharomyces cerevisiae*. The current study cannot confirm the effect of the growth phase on protein content because rotifers were collected at random from the stock culture and were therefore likely to have been at different phases.

The influence of protein content in the food of the rotifer, *B. plicatilis,* has been reported, and a positive correlation was found between the protein content of the food and that of the rotifers. Thus, the protein content of rotifers can be relatively similar to that of the diets they consume (Ben-Amotz et al., 1987). Ben- Amotz et al. (1987) reported the highest value of protein (55%) when rotifers were fed with baker's yeast *Saccharomyces cerevisiae* (protein content of yeast was 53%). In contrast, they reported the lowest protein content (28%) when rotifers were fed *Chlorella stigmatophora* (protein content of algae was 31%). Frolov et al. (1991) also reported similarity in protein content in food (*Monochrysis lutheri*) and rotifer, respectively, 48.1% and 47.4%. These publications provide clear linkages between protein content in food and similar protein content in rotifers. However, other studies in the literature have reported a weak linkage between food and rotifer protein content. For example, it was observed that protein contents of 46% and 30% were stored in rotifers and their food (*Saccharomyces cerevisiae*) respectively (Frolov et al.,1991)

In the current study, rotifers were fed with live freshwater algae *Chlorella vulgaris* (*C. vulgaris* cells were harvested during the exponential growth phase) and the protein content of *Chlorella*

vulgaris was found to be 12.90% (dry weight). In contrast to the literature, the protein content of *Chlorella vulgaris* has been reported in the range of 12.4% to 52.4% (dry weight) (Mahasneh., 1997; Illman et al., 2000; Bertoldi et al., 2008; Chia et al., 2013).

The effect of the temperature on protein content was examined by Scott and Baynes (1978). Their study estimated the protein content of saltwater rotifers to be between 49.1% and 58.8%. They concluded that the highest value of protein was found at low temperatures (18˚C), whilst the lowest value was found at high temperatures (28˚C). In the current investigation, the cultures of rotifers were incubated at a relatively high temperature (25 ±1˚C). This, in part, may explain the relatively low protein contents observed. It is highlighted that 25 ± 1 ˚C has been reported as optimal for the rotifers (Snell et al., 1991; Janssen et al., 1994; Sarma et al., 2006; Xi et al., 2007; Xu et al., 2015; Pan et al.,2016).

3.5.2 Lipid content

In the current trial, the lipid content in the freshwater rotifers was estimated to be 2.58 ± 0.44%. In contrast, several studies have reported that lipid content in rotifers ranged between 1.4 and 20% (Watanabe, 1978; Ben-Amotz et al., 1987; Carić et al., 1993). It was concluded that the lipid synthesis in rotifers depended on the quality of the algal species or diets used in the culture system (Watanabe, 1978; James and Abu-Rezeq, 1988). The lipid content in this chapter represented about 30% of the lowest value estimated by Carić et al. (1993). These authors studied the lipid content of the saltwater rotifers fed on varied species of algae and baker's yeast in different phases of growth, observing that the values of lipid range from 8.5 to 19.4%. The highest lipid value was found in the exponential phase, especially when the rotifers were fed using natural sea nanoplankton. A high value of lipid was also found in the death phase when rotifers were fed with baker's yeast. In contrast, lower values of lipid content, irrespective of food type, were observed in the death phase (Carić et al., 1993). Carić et al. (1993) showed that both factors, the diet and growth cycle, significantly influenced the lipid content of rotifers.

Significant interactions between the growth cycle and diet were found in all measurements of lipids. It was concluded that the diet significantly affected the content of lipids in all the phases of the growth cycle. As stated above, sampling rotifers at random in the current study precluded the assessment of the impacts of the growth cycle.

Furthermore, Ben-Amotz et al. (1987) studied the lipid content of saltwater rotifers that were fed various diets. Their results showed that the lowest value of lipid was 5% in rotifers fed on yeast, which is about twice the lipid content found in the current study. The relatively low lipid content in their study was likely related to the poor value of lipid (1%) that was found in the yeast, *Saccharomyces cerevisiae* (Ben-Amotz et al., 1987). On the other hand, the highest value of lipid content was about 20%, reported where the marine diatom, *Chaetoceros gracilis* (20.7% lipid content), was the food source.

Frolov et al. (1991) also tested the content of lipids in saltwater rotifers when using different food. They reported the lipid content of rotifers to correlate/match the lipid content of their food. For example, where *Phaeodactylum tricornutum* (10.1% lipid) was used as food, the lipid content in rotifers was 8.7%. A similar outcome was reported when rotifers were fed with *Nephrochloris salina* (lipid content 13.5%), with the lipid content of rotifers being 12%. A similar strong degree of correlation between the lipid content of rotifers and their food was noted by Watanabe (1978). Similarly, in the current trial, lipid content was found to be 2.58% and 3.32% for the rotifers and algae *Chlorella vulgaris* (*C. vulgaris* cells were harvested during the exponential growth phase), respectively. However, Chia et al. (2013) found that the percent total lipid yield of *C. vulgaris* was approximately 10.70% dry weight, which is three times the lipid value found by the present study in *Chlorella vulgaris*. As well, the content of lipids was found to be 5.90% in *C. vulgaris* (Converti et al., 2009), which represented about twice the value found here.

Regarding the influence of temperature on lipid content, Scott and Baynes (1978) observed the lipid content of saltwater rotifers to range from 5.4 to 5.6% and from 10.2 to 11.2% where rotifers were maintained for six days at 23 and 18 ˚C, respectively. They suggested that the values of lipid decrease with an increase in temperature.

3.5.3 Carbohydrate content

In the present study, the carbohydrate content in the freshwater rotifers was 3.35 ± 0.37%. This value was ~ 50% of the highest value of carbohydrate content found by Carić et al. (1993) in saltwater rotifers. Carić et al. (1993) observed the carbohydrate content of rotifers to vary with the growth phase and the type of feed, reporting carbohydrate values between 2% and 6.5%. Their study showed the high content of carbohydrates in the exponential phase, irrespective of diet. However, when rotifers were cultivated on baker's yeast *Saccharomyces cerevisiae,* the highest carbohydrate value was found in the death phase. In contrast, the lower values of carbohydrates were found in the death phase when rotifers were cultivated on different species of algae, such as *Dunaliella tertiolecta, Tetraselmis suecica*, *Nannochloropsis* sp., and *Phaeodactylum tricornutum.* Carić et al. (1993) confirmed that the growth cycle and the type of food can considerably affect the content of carbohydrates in the rotifers.

Ben-Amotz et al. (1987) measured the carbohydrate content of *B. plicatilis* cultured on different diets and observed values to range between 7% and 44%. Their lowest result is about twice the values in the present study. In their study, the highest value of carbohydrates was 44%, which was found when using the marine diatoms, *Chaetoceros gracilis* (49% carbohydrate content), as a diet. However, the lowest value (7%) was found when using the microalgae, *Isochrysis galbana* (11% of carbohydrate content). Ben-Amotz et al. (1987) concluded that the carbohydrate content of rotifers can be similar to that in their food. A similar result was obtained by Frolov et al. (1991) who used *Nephrochloris salina* as a food source. In their study,

the values of carbohydrates were 20.5% and 20.4% for the rotifers and *Nephrochloris salina,* respectively.

These results suggested that the carbohydrate content is primarily a consequence of the diet (Ben-Amotz et al., 1987; Frolov et al., 1991). However, the amount of carbohydrates for rotifers and their nutrition may vary. For example, the present examination suggested that the value of carbohydrates was 3.35% and 4.60% for rotifers and *C. vulgaris* (*C. vulgaris* cells were harvested during the exponential growth phase), respectively. However, some studies estimated the total content of the carbohydrate in *C. vulgaris* to be between 30.8% and 51% (Illman et al., 2000; Habib et al., 2003; Chia et al., 2013). Therefore, the result of this study represented only about one-tenth of the highest value documented for *C. vulgaris*. Ben-Amotz et al. (1987) found the carbohydrate content to be 10% and 20% for the rotifers and their food (C*hlorella stigmatophora*), respectively. Their result is consistent with the study of Frolov et al. (1991), who reported the values of carbohydrates to be 17.4% for the rotifers and 46.2% for their food (*Saccharomyces cerevisiae*).

The values for carbohydrates in rotifers have been assessed across different temperatures (Scott and Baynes, 1978). In their experiment, the carbohydrate content decreased with rising temperature from 18 ˚C to 28 ˚C. They recorded carbohydrate content in rotifers between 2.2 and 14.5% (Scott and Baynes, 1978). This finding is in accordance with the results obtained from the present study. Scott and Baynes (1978) suggested that at low temperatures, rotifers consume their food relatively slowly and maintain a high value of the biochemical composition. However, at high temperatures, rotifers consume their food quickly and tend to spend long periods under no food conditions. Therefore, the biochemical composition of the rotifers can decline.

3.6 Conclusion

Protein, lipid, and carbohydrate contents were quantified in freeze-dried samples of rotifer at two dilutions, full strength, and a 10-fold "X/10" dilution, over assay times of 0 to 60 mins. This method development was undertaken to establish optimal dilutions and assay times to determine total reserves of energy as protein, lipid, and carbohydrate, in freshwater rotifers. Data from these experiments support the methodical approach summarised in Table 3.3.

Table 3.3: Optimal method conditions for protein, lipid, and carbohydrates measurements

Target	Method	Dilution	Assay time
Protein	Bicinchoninic Acid	Full strength	60 min
Lipid	Nile Red fluorescence	Full strength	60 min
Carbohydrates	Phenol–Sulfuric Acid	Full strength	60 min

Applying these optimised assessment parameters, the proportion of the three energy stores in the freshwater rotifer *Brachionus calyciflorus* used in this study was established to be: 6.54 ± 0.45% protein, 2.58 ± 0.44% lipid, and 3.35 ± 0.37% carbohydrate. These values represent energy stored in rotifers that were not subjected to toxicant stress. The results suggest protein to be the most abundant energy store, followed by carbohydrate (about half that of protein) and then lipid (about a third that of protein). Benchmarking these results against existing literature indicated that the values of protein were much lower than in previous studies. Of the previous studies, the lowest value reported for protein store was 25.5% (Carić et al., 1993). Thus, the value determined by the present study for the freshwater rotifer *Brachionus calyciflorus* was 4-fold lower than the lowest values reported in the literature for protein content. On the other hand, the lowest values for lipid and carbohydrate contents in the literature were 1.4% (Watanabe, 1978) and 2% (Carić et al., 1993) respectively. These values were slightly lower

than the values determined in the present study (2.58% and 3.35%) for lipid and carbohydrate, respectively.

It is noted that the contents of protein, lipid, and carbohydrate are responsive to multiple factors. These include diet, the growth stage of the rotifers, and temperature. In the present study, the proportions of protein, lipid, and carbohydrate in *C. vulgaris* cells (*C. vulgaris* cells were harvested during the exponential growth phase) were, respectively, 12.90, 3.32, and 4.60%. In comparison with values published in the literature for *C. vulgaris,* these proportions are low. The literature supports strong proportionality between food and consumer energy store profiles. Thus, the low proportions of protein, lipid, and carbohydrate observed in rotifers in the present study are most likely linked to low proportions of these components in the food provided.

Another factor that may have influenced the low proportions of energy store components in rotifers is the growth cycle stage. In the present study, rotifers were randomly collected from the culture stock and, consequently, would likely have been of varying growth stages. The growth cycle stage has been shown to affect the chemical composition of saltwater rotifers in previous studies. Likely, this would also be the case for the freshwater rotifer *Brachionus calyciflorus* in the current study. The presence of juvenile rotifers in the sample used in the present research might be expected to suppress the value observed, as these individuals will not have reached their full potential in terms of energy store deposition.

CHAPTER FOUR

Chapter 4: Assessment of available energy (input energy) Ea, in freshwater rotifers exposed to sub-lethal concentrations of toxicants.

4.1 Introduction

Various methods have been established to estimate the impacts of contaminants on aquatic species abundance, production, populations, communities, and ecosystems (Hall et al., 1985; Argese et al., 1994; De Coen and Janssen, 1997). These methods can be divided into two groups, the cellular (sub-organismal) methods (Argese et al., 1994) and the ecosystem relevant methods (Hall et al., 1985). According to Goldstein (1981) the methods of sub-organismal endpoints require short exposure time (few days). However, they have disadvantages, as these biomarker-based endpoints can suffer from a lack of ecological relevance. Therefore, these methods can be difficult to rely on for the assessment of environmental impacts of chemical contaminants (Giesy and Graney, 1989). On the other hand, assessment methods with ecosystem-level endpoints have the advantage that they can study the effects that may be related to organismal and, hence, the population levels such as the structural changes or functional properties in aquatic communities. However, they have disadvantages, as these methods require long exposure times (few weeks), and therefore, they can be expensive (Suter, 1992).

Several theoretical principles were established to link toxic effects at the sub-organismal level with effects at the population-level (Nisbet et al., 1989). One of these is the “metabolic cost” hypothesis which was proposed by Calow and Sibly (1990). This hypothesis states that pollution stress produces metabolic changes in stressed organisms, which can cause a depletion of their energy reserves, and subsequently, adverse impacts on reproduction and growth can occur. The energy budget of organisms can be reduced as a result of pollution stress which encourages compensatory variations in the energy metabolism, as most of the energy budget is used by organisms for basal metabolism of reproduction and growth. Therefore, when the

energy expenditure increases for basal metabolism to overcome the toxic stress, this can cause a decrease in reproduction and growth (De Coen and Janssen., 2003). This means that the energy budget of stressed organisms can be considered as indicative of the organism's overall condition under stress (Calow, 1991; De Coen and Janssen, 2003). The rationale is that under stress, and in order to sustain homeostasis, energy is reallocated to different biochemical pathways, and this may reduce the energy available for other important ecological functions including growth and reproduction.

Perhaps the most effective application of this principle is the "scope for growth" (SFG). This concept combines different responses (e.g., excretion rate, respiration, feeding rates, and assimilation efficiency) in one integrated bioassay (Widdows and Salkeld, 1993). The scope for growth method is expressed as A - (R + U), where A is the energy absorbed (as measured by feeding rates or assimilation efficiencies or feeding rate efficiencies), R is the energy consumed (as measured by respiration rates), and U is the energy excreted (as measured by excretion rates of amino acid and ammonia) (Warren and Davies, 1967). This approach has been applied to assess toxicant stress for different organisms and some attempts have tried to associate these energy-based assessments with effects that can occur at higher levels of biological organization (Bayne et al., 1978). Scope for growth can measure the condition of an organism's energy; however, it is labour-intensive and tricky to be involved in routine environmental impact studies (De Coen and Janssen, 1997; Verslyckea et al., 2003). De Coen and Janssen (1997, 2003) and De Coen et al., (2001) have suggested an alternative biochemical method rather than the scope for growth (the physiological method). This approach is referred to as Cellular Energy Allocation (CEA) and can be applied by determining the energy allocation to assess the impacts of toxic stress on the cellular level of test organisms. In this technique, the energy budget of an organism can be quantified by measuring variations in the available energy budget (Ea) and the energy consumption (Ec). The energy consumption can

be assessed by evaluating the electron transport system activity (ETS) at the mitochondrial level. On the other hand, the available energy reserves for metabolism can be assessed by evaluating proteins, lipids, and carbohydrates contents for the test organism. The difference between the energy consumption (Ec) and the available energy reserves (Ea) represents the net energy budget of the organism tested (CEA) (De Coen et al., 1995; 1997; 2003). The cellular energy allocation technique can provide an integrated quantification of the energy budget of the organisms tested and can also help to clarify the action of different toxicants on exposed organisms (Verslycke and Janssen, 2002; Verslycke et al., 2003). Furthermore, since the physiological adjustment necessary for stress resistance mechanisms greatly affects the total energy budget (Sokolova et al., 2012; Sokolova, 2013), short-term changes in the balance of an organism's energy budget can be related to long-term outcomes such as reproduction and population size (De Coen and Janssen, 1997; Verslycke and Janssen, 2002; Verslycke et al.,2003).

The Cellular Energy Allocation (CEA) approach was originally established and validated for *Daphnia magna* (De Coen and Janssen, 1997; 2003); however, it can also be applied to other invertebrates and to vertebrates (De Coen and Janssen, 2003). The use of CEA to evaluate the energy budget of an animal in response to stressors is well established and being used in several invertebrates to test the impacts of toxicants on the organisms and populations (De Coen and Janssen 2003; Smolders et al., 2004). It has been used for Zebrafish *Danio Rerio* (Smolders et al., 2003), zebra mussels (Smolders et al., 2004), the shrimp *Neomysis integer* (Verslycke et al., 2003), and the aquatic oligochaetes such as the enchytraeids (Novais et al., 2013; Novais and Amorim, 2013; Gomes et al., 2015). These studies were performed with a supply of food during the exposure. However, Gomes et al. (2016a) performed a comparison study for the Cellular Energy Allocation (CEA) in the presence and absence of food in the *Enchytraeus albidus* (Oligochaeta) to explore the effect of food supply during toxicant exposure to sub-

lethal concentrations of cadmium. They hypothesized that chemicals such as cadmium (Cd) can affect food uptake by inhibiting the feeding rate and therefore the results of the energy budget can be related to uptake reduction and not to the direct toxicity of chemicals. However, food deficiency can be considered as a stressor, itself, which may lead to biased results. Nevertheless, they recommended the toxicant exposure in the absence of food, since it can be a more appropriate discriminant of chemical effects that leads to better understanding of chemical toxicity (Gomes et al., 2016a).

4.1.1 Aims of this chapter:

1. To measure the available energy in freshwater rotifers exposed to sublethal concentrations of toxicants in the absence of food by quantifying the total lipid, protein, and carbohydrate contents.
2. To establish dose responses relating protein, lipid, and carbohydrate content in freshwater rotifers to the concentrations of toxicant they were exposed to.
3. To evaluate the relative influence of four toxicants on protein, lipid, and carbohydrate contents in freshwater rotifers.

In this study, the monogonont rotifer *Brachinous calyciflorus* was used. It has been used as a useful test organism in numerous other experimental studies due to its biological characteristics (Snell and Janssen, 1995; Snell and Joaquim-Justo, 2007; Dahms et al., 2011; Moreira et al., 2016; Won et al., 2017). They have shown good sensitivity to some chemical toxicants, sometimes with greater sensitivity than other common model organisms (Snell and Joaquim-Justo, 2007; Dahms et al., 2011). In general, rotifers demonstrate the ability of bioaccumulation and biomagnification of toxicants (Dahms et al., 2011). Rotifers are also able to transfer chemicals from both water and sediments to higher trophic levels (Dahms et al., 2011). Nevertheless, there are limited numbers of ecotoxicological studies that used rotifers as a test

animal to evaluate the sublethal effects of exposure to chemical pollutants (Moreira et al., 2016). To date, no studies have reported on the use of rotifers within a cellular energy allocation framework. The present study sought to fill this knowledge gap.

4.2 Materials and Methods

4.2.1 Test animals, culture and husbandry

The monogonont rotifer, *B. calyciflorus*, was used as a test organism in this study. The cultures were established, produced and maintained as described in Chapter 2.

4.2.2 Experimental setup

Four toxicants were assessed for their impacts on the cellular energy allocation of rotifers. Two heavy metals, copper (Cu) and cadmium (Cd), and two pesticides, pentachlorophenol (PCP) and 3,4-dichloroaniline (3,4-DCA), were used in these experiments. Their sub-lethal concentrations (Table 4.1) were selected to be below the lethal thresholds that were determined in Chapter 2 (See Section 2.4.3 &Table 2.9).

Each toxicant had a separate set of cultures, rotifers cultures used in the experiments involving Cu and Cd were feed daily by adding live algae, but rotifers cultures used in the experiments involving PCP and 3,4-DCA were feed daily by adding live algae, and Roti-Rich as a food supplement. 1 mL of the Roti-Rich liquid, consisted of concentrates of phytoplankton, vitamin and mineral mix, and yeast, was diluted in 100 mL freshwater medium (EPA) and then 2 mL of the diluted liquid fed to the rotifers culture. This action was taken because it has been observed that the live algae was not enough to feed the rotifers, as discussed in Chapter 3, and depending on the results of the experiments involving Cu and Cd in this Chapter.

When the rotifer density reached the number of about 100 rotifers/mL in the cultures, they were harvested from the cultures after feeding as described in Chapter 2 and washed several times using deionised water to remove any food residues. Afterward, rotifers were introduced into

2000 mL conical flasks containing different sub-lethal concentrations of toxicants in a freshwater medium. The greatest care was followed to ensure 50 rotifers/mL were introduced into each flask. For each toxicant, experiment consisted of a control with no toxicant (only EPA medium) and four sub-lethal concentrations with three replicates for each treatment. In the case of 3,4-DCA, which was dissolved with a minimal amount of acetone, one control and another control + acetone treatments were used.

Table 4.1: Concentrations of the four toxicants used in CEA experiments

Toxicants	**Concentration mg/L**			
Cu	0.000006	0.00006	0.0006	0.006
Cd	0.000006	0.00006	0.0006	0.006
PCP	0.1	0.2	0.3	0.4
3,4-DCA	0.2	0.2	0.4	0.8

Exposures were conducted through four experiments; each toxicant had a separate set of exposure experiment. The rotifers were exposed to the toxicant concentrations for 24h at a temperature of 25 ˚C ±1 and day: night photoperiod cycle of 16:8h. Rotifers in the individual treatments and controls were not supplied with food during the exposure. At the end of the exposure period (after 24h), organisms of each replicate were harvested, rinsed, and freeze-dried, as mentioned in Chapter 3. After that, each dried replicate was weighed and stored at -80 ˚C. As explained in Chapter 3, the available energy fractions (reserves) were analysed in the homogenates of dried animals by spectrophotometrically determining of protein, lipid, and carbohydrate contents using the methods of bicinchoninic acid (BCA), the Nile red fluorescent, and the phenol–sulfuric acid respectively. Finally, all the energy fraction amounts were

transformed into energetic equivalents using the energy of combustion according to Gnaiger (1983), where protein = 24000 mj/mg, lipids = 39500 mj/mg, and carbohydrates = 17500 mj/mg.

4.2.3Analysis of data

The obtained results were statistically analysed using the SPSS software package (version 25). Significant differences between the observations in the treatments and control were analysed using one-way analysis of variance (ANOVA), after testing data for homogeneity of variance (Levene's test). Two-way ANOVA was used to compare the effects of the two heavy metals with each other under different stress levels as well as to compare the effects of the two organic toxicants to each other under different stress levels. Here, the toxicant type and the stress levels were the input two factors, while the total available energy, as percentage of control, was used as the independent variable. Differences were considered significant at $P \leq 0.05$.

4.3 Results

4.3.1The available energy fractions in *B. calyciflorus* under copper (Cu) stress

4.3.1.1 Protein contents

Protein contents under control conditions were 80.8 ±2 µg/mg of rotifers. While under Cu concentrations, protein amounts ranged from 74 ± 2.5 µg/mg under the lowest Cu concentration to 83.4 ± 2 µg/mg under the highest Cu concentrations. There was a significant effect of Cu exposure on protein levels of rotifers ($F_{4, 40}$, p = 0.042). However, pairwise comparisons showed that no significant differences between protein reserves under any of the Cu concentrations compared to control group (p values ranged from 0.074 to 0.82). By comparing the Cu concentrations to each other, rotifers exposed to the lowest Cu concentration had significantly lower proteins compared with both 0.6 µg/L and 6 µg/L groups (p = 0. 046 and 0.015 respectively) but was not different from the 0.06 µg/L group (p = 0.90). The rotifers

exposed to 0.06 µg/L had lower protein contents than those of 6 µg/L (p = 0. 0.02) but similar to those of 0.6 µg/L (p = 0.06). The two highest Cu concentrations had similar protein contents (p = 0.63) (Figure 4.1).

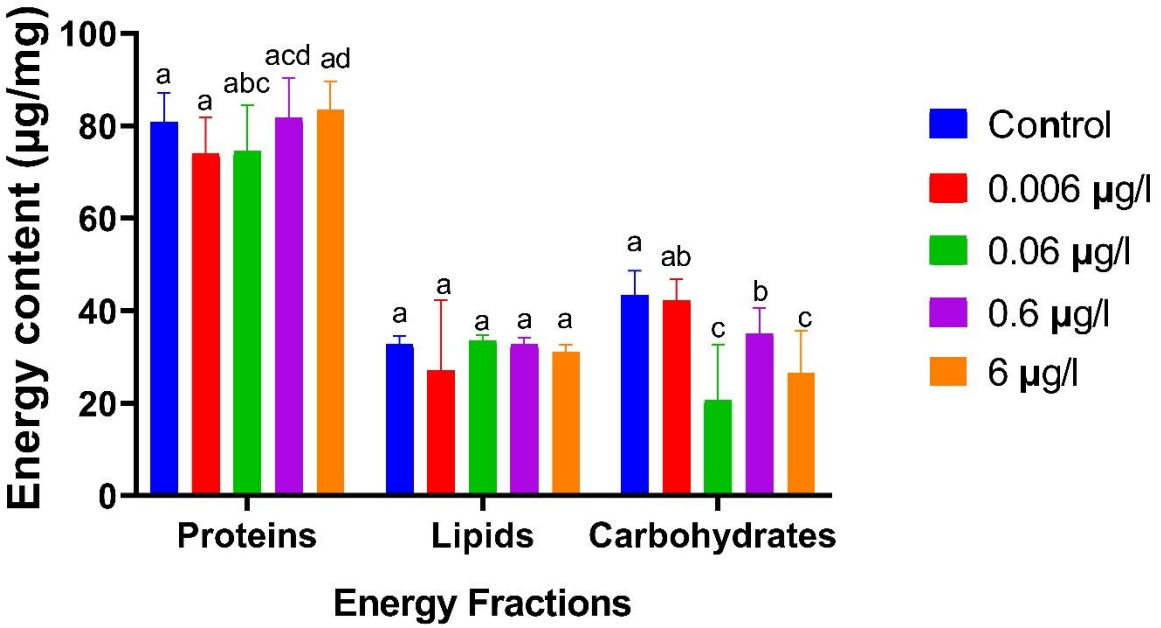

Fig. 4.1: The averages of the energy contents; protein, lipid, and carbohydrate (µg/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of copper (Cu). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cu concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3)

4.3.1.2 Lipid contents

Lipid contents under control conditions were 33 ± 2 µg/mg of rotifers. While under Cu concentrations, lipid amounts ranged from 27 ± 14 µg/mg under the lowest Cu concentration to 34.1 ± 1 µg/mg under the second lowest Cu concentration. There was no significant effect of exposure to Cu on the lipid contents of rotifers ($F_{4,40}$, p= 0.285). Lipid reserves were not significantly different under any of the Cu concentrations compared to the control group (P values ranged from 0.081 to 0.96). Furthermore, there was no significant differences between the Cu concentrations compared to each other (P values ranged from 0.051 to 0.79) (Figure 4.1).

4.3.1.3 Carbohydrate contents

Carbohydrate content under control conditions was 43 ± 5 µg/mg of rotifers. While under Cu concentrations, carbohydrate amounts ranged from 21 ± 11 µg/mg under the second lowest Cu concentration to 42 ± 5 µg/mg under the lowest Cu concentration. There was a significant effect of exposure to Cu on carbohydrate contents of rotifers ($F_{4, 40}$, p = 0.001). Compared to control, significant decreases in carbohydrate reserves by 52, 19, and 38% were observed for the concentrations of 0.06 (p = 0.001), 0.6 (p = 0. 028), and 6 µg/L (p = 0.001), respectively. The 0.006 µg/L concentration was not different from the control group (p = 0.75) and from the 0.6 µg/l group. In addition, the 0.006 µg/L group significantly differed from the 0.06 µg/L (p = 0.001) and 6 µg/L (p = 0.001) groups, which had comparably lower carbohydrate contents. Rotifers in 0.6 µg/L Cu had higher carbohydrate content relative to the 0.06 µg/L (p = 0.001) and 6 µg/L (p = 0. 027) groups that had similar protein contents (p = 0.118) (Figure 4.1).

4.3.1.4 Total of available energy

Energy fractions were transformed into energetic equivalents using the energy of combustion according to Gnaiger (1983), where protein = 24000 mj/mg, lipids = 39500 mj/mg, and carbohydrates = 17500 mj/mg. Under the control conditions, energy equivalent to protein reserves were 1940 ± 149 mj/mg of rotifers. Under copper exposure, energy equivalent to protein reserves ranged from 1776 ± 186 mj/mg under the lowest Cu concentration (0.006 µg/L) to 2003 ± 147 mj/mg under the highest Cu concentrations (6 µg/L) (Figure 4.2).

Under the control conditions, energy equivalent to lipid reserves were 1297 ± 61 mj/mg. Under copper exposure, lipid reserves ranged from 1066 ± 451 mj/mg under the lowest Cu concentration to 1326 ± 45 mj/mg under the 0.06 µg/L concentration. Energy equivalent to carbohydrate reserves for the control group were 759 ± 81 mj/mg. For carbohydrates, under

copper exposure, the energy reserves ranged from 363 ± 197 mj/mg under the 0.06 µg/L concentration to 739 ± 76 mj/mg under the 0.006 µg/L concentration (Figure 4.2).

Combining Ea stores held in protein, lipid, and carbohydrate (protein-Ea + lipid-Ea + carbohyderate-Ea) enables an overall Ea to be determined (Fig 4.3). The Energy available (Ea), as the sum of the three energy fractions, was 3997 ± 228 mj/mg for the control group. Under Cu concentrations, the Ea ranged from 3475 ± 373 mj/mg at 0.06 µg/L to 3864 ± 285 mj/mg at 0.6 µg/L concentration. The amount of Ea was not significantly affected by exposure to Cu ($F_{4, 40}$, p = 0.083). No significant differences were observed in Ea of rotifers under the two highest Cu concentrations (0.6 µg/L and 6 µg/L) when compared to the control group, but the lowest concentrations (0.006 µg/L and 0.06 µg/L) had significantly lower Ea content compared with control (p = 0.044 and 0.012 respectively). Where these significant differences were discernible via multiple pairwise comparison; in reality, the absolute difference in Ea was not very large – maximum divergence form the control was about 13% of the control Ea value. Given the biological origin of the samples, such "natural" variation is to be expected. Thus, while significant differences were detected; in real terms, Cu (across the range 0.006 to 6 µg/L) had no impact on Ea and this was further evidenced by the lack of significant effect of Cu concentrations ($F_{4, 40}$, p = 0.083). On the other hand, Ea was not significantly different in the Cu exposed rotifers at all the concentrations used when compared to each other (Figure 4.3).

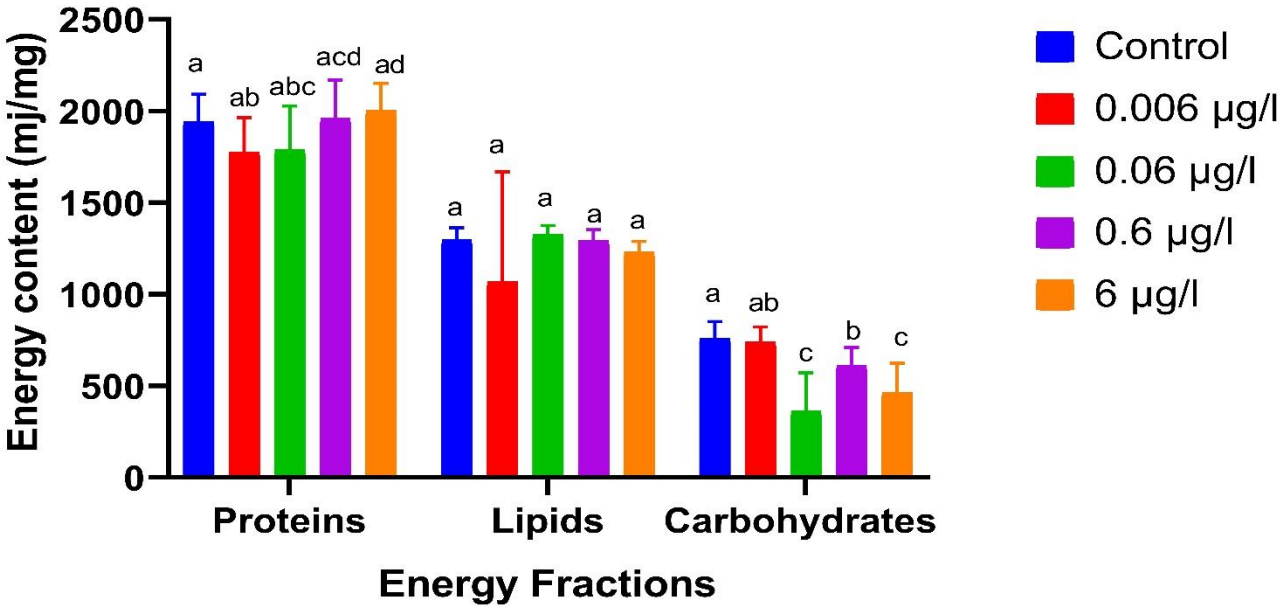

Fig.4.2: The averages of the available energy fractions; protein, lipid, and carbohydrate reserves (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of copper (Cu). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cu concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3).

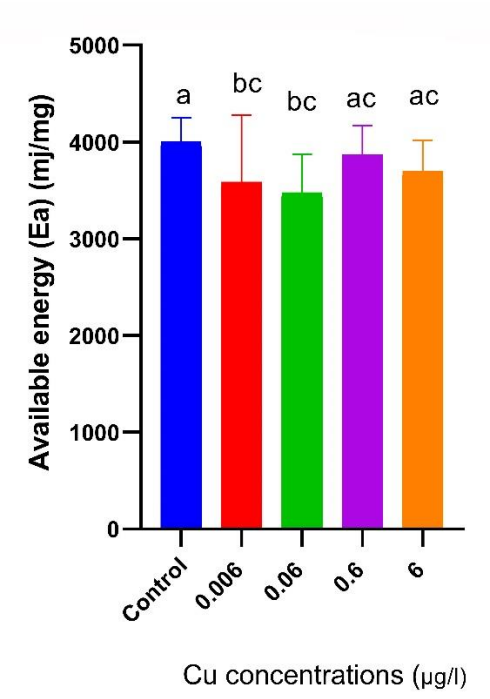

Fig. 4.3: The averages of the total available energy in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of copper (Cu). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cu concentrations. Results are expressed as average ± standard deviations (N=3).

4.3.2 The available energy fractions content in *B. calyciflorus* under the stress of cadmium (Cd)

4.3.2.1 Protein content

Protein content under control conditions was 82 ± 6 µg/mg of rotifers. While under Cd exposure, protein contents ranged from 67 ± 3 µg/mg under the highest Cd concentration to 82 ± 3 µg/mg under the lowest Cd concentration. There was a significant effect of Cd exposure on protein levels in rotifers ($F_{4, 40}$, P = 0.005). Pairwise comparisons showed that protein content in 0.06, 0.6, 6 µg/L Cd were significantly lower than in the control group (p = 0.008, 0.008, 0.004, respectively) and the lowest Cd exposure concentration (0.006 µg/L; p = 0.021, 0.022, 0.011 respectively), which itself was not different from the control (p = 0.688). Moreover, no significant differences were observed between protein reserves of rotifers exposed to Cd concentrations of 0.06, 0.6 and 6 µg/L (Figure 4.4).

4.3.2.2 Lipid contents

Lipid content under control conditions was 33 ± 3 µg/mg. While under Cd concentrations, lipid amounts ranged from 33 ± 6 µg/mg under Cd concentration of 0.06 µg/L to 42 ± 4 µg/mg under Cd concentration of 0.6 µg/L. There was a significant effect of Cd concentrations on lipid levels ($F_{4, 40}$, P = 0.003). Pairwise comparisons showed that rotifers under Cd concentrations of 0.6 and 6 µg/L had significantly higher lipid contents compared with both the control (p = 0.002 and 0.005 respectively) and the 0.06 µg/L Cd treatment (p = 0.003 and 0.007 respectively). On the other hand, protein contents in those two treatments were not significantly different from those in the lowest Cd concentration, 0.006 µg/L (p = 0.050 and 0.089 respectively), which was also not significantly different from those in the control (p = 0.225) (Figure 4.4).

4.3.2.3 Carbohydrate content

Carbohydrate content was 35 ± 10 µg/mg for the control group. Carbohydrate reserves in rotifers exposed to Cd ranged from 38 ± 12 µg/mg under Cd concentration of 0.6 µg/l to 47 ± 13 µg/mg under Cd concentration of 6 µg/L. There was no significant effect of Cd concentrations on carbohydrate contents ($F_{4, 40}$, p = 0.145). Pairwise comparisons showed that all Cd concentrations were not significantly different from the control group or from each other, except for the 6 µg Cd/L which had significantly higher lipids compared with the control group (p = 0.017) (Figure 4.4).

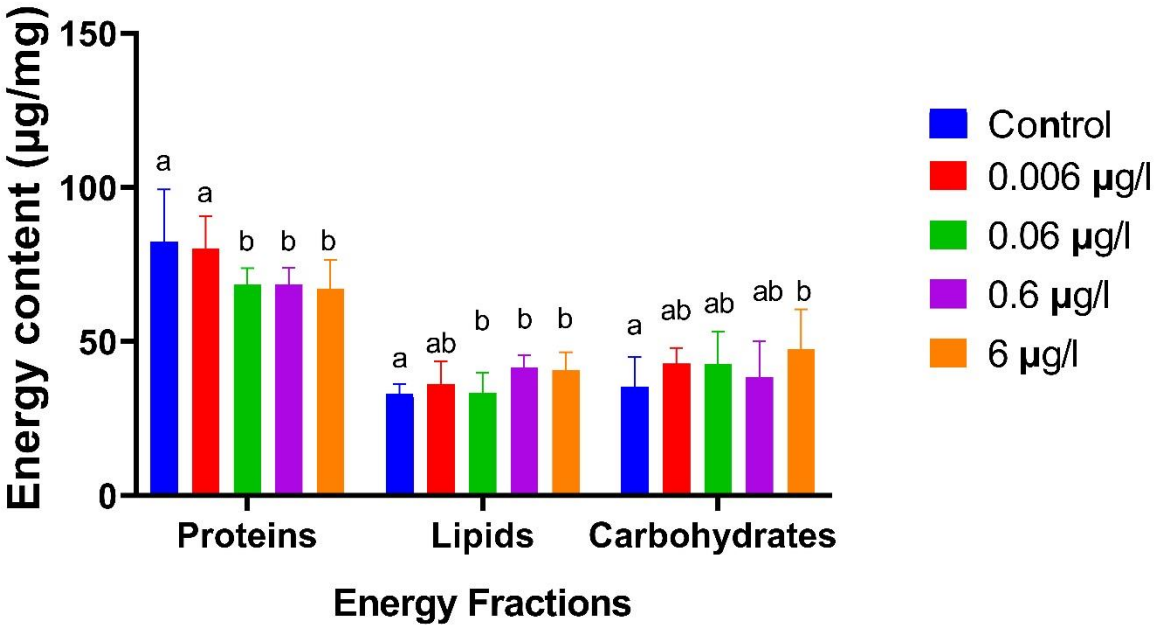

Fig. 4.4: The averages of the energy contents; protein, lipid, and carbohydrate (µg/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of cadmium (Cd). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cd concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3)

4.3.2.4 Total available energy

All the energy fractions were transformed into energetic equivalents using the energy of combustion according to Gnaiger (1983), where protein = 24000 mj/mg, lipids = 39500 mj/mg and the carbohydrates = 17500 mj/mg. Protein reserves under the control group was 1973 ± 386 mj/mg. Protein reserves under the Cd concentrations ranged from 1612 ± 211 mj/mg under the highest Cd concentration to 1925 ± 234 mj/mg under the lowest Cd concentration. Lipid reserves under control conditions were 1305 ± 112 mj/mg. Lipid levels in the treated rotifers under Cd concentration ranged from 1319 ± 241 mj/mg at 0.06 µg/L to 1640 ± 147 mj/mg at 0.6 µg/L. Carbohydrate reserves were 618 ± 167 mj/mg for the control group. Carbohydrate reserves in rotifers exposed to Cd ranged from 673 ± 193 mj/mg under 0.6 µg/L to 830 ± 214 mj/mg under 6 µg/L (Figure 4.5). The energy available in rotifers under control conditions were 3897 ± 418 mj/mg. Under Cd exposure conditions, it ranged from 3710 ± 463 mj/mg under the 0.06 µg/L to 4108 ± 248 mj/mg under the 0.006 µg/L. No significant effect of Cd concentrations was observed on the Ea of rotifers ($F_{4, 40}$, p= 0.218).

Pairwise comparisons showed no differences observed in E_a of treated rotifers under all Cd concentrations when compared to the control group. Furthermore, E_a was not significantly different in the Cd treated rotifers at all the concentrations when compared to each other, except for the 0.06 µg/L concentration that was significantly lower than the 0.006 µg/L concentration (p = 0.031), and these two groups were not significantly different from all other treatments (Figure 4.6). Thus, the ANOVA results showed that Cd (across the range 0.006 to 6 µg/L) had no impact on the Ea content of the rotifer *B. calyciflorus*.

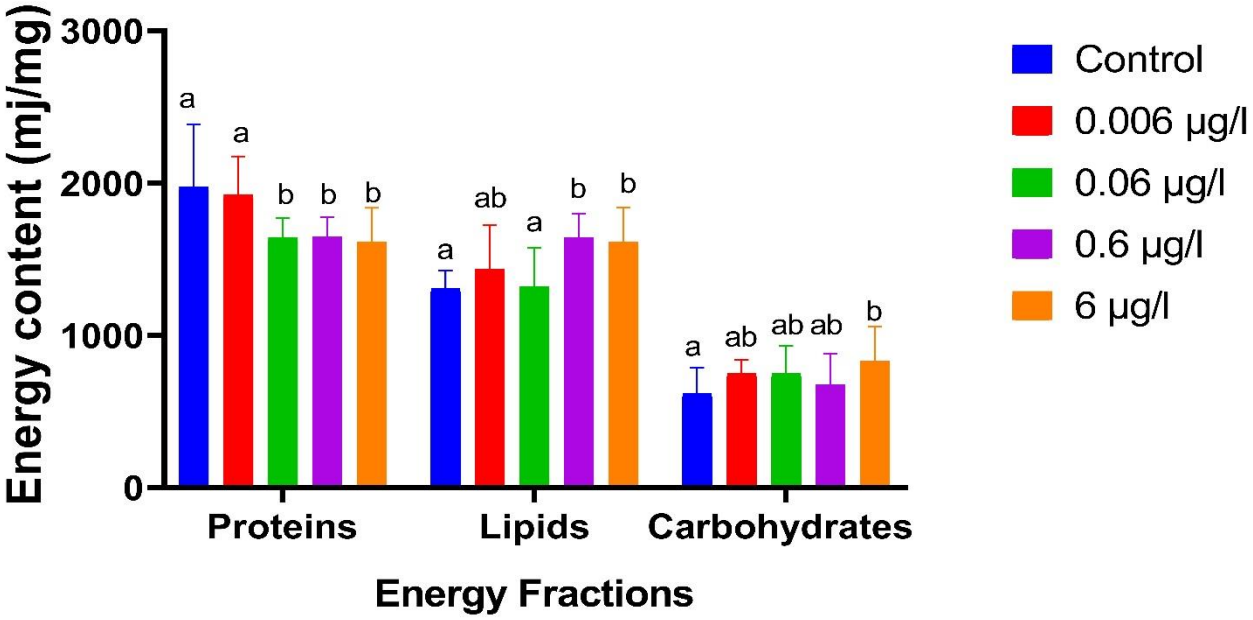

Figure 4.5: The averages of the available energy fractions; protein, lipid, and carbohydrate reserves (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of cadmium (Cd). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between Cd concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3).

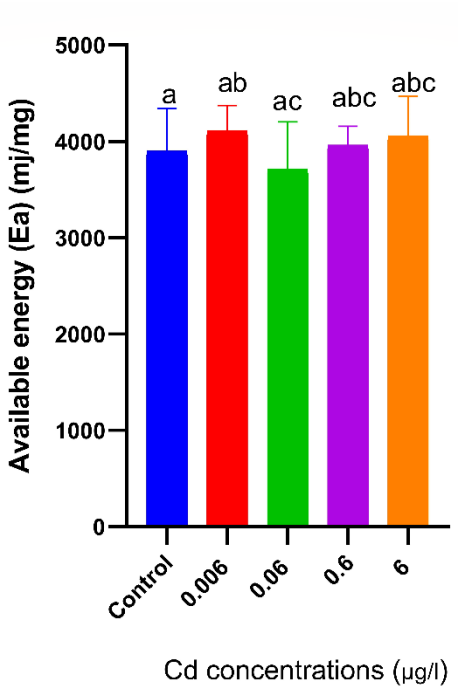

Fig. 4.6: The averages of the total available energy (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of cadmium (Cd). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between Cd concentrations. Results are expressed as average ± standard deviations (N=3).

4.3.3 Measurements of available energy in *B. calyciflorus* under stress of pentachlorophenol (PCP)

4.3.3.1 Protein contents

Protein content for rotifers in the control group was 204 ± 5 µg/mg. Under PCP exposure, the protein content ranged from 143 ± 1 µg/mg under PCP concentration of 0.4 mg /L to 177 ± 7 under PCP concentration of 0.1 mg PCP/L. Protein reserves steadily decreased with increasing PCP concentration ($F_{4,40}$, $p = 0.001$). Indeed, when compared with control conditions, protein reserves were significantly reduced by 13, 20, 28, and 30% in the treated rotifers under PCP concentrations of 0.1, 0.2, 0.3, and 0.4 mg/L respectively ($p = 0.001$ for all concentrations). Protein levels under 0.1 mg PCP/L were significantly different from those of 0.2 ($p = 0.041$), 0.3 ($p = 0.001$), and 0.4 mg/L ($p = 0.001$). Also, rotifers exposed to 0.2 mg PCP/L significantly differed in protein contents when compared with those of 0.3 ($p = 0.037$) and 0.4 mg PCP/L (p

= 0.008) concentrations. However, protein levels in the treated rotifers under the 0.3 mg PCP/L and the 0.4 mg PCP/L concentrations were not significantly different (p = 0.527) (Figure 4.7).

4.3.3.2 Lipid content

Lipid content was 103 ± 12 µg/mg for the control group. Under PCP exposure, lipid contents ranged from 59 ± 17 µg/mg under PCP concentration of 0.2 mg/L to 75 ± 18 µg/mg under PCP concentration of 0.1 mg/L. The exposure to PCP concentrations significantly affected the lipid contents in the exposed rotifers (F4,40, p = 0.001). Compared with the control, lipid contents in the PCP exposed rotifers were significantly reduced by 27, 42, 36, and 30% at 0.1, 0.2, 0.3, and 0.4 mg PCP/L, respectively (p = 0.001 for all concentrations). Moreover, lipid content did not differ significantly between individual treatments, except for the exposure to 0.2 mg PCP/L which had significantly lower lipid levels than rotifers exposed to 0.1 mg PCP/L (p =0.016) (Figure 4.7).

4.3.3.3 Carbohydrate content

Carbohydrate content was 132 ± 12 µg/mg for rotifers in the control group. Under PCP exposure, carbohydrate contents ranged from 108 ± 10 µg/mg under highest PCP concentration to 129 ± 24 µg/mg in rotifers kept under the lowest PCP concentration. There was a significant effect of PCP exposure on carbohydrate contents in the exposed rotifers (F4.40, p = 0.031). Compared to the control, rotifers exposed to PCP showed decreasing carbohydrate content when the PCP concentration increased, but this decrease in carbohydrates was significant only at the two highest PCP concentrations (0.3 mg/L, p = 0.024; 0.4 mg/L, p = 0.007). Moreover, no significant differences were observed in carbohydrate reserves of rotifers between the treatments, except for 0.4 mg PCP/L that had significantly lower carbohydrate reserves compared with that of the lowest PCP concentration (p = 0.020) (Figure 4.7).

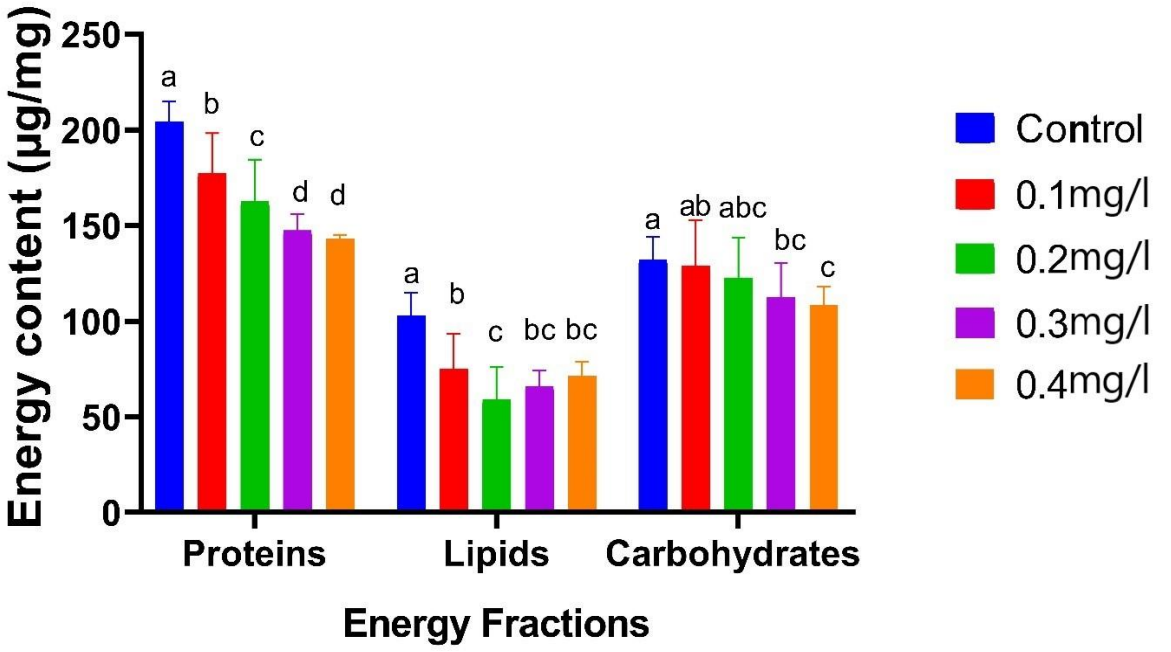

Fig. 4.7: The averages of energy contents; protein, lipid, and carbohydrate (µg/mg) measured in the rotifer *B. calyciflorus* after 24 hrs exposure to different concentrations of pentachlorophenol (PCP). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between PCP concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3).

4.3.3.4 Total of available energy

All the energy fractions were transformed into energetic equivalents using the energy of combustion according to Gnaiger (1983, where protein = 24000 mj/mg, lipids = 39500 mj/mg, and carbohydrates = 17500 mj/mg. The determined average protein reserves for the control group was 4905 ± 83 mj/mg. Under the PCP exposure, the protein contents ranged from 3431 ± 17mj/mg at 0.4 mg PCP/L to 4256 ± 167 at 0.1 mg PCP/L. Lipid reserves were 4059 ± 158 mj/mg for the control group. Under PCP exposure, lipid contents ranged from 2335 ± 224 mj/mg at 0.2 mg/L to 2965 ± 242 mj/mg at 0.1 mg/L. Carbohydrate reserves were 2313 ± 69 mj/mg for the control group. And under PCP exposure, carbohydrate reserves ranged from

1895 ± 56 mj/mg at highest PCP concentration to 2252 ± 140 mj/mg at the lowest PCP concentration (Figure 4.8).

The total available energy for the control group was 11279 ± 271 mj/mg. Under exposure to PCP, the available energy ranged from 8105 ± 147 mj/mg at 0.3 mg PCP/L to 9474 ± 513 mj/mg at 0.1 mg PCP/L. The available energy in rotifers was significantly affected by the exposure to PCP ($F_{4,40}$, $p = 0.001$). The Ea content steadily decreased with increasing PCP concentration in compared with control conditions, and rotifers exposed to PCP showed significant reduction in Ea by 16, 25, 28, and 27% at concentrations of 0.1 ($p = 0.001$), 0.2 ($p = 0.001$), 0.3 ($p = 0.001$), and 0.4 ($p = 0.001$) mg PCP/L respectively. The E_a levels of the PCP-treated rotifers were not significantly different when compared between all treatments, except for the lowest PCP concentrations that had significantly higher Ea levels compared to that of 0.2 ($p = 034$), 0.3 ($p = 0.009$), and 0.4 ($p = 0.010$) mg PCP /L (Figure 4.9). These results indicate that the PCP was negatively impacting the Ea through the gradient of concentrations.

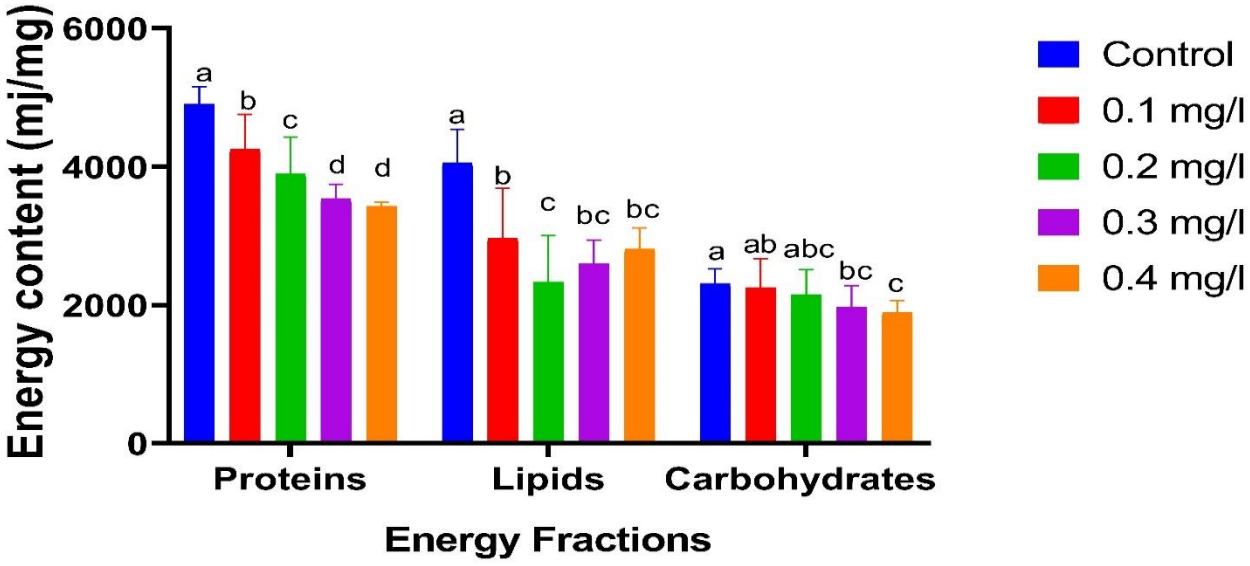

Figure 4.8: The averages of available energy fractions; protein, lipid, and carbohydrate reserves (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of pentachlorophenol (PCP). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between PCP concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3).

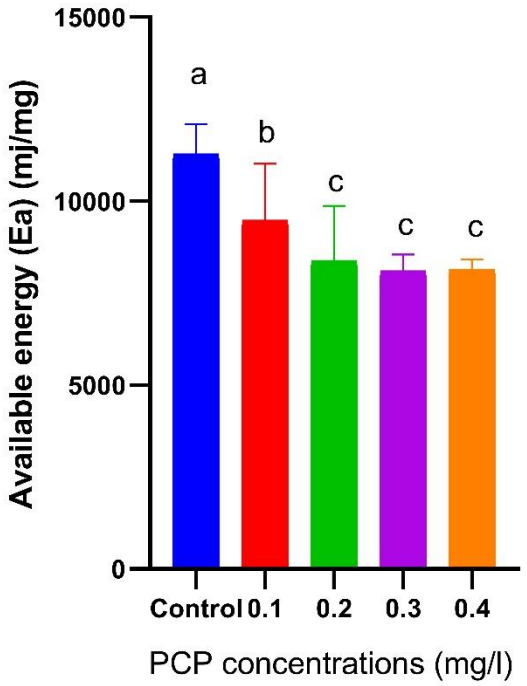

Fig. 4.9: The averages of the total available energy (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of pentachlorophenol (PCP). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between PCP concentrations. Results are expressed as average ± standard deviations (N=3).

4.3.4 Measurements of available energy in *B. calyciflorus* under stress of 3,4-dichloroaniline (3,4-DCA)

4.3.4.1 Protein contents

Protein content was 173 ± 6 µg/mg for rotifers in the control group and 172 ± 1 µg/mg for the control with acetone group. Under 3,4-DCA exposure, protein reserves ranged from 130 ± 4 µg/mg at 0.8 mg/L to 170 ± 5 µg/mg at 0.2 mg/L. The protein contents in rotifers were significantly affected by exposure to 3,4-DCA ($p = 0.001$). No significant difference was observed between the two control groups (with / without acetone). Moreover, protein levels in the rotifers exposed to the lowest concentration (0.2 mg/L) were not significantly different from those of the two control groups.

On the other hand, protein reserves decreased significantly with the further increase in DCA concentrations. Protein levels were significantly reduced by 14.3, 20.7, and 24.8% by exposure to 0.4, 0.6, and 0.8 mg/L, respectively ($p = 0.001$ for all concentrations) when compared with the control. Protein levels in the treated rotifers exposed to 0.4, 0.6, and 0.8 mg/L of 3.4-DCA decreased by 20.1, 19.3, and 23.5%, respectively, when compared to the 0.2 mg/L group ($p = 0.001$ for all concentrations). A similar trend was obtained for protein levels of the rotifers treated with 0.8 mg/L, which reduced protein levels by 12.2 % ($p = 0.002$) when compared with the 0.4 mg/L group. However, both of these two groups were not different from protein levels at 0.6 mg/L ($p = 0.207$ and 0.052 respectively) (Figure 4.10).

4.3.4.2 Lipid content

Lipid content was 47 ± 13 µg/mg and 42 ± 7 µg/mg for the control and the control with acetone groups, respectively. Under 3,4-DCA exposure, the lipid content ranged from 23 ± 7 µg/mg under 3,4-DCA concentration of 0.8 mg/L to 47 ± 9 µg/mg under 3,4-DCA concentration of 0.2 mg/L. Similar to proteins, the lipid contents in the rotifers were also affected by the exposure to the 3,4-DCA ($p = 0.001$). No significant difference was observed between the two control groups. Moreover, lipid contents in the rotifers exposed to the lowest 3,4-DCA concentration (0.2 mg/L) were not significantly different from those of the two control groups. While all other concentrations showed significantly lower lipid contents when compared to the control. In the case of the control with acetone, the 0.4 and 0.6 mg/L treatments did not differ significantly in lipid content, while the 0.8 mg/L treatment showed significantly lower lipid contents in comparison with the control plus acetone group ($p = 0.011$).

By comparing lipid content between treatment groups, the lowest 3,4-DCA concentration (0.2 mg/L) group had the highest lipid contents compared to the other higher concentrations ($p = 0.026$, 0.023, and 0.001 for 0.4, 0.6, and 0.8 mg/L, respectively). On the contrary, the highest 3,4-DCA concentration (0.8 mg/L) group had the lowest lipid contents compared with the three

other lower concentrations (p = 0.001, 0.002, and 0.002 for 0.2, 0.4, and 0.6 mg/L, respectively). The two middle concentrations (0.4 and 0.6 mg/L) showed similar lipid contents (p = 0.96) (Figure 4.10).

4.3.4.3 Carbohydrate content

Carbohydrate content was 126 ± 31 µg/mg and 135 ± 37 µg/mg for control and control with acetone groups, respectively. Under 3,4-DCA exposure, carbohydrate contents ranged from 52 ± 11 µg/mg under the highest 3,4-DCA concentration (0.8mg/L) to 109 ± 26 µg/mg at the lowest 3,4-DCA concentration (0.2 mg/L). Similar to proteins and lipids, exposure to 3,4-DCA significantly affected rotifer carbohydrate contents (p = 0.001). Pairwise comparisons showed that no significant differences were observed between the two control groups (p = 0.48). Moreover, carbohydrate levels in the rotifers exposed to the lowest 3,4-DCA concentration were not significantly different from those of the control group (p =0.19) but significantly differed from those of the control with acetone group (p = 0.049). On the other hand, when the concentration of 3,4-DCA increased more than 0.2 mg/L, carbohydrate reserves were significantly decreased by 32.8, 35.2, and 58% for 0.4, 0.6, and 0.8 mg/L, respectively, relative to the control group (p = 0.003, 0.001, and 0.001 respectively). Furthermore, the three concentrations also significantly reduced the carbohydrate contents when compared with the control with acetone group (p = 0.001 for all concentrations).

Comparing the exposed rotifers with each other, the group exposed to the lowest 3,4-DCA concentration had the highest carbohydrate contents compared to 0.6 (p = 0.043) and 0.8 (p = 0.001) mg/L, but it was not significantly different from that of 0.4 mg/L (p = 0.070). Whereas the group exposed to the highest 3,4-DCA concentration had the lowest carbohydrate contents compared with the other three lower concentrations (p = 0.001, 0.017, and 0.029 for 0.2, 0.4, and 0.6 mg/L respectively). The two middle concentrations (0.4 and 0.6 mg/L) had similar carbohydrate levels (p = 0.81) (Figure 4.10).

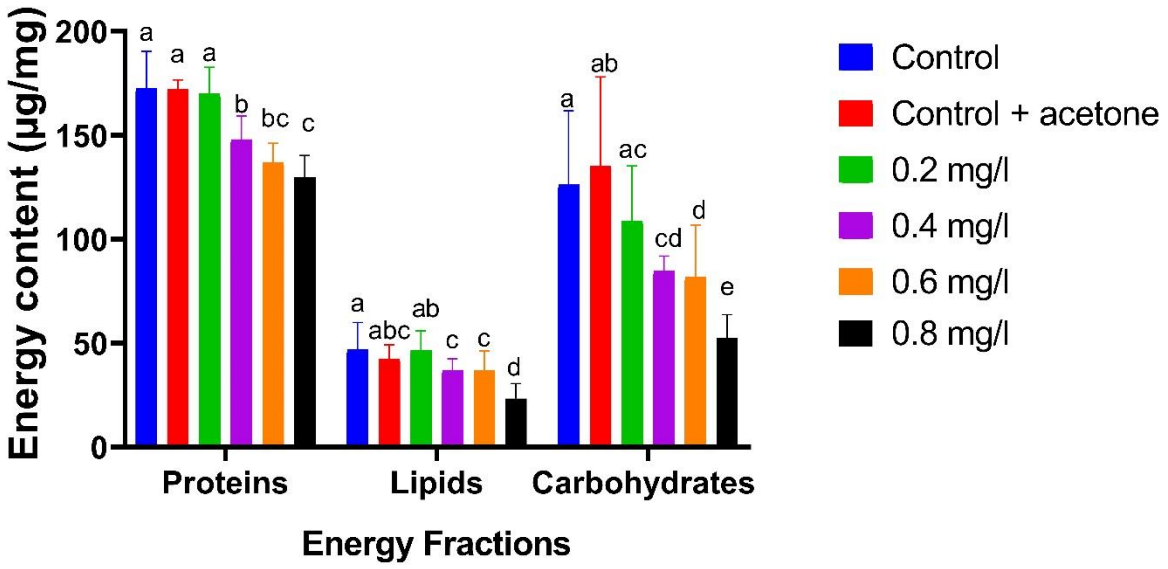

Figure 4.10: The averages of the energy contents; protein, lipid, and carbohydrate (µg/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of 3,4 dichloroaniline (3,4-DCA). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between PCP concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3)

4.3.4.4 Total of available energy

All the energy fractions were transformed into energetic equivalents using the energy of combustion according to Gnaiger (1983), where protein = 24000 mj/mg, lipids= 39500 mj/mg, and carbohydrates = 17500 mj/mg. Protein reserves were 4140 ±143 mj/mg for the control group and were 4133 ± 35 mj/mg for the control with acetone group. Under 3,4-DCA exposure, protein reserves ranged from 3115 ± 83 mj/mg at concentration of 0.8 mg/L to 4075 ± 102 mj/mg at concentration of 0.2 mg/L. Lipid reserves were 1851 ± 171 mj/mg and 1677 ± mj/mg for the control and the control with acetone groups, respectively. Under 3,4-DCA exposure, the lipid reserves ranged from 922 ± 93 mj/mg at 0.8 mg/L to 1841 ± 122 mj/mg at 0.2 mg/L. Carbohydrate reserves were 2207 ± 208 mj/mg and 2367 ± 249 mj/mg for control and control with acetone groups, respectively. Under the 3,4-DCA exposure, carbohydrate contents ranged

from 916 ± 65 mj/mg at the highest concentration (0.8mg/L) to 1905 ± 153 mj/mg at the lowest concentration (0.2 mg/l) (Figure 4.11).

The amount of energy available (Ea) was 8200 ± 464 mj/mg and 8179 ± 314 mj/mg for control and control with acetone groups, respectively. Under the 3,4-DCA exposure, the Ea ranged from 4954 ± 224 mj/mg at the highest concentration (0.8 mg/L) to 7823 ± 281 mj/mg at the lowest concentration (0.2 mg/L). The exposure to 3,4-DCA had a significant effect on the Ea ($p = 0.001$). No significant difference was observed between the two control groups ($p = 0.96$). Moreover, Ea levels in the rotifers under the lowest 3,4-DCA concentration (0.2mg/L) were not significantly different from those of the control ($p = 0.39$) and control with acetone ($p = 0.41$) groups. On the contrary, the energy available decreased significantly with increasing 3,4-DCA concentrations more than 0.2 mg/L, since Ea levels were significantly reduced by 20, 24, and 39% in the rotifers exposed to concentrations of 0.4, 0.6 and 0.8 mg/L, respectively, relative to the two control groups ($p = 0.001$ for all concentrations).

Comparing the Ea 3,4-DCA concentrations between treatments, the lowest 3,4-DCA concentration (0.2 mg/L) group showed the significant highest Ea contents compared with the other three higher concentrations ($p = 0.004$, 0.001, and 0.001 for 0.4, 0.6, and 0.8 mg/L respectively). On the contrary, the highest 3,4-DCA concentration (0.8 mg/L) group showed the significantly lower Ea contents when compared with the other three lower concentrations ($p = 0.001$, 0.001, and 0.008 for 0.2, 0.4, and 0.6 mg/L respectively). The two middle concentrations (0.4 and 0.6 mg/L) showed similar Ea contents ($p = 0.45$) (Figure 4.12). These results indicate a significant adverse impact of 3,4-DCA on Ea through the gradient of concentrations.

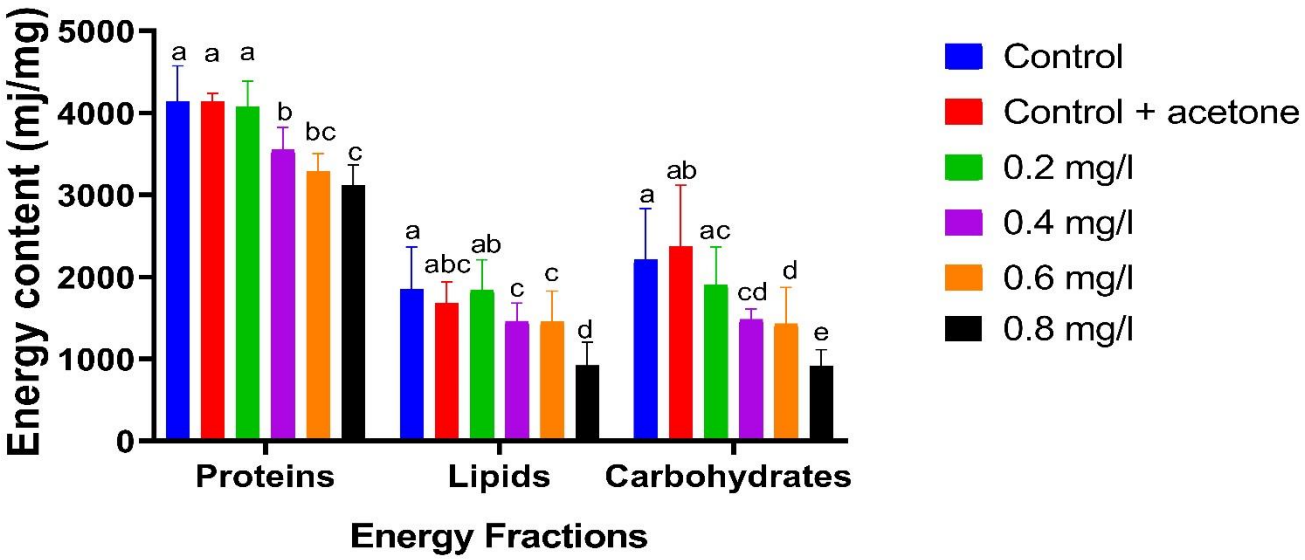

Fig. 4.11: The averages of the available energy fractions; protein, lipid, and carbohydrate reserves(mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of 3,4- Dichloroaniline (3,4-DCA). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between 3,4-DCA concentrations for each energy fraction. Results are expressed as average ± standard deviations (N=3)

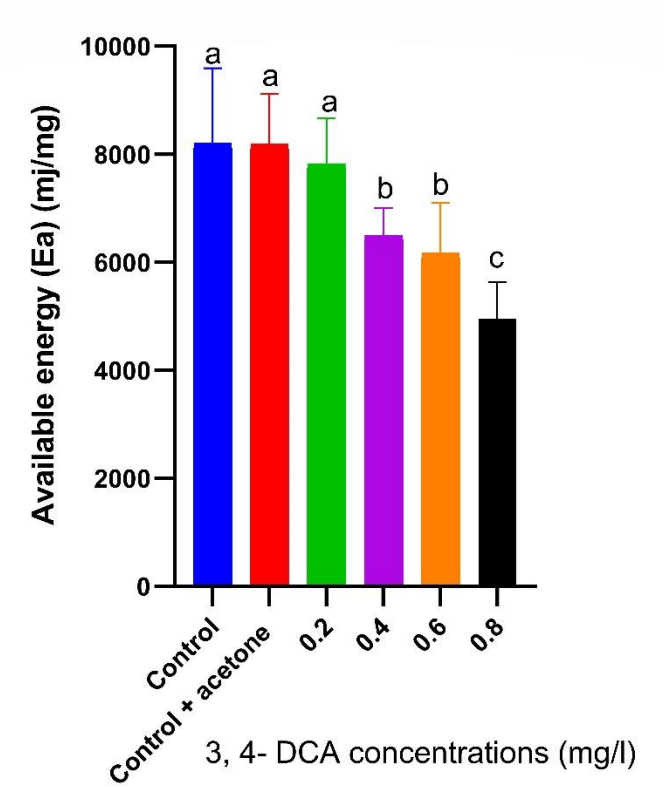

Fig. 4.12: The averages of the total available (mj/mg) measured in the rotifer *B. calyciflorus* after 24 h exposure to different concentrations of 3,4- Dichloroaniline (3,4-DCA). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between 3,4-DCA concentrations. Results are expressed as average ± standard deviations (N=3).

4.3.5 Available energy as percentage of control

To more easily compare the 4 toxicants, Ea values were converted to a percentage of the control values of the individual toxicants. Toxicant concentrations were also converted to a designated stress level, which were derived from the range finding experiments conducted in Chapter 2. In order to aid the discussion (avoiding incongruent concentration values), the four stress levels applied in each experiment were designated from low stress to high stress, respective to toxicant concentration from the lowest to highest concentration. An intuitive colour alert scale was used, wherein *green-stress* was the lowest stress level, *yellow-stress* next, then *orange-stress,* and finally *red-stress*, which was the highest stress level, linked to the highest toxicant concentration.

Under the exposure to heavy metals Cu and Cd, Ea showed very little change across the stress gradient applied and was not significantly different ($F_{3,63}$, $p = 0.061$). However, there was a significant difference between the effects of the two toxicants, as Cu led to an overall significantly lower available energy compared to Cd ($F_{3,63}$, $p = 0.001$). Pairwise comparisons showed that this difference was driven by the results of the two toxicants only under the green- and the red-stress levels used (Figure 4.13). There was no interaction between the toxicant type and the stress levels.

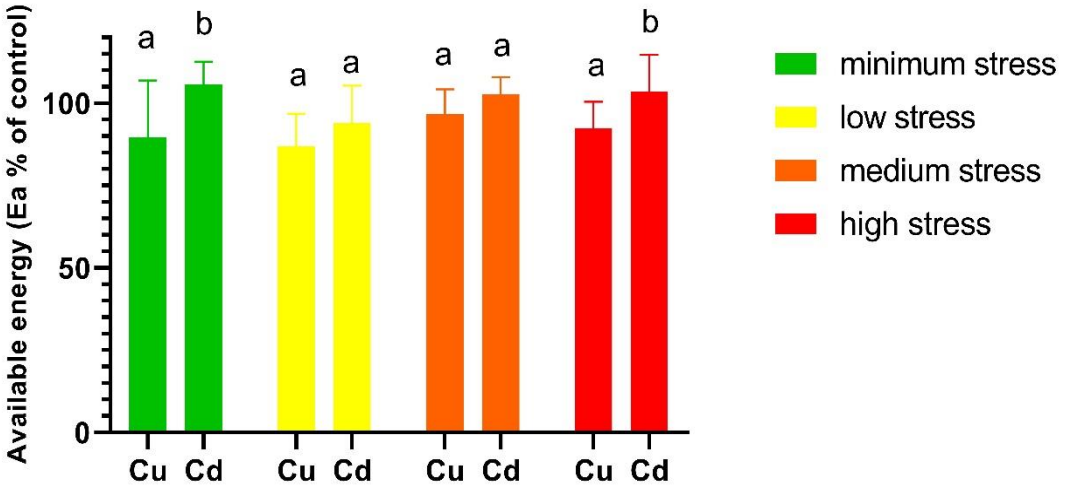

Fig. 4.13: The available energy as percentage of control values in rotifers exposed to Cu and Cd stress.

Under exposure of organic toxicants, there was a clear trend of decreasing Ea with the magnitude of stress, and there was a significant effect of stress levels ($F_{3,63}$, $p = 0.001$) (Figure 4.14). Furthermore, there was no effect of toxicant type, as both toxicants had, overall, similar effects on the total available energy ($F_{1,63}$, $p = 0.77$). For PCP, decreases in Ea were marked under yellow, orange, and red stress levels. While for 3,4-DCA, decreases in Ea were marked under the red stress level. For PCP, Ea values decreased to around 73-75% of the control value under these higher stress levels. While for 3.4-DCA, Ea decreases were greater, with higher stress levels decreasing Ea to 60% of the control value (Figure 4.14). There was a significant interaction effect between the stress level and the toxicant type ($F_{3,63}$, $p = 0.005$), as under red stress level, 3,4-DCA significantly reduced the Ea compared to the PCP.

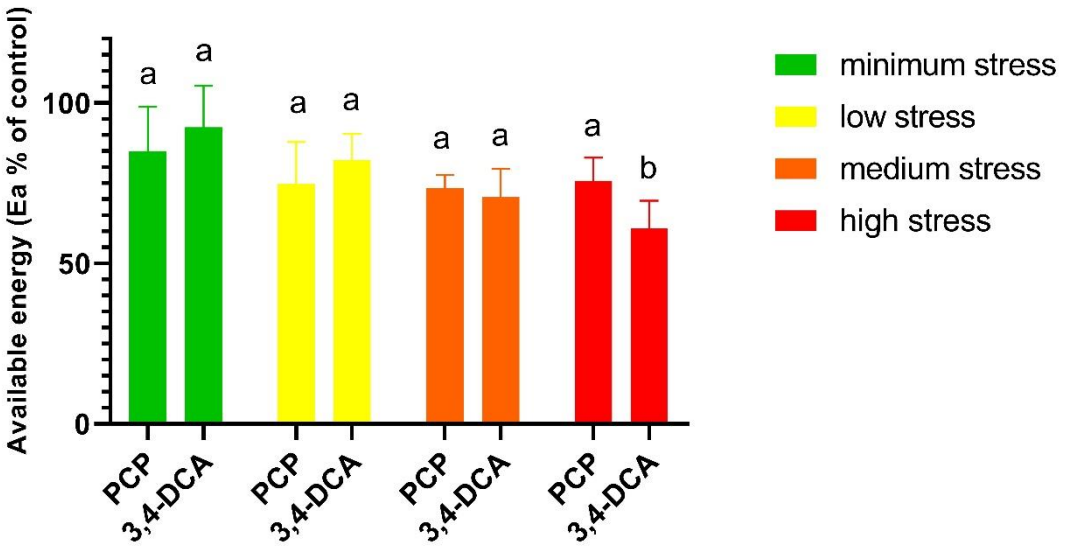

Fig. 4.14: The available energy as percentage of control contents in rotifers exposed to different pesticides stress magnitudes which expressed as percentage of chemical concentration at control.

4.4 Discussion

While some difference in amounts of energy store components were observed for Cu and Cd, the overall Ea under stress conditions showed no real impact when compared to the control (Figures from 4.1 to 4.6). In order to streamline further discussion, focus was placed on PCP and 3,4-DCA. Both of these toxicants elicited considerable changes to protein, lipid and carbohydrate in rotifers subjected to gradients of toxicant stress.

4.4.1 Effects of chemical toxicants on the allocation of energy fractions

4.4.1.1Proteins

Protein reserves represented the highest proportion of the three energy proportions measured under both no stress and with stress applied. Energy equivalent to protein reserves under control conditions ranged from 4140 mj/mg at 3,4-DCA control to 4905 mj/mg at PCP control group. Similar protein contents under control conditions were reported for daphnids, which had energy content of 4050 mj/mg linked to proteins (De Coen and Janssen, 2003).

In this study, energy reserves linked to protein were observed to be significantly affected by chemical exposure to organic toxicants (PCP and 3,4- DCA) but not to heavy metals (Cu and Cd), when compared to the control (Figures 4.2, 4.5, and 4.8). Under the red-stress concentration of organic compounds, protein reserves considerably decreased relative to the control, where they ranged from 3115 mj/mg under 3,4-DCA to 3431 mj/mg under PCP. The result of protein energy reserves observed in this study was higher than the maximum values reported in the literature for daphnids, which was 1580 mj/mg when exposed to 32 µg/L of mercury (De Coen and Janssen, 1997). These differences might indicate a lower toxic effect of the organic compound compared to mercury effect on daphnids. Another possible explanation might be that the two zooplankton groups respond differently to chemical toxicity, where the stress resistance mechanisms might cause more depletion in the energy components of daphnids than rotifers (De Coen and Janssen, 1997; Jeon et al., 2013). This also differs from one toxicant to another, as mercury seemed to have more toxic effects (De Coen and Janssen, 1997).

A similar reduction in energy reserves linked to protein was reported in daphnids and enchytraeids under chemical exposure (Sancho et al., 2009; Villarroel et al., 2013, Silva et al., 2020). This reduction in total protein content and, therefore, protein-linked energy reserves may be related to the important role of protein reserves as a vital source of energy under stress. Drawing upon these reserves enables animals to support the increased demands for energy necessary for survival (Sancho et al., 2009; Villarroel et al., 2013, Silva et al., 2020).

4.4.1.2 Lipids

Lipid reserves constitute the second largest energy proportion of rotifers in this study. Under the highest chemical concentration in this study, the Ea of lipids ranged from 922 mj/mg at 0.8 mg/L of 3,4-DCA to 2815 mj/mg at 0.4 mg/L of PCP. Daphnids had lipid reserves at 0.75 mg/L of lindane (1247 mj/mg) similar to that of 3,4-DCA (De Coen and Janssen, 1997), but this value was lower than that reported for PCP in this study, suggesting a lower toxic effect of 0.4 mg PCP/L compared with 0.75 mg lindane/L.

For the two organic toxicants used in this study, the lipid contents decreased under the red stress level concentrations. This reduction in lipid contents as a result of chemical exposure was previously reported in other studies on invertebrates (De Coen and Janssen, 1997, 2003; Soetaert et al., 2007; Sancho et al., 2009). For example, in their study, De Coen and Janssen (2003) showed that lipid contents were the most sensitive fraction under the exposure of four out of six chemical contaminants. These observations could be explained by the fact that lipids are a highly efficient energy reserve, hence are consumed first by stressed organisms (De Coen and Janssen, 1997; Muyssen and Janssen, 2001; Soetaert et al., 2007; Amorim et al., 2012). Furthermore, lipid consumption can be very useful to support mechanisms that repair cellular and tissue damage under chemical exposure (Villarroel et al., 2013). Our results indicate that organic compounds negatively affected both proteins and lipids more than the heavy metals used in the present study. This may be because the concentrations of heavy metals used in this study were of low potency.

4.4.1.3 Carbohydrates

Carbohydrate reserves constitute the lowest energy fraction of rotifers in this study for every toxicant. Ea linked to carbohydrate reserves under control conditions ranged from 2207 mj/mg for the 3,4-DCA control to 2313 mj/mg for the PCP control group. Similar Ea of carbohydrate

reserves under control conditions were previously reported in studies on daphnia, where the carbohydrate energy content was 1321 mj/mg (De Coen and Janssen, 2003).

Under the highest chemical concentration in this study, carbohydrate contents highly decreased relative to the control and ranged from 916 mj/mg at 0.8 mg/L of 3,4-DCA to 1895 mj/mg at 0.4 mg/L of PCP. This range reported in our study is higher than what was reported for daphnids in the literature, where under the highest toxicant concentrations, carbohydrate energy content reached a maximum of 474 mj/mg at 0.32 mg/L of chromium (De Coen and Janssen, 2003). The general trend of carbohydrates allocation decreased with increasing toxicant concentration. Under the exposure to organic toxicants (PCP and 3.4- DCA), carbohydrate energy reserves significantly decreased under high toxicant concentrations compared to control conditions. The reduction in carbohydrate reserves was previously reported in different studies that evaluated the impacts of exposure to chemical contaminants in aquatic invertebrates (Sancho et al., 2009; Vandenbrouck et al., 2009; Novais and Amorim, 2013; Gomes et al., 2016). Carbohydrates consumption by the stressed organisms represents the main resource for energy used in metal detoxification mechanisms (De Coen et al., 2001). Furthermore, the depletion of carbohydrate contents may highlight its important role as a rapidly mobilizable metabolic fuel used to cover a rapid increase in energy demand under chemical stress.

4.4.2 Effect of chemical toxicants on the total available energy

When comparing the percentage Ea of control results of the four toxicants used, the two organic toxicants highly reduced the Ea content, especially under the red stress level concentrations more than the two heavy metals. De Coen and Janssen, (1997), when comparing the pesticide, lindane, with the heavy metal, mercury, reported similar effect of the two toxicants on the degree of Ea reduction under the highest concentration used for both toxicants. The same observation was reported by De Coen and Janssen, (2003), who found similar trends for the

heavy metal, chromium, reducing percentage Ea of control (Cr, 19%) when compared with a pesticide (tributyltin, 33%) and with a surfactant (linear alkylbenzene sulfonic acid, 33%). They found that all the three toxicants reduce the Ea content with different, but not highly dissimilar, percentages.

The difference between our results and the above results can be due to two factors. Firstly, the response of daphnia is different from that of rotifers to the chemical exposure. Secondly, we are using lower concentrations compared with those studies. However, our results elucidate that rotifer exposure to chemical contaminants is impacting the organism's energy budget, since the energy reallocation to stress resistance mechanisms will minimize the energy available for other biological processes. The determination of the available energy content in organisms under chemical exposure provides a good indication for the overall condition of the stressed organisms.

4.5 Conclusion

Results indicated Cu and Cd to have very little influence on protein, lipid, and carbohydrate levels in rotifers. Thus, variations in Ea under Cu and Cd stress were not observed. It is likely that the Cu/Cd levels were below the threshold to impact energy stores in the rotifers. The levels of Cu/Cd were established using data presented in Chapter two. This chapter established significant impacts both above and below lethal limits and indicated concentration thresholds (LC_{50}) of 0.012 mg/L and 1.45 mg/L for Cu and Cd, respectively. In designing the experiments for Chapter 4, upper concentrations for Cu and Cd were set to the sublethal limits at 0.006 and 0.006 mg/L, respectively. With hindsight, these concentrations could have been set higher, closer to the thresholds identified in Chapter 2.

In contrast, results obtained for PCP and 3,4-DCA showed real and significant toxicant impacts on protein, lipid, and carbohydrate energy stores in rotifers. These changes underpinned large

shifts in Ea, particularly under orange and red stress levels. It is noted that the red stress levels for PCP and 3,4-DCA were 0.4 and 0.8 mg/L, and these values were closer to the threshold values defined in Chapter 2. In the presence of PCP and 3,4-DCA at red stress levels, Ea was reduced to 60-70% of the control values. These results highlight the opportunity to use a CEA approach to consider toxicant stress on rotifers. However, Ea is only half of the energy balance equation. To fully appreciate toxicant impact on CEA, energy consumed (Ec) must be considered. This is the subject of the following chapter.

CHAPTER FIVE

Chapter 5: Benchmarking Cellular Energy Parameters for Aquatic Organisms

5.1 Introduction

Cellular energy allocation (CEA) is a widely used approach to assess the energy cost associated with exposure to contaminants. CEA is calculated on the cellular level as the balance point between available energy (Ea: lipid, carbohydrate, and protein contents) and the consumed energy (Ec, as ETS) (De Coen and Janssen, 1997). CEA can give a good indication of an organism's overall condition under stress (Calow, 1991). The use of CEA to evaluate the energy budget of an animal in response to stressors is well established and has been applied to several invertebrates to test the impacts of toxicants on organisms and populations, as CEA may also be linked to effects on higher levels of biological organization (De Coen and Janssen, 2003; Smolders et al., 2004; Silva et al., 2019). The rationale is that under stress, energy is reallocated to different biochemical pathways to sustain homeostasis, and this may reduce the energy available for other important ecological functions including growth and reproduction. Therefore, it is useful to characterize the effect of toxicants on the energy-related parameters to identify the impacts of their toxicity on the cellular, organismal, and population levels. Furthermore, as the physiological adjustment necessary for stress resistance mechanisms is highly dependent on the energy budget, the CEA approach can be used at the population level (Sokolova, 2013; 2021).

The CEA approach has been widely applied in many ecotoxicological studies since it was proposed by De Coen and Janssen (1997). Trends of correlation and the relationship of Ec and CEA with Ea have been established for a range of taxa. These correlations allow the assessment of whether the effect of increasing toxicant concentrations on the energy budget parameters varies with the sensitivity of different species, which of these species can demonstrate low or high responses, and whether these responses can be affected by experimental conditions. In other words, these correlations, when known, will provide insights about the global response

trends of the energy budget parameters to stressors among different species. This will help us to ascribe one parameter when knowing the other. To do this, a systematic review of the literature is required.

Thus, this chapter aims to make a systematic review of the current literature on CEA using data of cladoceran daphnids, which are extensively used as a bioindicators in ecotoxicological studies for aquatic ecosystem (Lari et al., 2016). In addition, to compliment this synthesis, further studies that considered enchytraeids and some microalgae species are included. The assembled data was used to quantitatively examine Ec, Ea, and CEA linkages.

The conclusions of the literature-based approach were then used to enable CEA assessment for rotifers to be completed using Ea values measured (Chapter 4) and Ec values justified through the literature review presented in this chapter. Owing to curtailment of laboratory work under COVID restrictions, the opportunity to develop and apply Ec assays was not feasible. The synthesis presented in this chapter represents a possible solution to the prevailing situation. Furthermore, the synthesis of Ea/Ec and CEA data represents a useful contribution to knowledge.

5.2 Materials and methods

To conduct the systematic review of studies that have calculated the cellular energy allocation in rotifers, data in literature was identified using the following search terms, (“cellular energy allocation” AND “rotifer”), (“energy allocation” AND “rotifer”), (“heavy metals” AND “rotifer”), (“pesticides” AND “rotifer”), (“herbicides” AND “rotifer”), (“toxicants” AND “rotifer”), and (“stress” AND “rotifer”), on Google Scholar and Scopus. The search results were first screened for their titles and abstracts, and a total of 104 papers were downloaded. Thereafter, the search terms “energy”, “allocation”, and “caloric” were used to further screen the 104 papers, resulting in 55 papers. in the next phase comprised reading of the materials and

methods and/or results (Table 5.1). This phase resulted in excluding all 55 papers, as none was found that calculated CEA or Scope for growth (SFG), or at least energy-related parameters (lipids, proteins, and carbohydrates) (Table 5.2).

A similar literature survey was undertaken for studies of daphnids. As there were more studies that calculated the CEA for daphnids, a lower number of search terms were used. Only four search terms were used, as follows: ("cellular energy allocation" AND "daphnia"), ("heavy metals" AND "daphnia"), ("pesticides" AND "daphnia"), and ("herbicides" AND "daphnia"). Search results were firstly checked for their titles and abstracts, and a total of 134 papers were downloaded. Thereafter, the search terms "energy", "allocation", and "caloric" were used to screen the 134 papers. This resulted in 73 papers passing to the last screening phase that included reading materials and methods and/or results (Table 5.1). This phase resulted in 20 papers that calculated the CEA or Scope for growth (SFG), or at least the energy reserves (lipids, proteins, and carbohydrates). The identified papers passed to the data extraction phase (italic font in Table 5.3).

A third literature search was conducted to identify further studies that had calculated the CEA in species other than rotifers and daphnia. This time, a restricted search term, "cellular energy allocation", was used. A total of 16 studies were selected from the search results to complement the daphnia papers identified above. Those studies were related to amphipods (n = 2), isopods (n = 3), microalgae (n = 4), and enchytraeids (n = 7). 8 papers passed to the data extraction phase (italic font in Table 5.4). Studies on larger animals (e.g. gastropods) were excluded.

Table 5.1: Literature survey methodology and number of papers outcome

Organism	Rotifers	Daphnia	N/A
Primary search phrases:	cellular energy allocation, energy allocation, heavy metals, pesticides, herbicides, toxicants, and stress (+ AND rotifers)	cellular energy allocation, heavy metals, pesticides, and herbicides (+ AND daphnia)	cellular energy allocation
No. primary papers identified	104	134	-
Secondary search phrases:	energy, allocation, and caloric	energy, allocation, and caloric	-
No. secondary papers identified	55	73	16
No. papers for data extraction	0	20	8
Total number of papers used in CEA benchmarking (No. data sets)	0	12 (18)	8 (14)

Table 5.2: Rotifer papers identified after using primary and secondary search criteria

Stress type	References
Caloric restriction (starvation)	Kirk, 1997; Kirk et al., 1999; Stelzer, 2001Shertzer and Ellner, 2002; Lürling, 2006; Nandini et al., 2007; Gribble and Mark Welch, 2013
Heavy metals	Sarma et al., 2000; Ríos-Arana et al., 2007; Jose Luis Gama-Flores et al., 2007; Rehman et al., 2008; Sarma et al., 2008, 2010; Klimek et al., 2013; Hernández-Ruiz et al., 2016; Kim et al., 2016; Pan et al., 2016; Snell et al., 2017; Mashjoor et al., 2018;El-Sayed and El-Khodary, 2019; Jeong et al., 2019a, b; Byeon et al., 2020; Xu et al., 2020; Han et al., 2021; Schanz et al., 2021
Heavy metals in combination with other stressors.	Cecchine and Snell, 1999; Luna-Andrade et al, 2002;José Luis Gama-Flores et al., 2005; Filenko and Samoylova, 2008; Li et al., 2014; Kang et al., 2021; Rebolledo, Páez-Osuna, et al., 2021; Rebolledo, Rico-martínez, et al., 2021
Insecticides	Guo et al., 2012a, b; Guo and Chen, 2015; Chen and Guo, 2015; Gharaei et al., 2020
Pesticides	Xi et al., 2007; Huang et al., 2013; Wang et al., 2015; Park et al., 2017; M. C. Lee et al., 2019; Yang et al., 2021
Microplastics	Jeong et al., 2016; Park et al., 2018; Xue et al., 2021; Liang, Yang, et al., 2021; Drago and Weithoff, 2021
Surfactants	Williams et al., 2016
Microcystin	Liang et al., 2017; Liang et al., 2020; Liang et al., 2021

Table 5.3: Daphnids' papers identified after using primary and secondary search criteria. Italic underlined papers are those which passed to the data extraction phase.

Stress type	Reference
Starvation	Glazier and Calow, 1992; Peeters et al., 2010; (Nguyen et al., 2021
Heavy metals	Arambašić et al., 1995; Sorvari and Sillanpää, 1996; *De Coen and Janssen, 1997;* Cañizares-Villanueva et al., 2000; Burba, 1999; Knops et al., 2001; De Coen et al., 2001; Muyssen and Janssen, 2001; Muyssen et al., 2002*; De Coen and Janssen, 2003*; Canli, 2005*; Baillieul et al., 2005; Smolders et al., 2005; Bossuyt et al., 2005;* Canli, 2006; Guan and Wang, 2006; K. T. Kim et al., 2006; Theegala et al., 2007; Murano et al., 2007*; Soetaert et al., 2007*; Connon et al., 2008; *Zeman et al., 2008; Vandenbrouck et al., 2009;* Lim et al., 2009; Muyssen et al., 2010*; Li et al., 2011*; Tang et al., 2011*; Arzate-Cárdenas and Martínez-Jerónimo, 2012a,* 2012b; Kolkmeier and Brooks, 2013; Rosenfeldt et al., 2014*; Adam et al., 2015*; Stevenson et al., 2017; Rogalski, 2017; L. Li et al., 2017; Lari et al., 2017; Kim et al., 2018; Pacheco et al., 2018; Cui et al., 2018; Yingying Liu et al., 2018; Gao et al., 2018; T. Zhou et al., 2019; Brun et al., 2019; Arreguin Rebolledo et al., 2020; Yuan et al., 2020; Gust et al., 2021; Niemuth et al., 2021; Hansul et al., 2021; *Tourinho et al., 2021*
Pesticides	Pieters et al., 2006; *Martínez-Jerónimo et al., 2013; Villarroel et al., 2009; Sancho et al., 2009; Jeon et al., 2013;* Gaete et al., 2013; *Silva et al., 2019;* Moreira et al., 2020; Yanhua Liu et al., 2022
Humic	Steinberg et al., 2010; Bouchnak and Steinberg, 2013; *Saebelfeld et al., 2017*
Herbicides	*Villarroel et al., 2013*; Başalan Över et al., 2021
Multiple contaminants	Pablos et al., 2015; Y. C. Lee et al., 2016; Kim et al., 2017
Thermal stress	Samanta et al., 2020
Microcystis	Bojadzija Savic et al., 2020; Q. Zhou et al., 2020
Fullerene	Lv et al., 2017
Tetracycline	Kim et al., 2014

Table 5.4: Papers identified from the third literature search. Italic underlined papers are those which passed to the data extraction phase.

Taxa	References
Amphipods	Olsen et al., 2007, 2008
Aquatic worms	Stomperudhaugen et al., 2009; *Novais et al., 2013; Novais and Amorim, 2013;* Gomes et al., 2015a; *Gomes et al., 2015b; Gomes et al., 2016; Patrício-Silva and Amorim, 2016*
Isopods	Ferreira, Morgado, et al., 2015; Ferreira, Cardoso, et al., 2015; Ferreira et al., 2016
Microalgae	*Aderemi et al., 2018; Duarte et al., 2020; Feijão et al., 2020; Silva et al., 2020*

In the summary, of current data from the secondary phase of papers considered, comparable datasets that conducted complete cellular energy allocation determination and/ or measured the energy reserves (protein, lipid, and carbohydrates) under chemical stress was evident for all taxa. To only focus on acute responses that are comparable to our exposures, studies that used an exposure period of more than 120h were excluded from the data collection. However, where

such papers contained useful control values, these were used to benchmark Ec % of control vs the Ea % of control (in daphnia only). Only papers that used chemical contaminants were evaluated. This reduced the number of papers that reached the data extraction phase to 20 for different species of daphnia (but dominated by *D. magna*) and 9 for other species. In studies that used multiple time point measurements, only the 24h or 48h results were included.

To extract data from the selected papers, the online tool "web plot digitizer" was used to extract the data from images of the existing graphs. Screenshots were made of every graph, saved as an image using the Paint software, and then uploaded online to the web plot digitizer for data to be extracted. The data was organized, collected, and saved in spreadsheets for later analysis.

Owing to between-study methodological differences in concentrations used for each toxicant, this study normalized the concentration relative to the maximum concentration used in each dataset, and therefore within each study, the control represented the 0% stress level while the highest concentration represented the 100% of stress magnitude. Therefore, in studies involving daphnia, as it had the highest number of papers compared with other species, only datasets that had at least three toxicant concentrations in addition to the control were included. This was done to ensure that the responses observed were spread across a range of stress magnitudes. Furthermore, all extracted data of Ec, Ea, and CEA were normalized relative to the within-study control group data to calculate the linkage between Ec % of control vs Ea % of control and CEA % of control vs Ea % of control.

After applying the above-mentioned criteria for article inclusion, 12 papers for daphnia that had 18 data sets, 5 papers with 7 data sets for the enchytraeids, and 4 papers with 7 data sets for microalgae were included in the data analysis.

In the included literature, most of the results involving daphnia were expressed as mj/organism. To enable cross comparison, these values were converted to mj/mg. This unit conversion also

aligned the analysis here with results presented in Chapter 4. There were limitations to calculating the average mass of daphnia under stress in the literature. However, due to limited information available, Ea and Ec results were extracted from only two papers (Baillieul et al., 2005 and Tourinho et al., 2022). As before, these were provided in mj/ mg and mj/ organism. In order to reconcile units and plot linear regression relationships, Ea and Ec values were converted from mj/organism to mj/mg in the studies involving daphnia (Figure 5.1). For Ea unit conversion, the following equation was used:

$Ea_{mj/mg} = (1.5943 \times Ea_{mj/organism}) + 123.64$ (Figure 5.1, A).

For Ec unit conversion, all the $Ec_{mj/organism}$ data were firstly converted to $Ec_{mj/organism}$ per hour, and the following equation then applied:

$Ec_{mj/mg} = (0.6625 \times Ec_{mj/organism}) + 3.7188$ (Figure 5.1, B).

The results of enchytraeids were obtained in mj/mg. For microalgae, the results were expressed as $mj/10^6$ cells. For every microalga species, it was assumed that 10^6 cells of each microalga were equal to 1 mg, although there is some limitation to this approximation approach.

All Ea graphs for numerical linkages were calculated for the responses per 24h exposure, while Ec graphs were calculated for oxygen consumption per 1h. The quantification of cellular energy allocation in the included studies was done by integrating the Ea and Ec datasets following two methods:

(a) According to De Coen and Janssen (1997), where the Ea value was calculated by integrating the change in the different energy reserve fractions over the exposure period. Similarly, the Ec value was obtained by integrating the change in energy consumption (electron transport system activity) over the exposure period. The total net energy budget was then calculated by subtracting the Ec from the Ea and dividing the result over the time of exposure.

(b) According to Verslycke et al. (2004), where the Ea, Ec, and CEA values were calculated as follows:

Ea (available energy) = Ea-protein + Ea-lipid + Ea-carbohydrates (mj /mg)

Ec (energy consumption) = ETS activity (mj /mg/ h)

CEA (cellular energy allocation) = Ea/Ec

In both methods, all energy fractions were transformed into energetic equivalents using their respective energy of combustion (17,500 mj/mg glycogen, 24,000 mj/mg protein, and 39,500 mj/mg lipid; Gnaiger, 1983). The ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein, and sugar mixture (484 kJ /mol O_2). In Chapter 6, we use the method of Verslycke et al. (2004) to calculate the CEA in the current study, so we transformed all Ec values found in the literature to be per hour in order to be easily aligned with our results.

5.2.1 Data analysis:

All individual (per taxa) and overall (all taxa grouped) linear regression slopes were compared statistically with a horizontal line (i.e., line of zero gradient) to evaluate the decrease or increase of energy-related parameters under different stress magnitude. Datasets were analysed using the SPSS software package (version 25). Differences were considered significant at $P < 0.05$.

5.3 Results and discussion

5.3.1 Conversion of energy values of daphnia from mj/organism to mj/mg

Ea and Ec values from mj/organism to mj/mg of daphnia were used to reconcile units and plot linear regression relationships (Figure 5.1). For Ea unit conversion, the following equation was obtained from the plotted results:

$Ea_{mj/mg} = (1.5943 \times Ea_{mj/organism}) + 123.64$ (Figure 5.1, A).

For Ec unit conversion, the following equation was obtained after all the $Ec_{mj/organism}$ data was firstly converted to be per hour:

$Ec_{mj/mg} = (0.6625 \times Ec_{mj/organism}) + 3.7188$ (Figure 5.1, B).

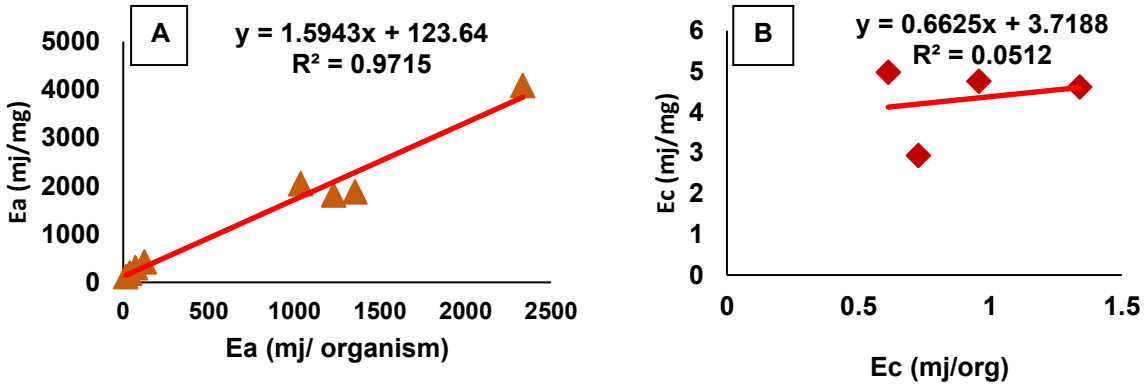

Fig. 5.1: The relationship between Ea in mj/organism and Ea mj/mg (A), and the relationship between Ec in mj/organism and Ea mj/mg (B) of daphnia.

5.3.2 Energy reserves

Available energy reserves were measured by quantifying the total lipid, protein, and carbohydrate content in all the included papers. Data of each energy reserve were collected and integrated for all taxa and a relationship between the energy reserves and the stress magnitude was conducted. The energy reserves parameters were measured in 24 data sets out of 33 used, 13 were on daphnia, 7 were on enchytraeids, and 4 were on microalgae. The equation appeared on all the following graphs represents the overall trendline regression.

5.3.2.1 Proteins

The overall trendline linear regression (all taxa grouped) (Figure 5.2) showed that the protein contents (mj/mg/24h) significantly decreased with increasing stress magnitude ($p = 0.019$) which may indicate the importance of proteins as an energy source under stress. Furthermore, taken on a by taxa basis, protein content consistently decreased with increasing stress magnitude for both daphnia ($p = 0.001$) and enchytraeids (0.045), while was not affected for microalgae ($p = 0.33$). A reduction in total protein contents indicates that protein reserves may undergo proteolysis which produces free amino acids that are used in energy production during stressful conditions. To fulfil energy requirements during exposure to toxicants, animals increase their energy demands and this may result in the catabolism of protein reserves (Sancho et al., 2009, Villarroel et al., 2013, Silva et al., 2020). Comparing the three included taxa with each other, daphnia and microalgae had the highest protein levels followed by enchytraeids, but the rate of protein depletion under increasing stress magnitude was daphnia > enchytraeids with no depletion in microalgae (Figure 5.2).

In daphnia, the Ea associated with proteins in control groups ranged from 319 mj/mg/ 24h (Villarroel et al., 2013) to 4050 mj/mg/ 24h (De Coen and Janssen, 2003). And at 100% stress, Ea-protein ranged from 188 mj/mg/ 24h (Villarroel et al., 2013) to 1580 mj/mg/ 24h (De Coen andJanssen, 1997). In enchytraeids, Ea associated with proteins in control groups ranged from

17 mj/mg/ 24h (Gomes et al., 2016) to 652 mj/mg/ 24h (Novais and Amorim, 2013) and from 94 mj/mg/ 24h (Gomes et al., 2015) to 768 mj/mg/ 24h (Novais and Amorim, 2013) under 100% stress24h. In microalgae, Ea associated with proteins in control groups ranged from 711 mj/mg/ 24h to 2617 mj/mg/ 24h; while under 100% stress, Ea-protein ranged from 847 mj/mg/ 24h to 1503 mj/mg/ 24h (Aderemi et al., 2018). The overall results showed that protein was depleted under stress conditions across different taxa.

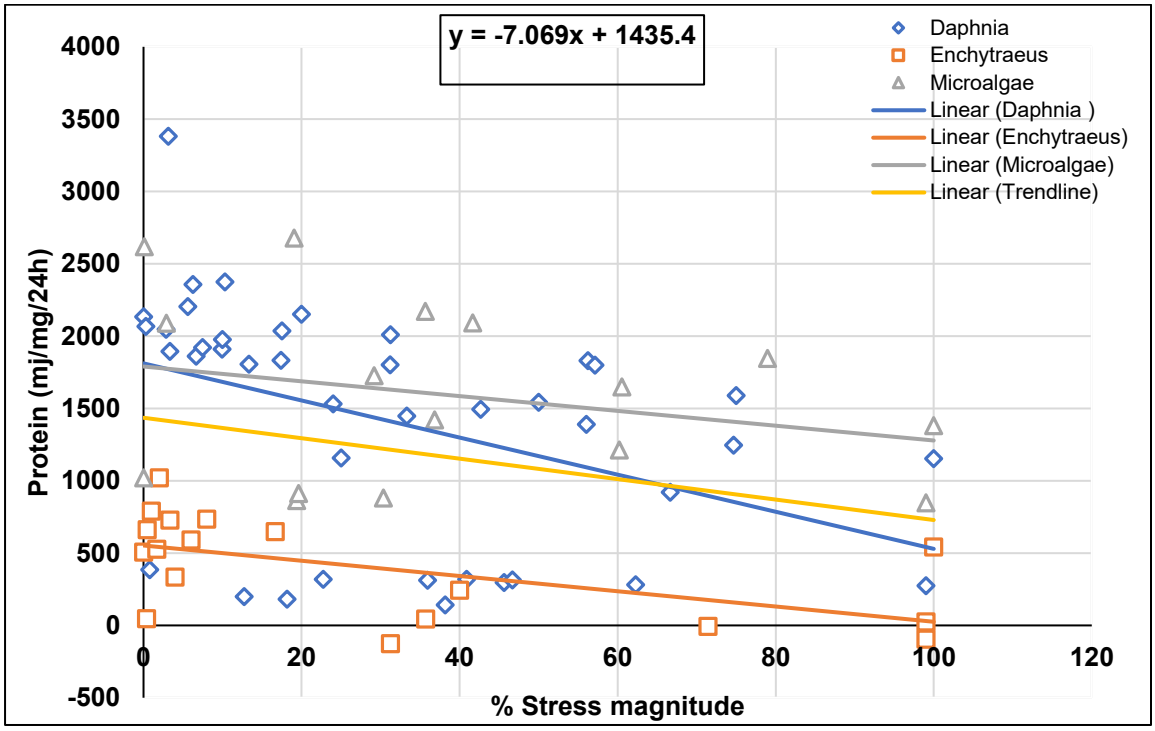

Fig. 5.2: Values of proteins expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

In order to facilitate data comparison between published data and those data obtained in this study (Chapter 4), focus was placed only on results of PCP and 3,4-DCA. Both toxicants elicited significant changes to protein, lipid and carbohydrate contents in rotifers under exposure (Figures from 4.7 to 4.12) while Cu and Cd showed no real impact on the overall Ea under stress conditions relative to the control (Figures from 4.1 to 4.6).

Comparing the Ea-protein data of PCP (Figure 4.7) and 3,4-DCA (Figure 4.11) with data obtained from literature (Figure 5.2), Ea associated with protein levels in rotifers under PCP and 3,4-DCA exposure showed decreased consistently with increasing stress similarly to published results for the three taxa (Figure 5.2). However, the amount of Ea-protein in rotifer was higher than Ea-protein in all the three taxa studied in this chapter. While the overall regression equation (Figure 5.2) showed that the amount of Ea-protein reserve under 0% stress was about 1435 mj/mg, which was reduced to around 728 mj/mg under the highest stress levels, the amount of Ea-protein in rotifers under 0% stress was 4905 and 4110 mj/mg, which was reduced to 3431 and 3115 mj/mg for PCP and 3,4-DCA, respectively. These differences in Ea-protein amounts may be due to taxa-specific responses, as there were differences between the three taxa as well as with rotifers. Furthermore, difference in Ea values is expected due to using different methodologies to calculate the Ea content. Authors either calculate the difference in Ea content during the exposure time only or they calculate the total Ea content at the end of the exposure time only. Those explanations are proffered for all further differences in Ea associated to energy reserves in this chapter.

5.3.2.2 Lipids

Lipid reserves (mj/mg/24h) overall (all taxa grouped) showed no change in increasing stress magnitude ($p = 0.34$). Taken on a by taxa basis, lipid content consistently decreased with increasing stress magnitude for daphnia ($p = 0.011$), while it was not changed for both enchytraeids (0.0.49) and microalgae (0.64). This decrease in lipids in daphnids under stress conditions was previously reported by De Coen and Janssen, (2003), where lipids were found to be the most impacted CEA parameter under the exposure of four out of six chemical contaminants. Moreover, lipids have been well documented to be the first energy source for consumption by organisms exposed to toxicant stress because they are a highly efficient energy reserve (De Coen and Janssen, 1997; Muyssen and Janssen, 2001; Soetaert et al., 2007;

Amorim et al., 2012). In addition, the reduction in the lipid fraction may be a response to oxidative damage of the cell membranes that occurs under exposure to chemical toxicants (Novais et al., 2011), leading to higher levels of lipid peroxidation. Lipid catabolism can produce lipoproteins which are utilised to repair the cellular and tissue damage as well as providing energy directly to the cells for energy (Villarroel et al., 2013). Collectively, all of these mechanisms of lipid utilisation lead to lipid depletion over time under chemical exposure. This was apparent in the collated data as, taken on a by taxa basis, there was a consistent decrease in lipid content with increasing stress magnitude in daphnids.

Comparing lipid contents between the three included taxa, daphnia had the highest lipid (mj/mg/24h) levels followed by microalgae and then enchytraeids. The rate of Ea-lipid depletion in increasing stress was daphnia > enchytraeids, while in microalgae Ea-lipid content was observed to slightly (not significant) increase with increasing stress (Figure 5.3). This response of microalgae was explained as the increase of some plastidial lipid classes essential for the maintenance of the electron transport mechanisms and the quinone pool structure and function under stress (Duarte et al., 2020).

In daphnia, Ea associated with lipids in control groups ranged from 375 mj/mg/ 24h (Sancho et al., 2009) to 3604 mj/mg/ 24h (De Coen and Janssen, 2003) and from -907 mj/mg/ 24h (Soetaert et al., 2007) to 1247 mj/mg/ 24h (De Coen and Janssen, 1997) at 100% stress. For the enchytraeids, Ea associated with lipids in control groups ranged from 144 mj/mg/ 24h (Gomes et al., 2015) to 576 mj/mg/ 24h (Novais and Amorim, 2013) and from 47 mj/mg/ 24h (Novais et al., 2013) to 480 mj/mg/ 24h (Novais and Amorim, 2013) at 100% stress. In microalgae, Ea associated with lipids in control groups ranged from 1076 mj/mg/ 24h to 2607 mj/mg/ 24h and from 1258 mj/mg/ 24h to 3103 mj/mg/ 24h (Aderemi et al., 2018) at 100% stress. The overall results showed that lipid was affected and depleted under high stress exposure in daphnia (Figure 5.3).

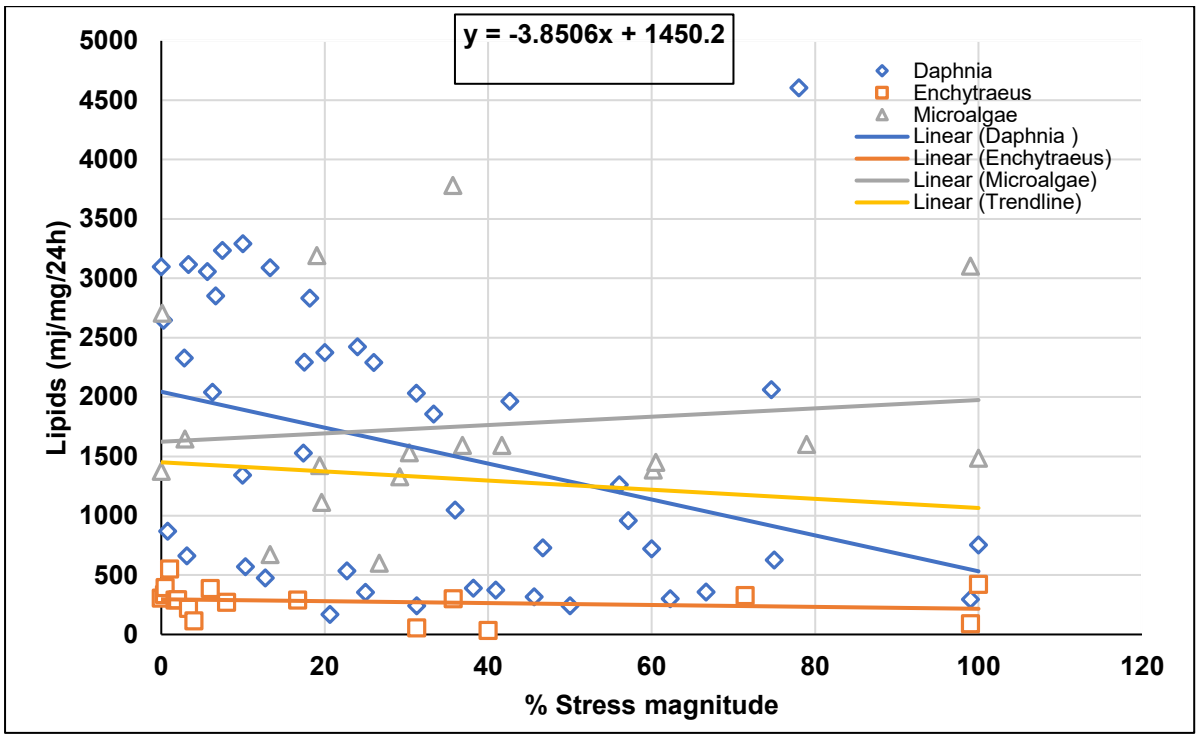

Fig. 5.3: Values of lipids expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

Comparing the Ea-lipid data of PCP (Figure 4.7) and 3,4-DCA (Figure 4.11) with data obtained from the literature (Figure 5.3), Ea associated with lipid levels in rotifers under PCP and 3,4-DCA exposures decreased under increasing stress magnitude similar to the overall results of the collated data for the three taxa (Figure 5.3). The amount of Ea-lipid in rotifer under 3,4-DCA exposures was similar to the overall (all taxa grouped) Ea-lipid in this chapter, as under 3,4-DCA exposure, the Ea-lipid reserves ranged from 1851 under 0% stress to 922 mj/mg under 100% stress. Ea-lipid in rotifers under the PCP was higher than both the exposure to 3,4-DCA, and the overall results from the regression equation of the collated data (Figure 5.2), which showed that the amount of Ea-lipid under 0% stress was about 1450 mj/mg, which is reduced to around 1065 mj/mg under the highest stress levels.

5.3.2.3 Carbohydrates

Carbohydrate levels (mj/mg/24h) were, overall, decreased by increasing the chemical stress magnitude across all taxa ($p = 0.032$) (Figure 5.4). However, carbohydrate content was significantly decreased in decreasing stress magnitude for daphnids ($p = 0.001$), while enchytraeids ($p = 0.44$) and microalgae ($p = 0.72$) showed no changes in increasing stress. One of the reasons for the observed reduction in carbohydrates is that the toxicants increase the glycolytic activity, leading to carbohydrate catabolism. Carbohydrates represent the main source of energy because they can easily be incorporated in pathways to produce ATP for mechanisms that could be involved in metal detoxification (De Coen et al., 2001). Furthermore, the early depletion of carbohydrates is consistent with its role as a rapidly mobilizable metabolic fuel used to cover a rapid increase in energy demand under stress (Sancho et al., 2009, Vandenbrouck et al., 2009; Novais and Amorim, 2013; Gomes et al., 2016). In contrast, depletion of lipids and proteins might occur later during the chemical exposure.

Comparing carbohydrate content between the three included taxa, daphnia had the highest carbohydrate levels followed by microalgae and then the enchytraeids. The gradient of the depletion under increasing stress magnitude followed the same order, as in daphnia, there was depletion of Ea-carbohydrates under higher stress while there was no effect for both enchytraeids and microalgae.

In daphnia, Ea associated with carbohydrates in control groups ranged from 128 mj/mg/24h (Sancho et al., 2009) to 1321 mj/mg/24h (De Coen and Janssen, 2003). At 100% stress, Ea-carbohydrates ranged from 43 mj/mg/24h (De Coen and Janssen, 1997) to 474 mj/mg/24h (De Coen and Janssen, 2003). In the enchytraeids, the Ea associated with carbohydrates at control groups ranged from -11 mj/mg/24h (Gomes et al., 2016) to 75 mj/mg/24h (Novais et al., 2013) and from -62 mj/mg/24h (Novais and Amorim, 2013) to 125 mj/mg/24h (Gomes et al., 2015)

at 100% stress. In microalgae, Ea associated with carbohydrates ranged from 105 mj/mg/24h to 945 mj/mg/24h (Aderemi et al., 2018) for the control groups and from 146 mj/mg/24h to 842 mj/mg/24h (Aderemi et al., 2018) at 100 % stress.

Overall, the regression equation showed that the amount of carbohydrates reserves under 0% stress was about 495 mj/mg, which was reduced to around 150 mj/mg under the highest stress levels. This result indicates that, similar to both Ea-proteins and Ea-lipids, Ea-carbohydrates were reduced by increasing the stress magnitude. This may show that carbohydrate content can be an easily available source of energy, hence it is usually the favourite used under stress conditions (Samanta et al., 2020).

Comparing the Ea-carbohydrates data under PCP (Figure 4.7) and 3,4-DCA exposures (Figure 4.11) with data obtained from literature (Figure 5.4), Ea associated with carbohydrate levels in rotifers under PCP and 3,4-DCA exposures tended to decrease under increasing stress similar to the overall results of the collated data of the three taxa (Figure 5.4). The amount of Ea-carbohydrates in rotifer under both PCP and 3,4-DCA exposures was higher than the value of the overall (all taxa grouped) Ea-carbohydrates in this chapter. Furthermore, the overall results from regression equation of the collated data (Figure 5.4) showed that the amount of Ea-carbohydrates reserves under 0% stress was about 495 mj/mg, which was reduced to around 150 mj/mg under the highest stress levels. Under PCP and 3,4-DCA exposures, carbohydrate reserves were 2313 and 2207 mj/mg under 0% stress and 1895 and 916 mj/mg under 100% stress for rotifers under PCP (Figure 4.8) and 3,4-DCA exposure, respectively (Figure 4.11).

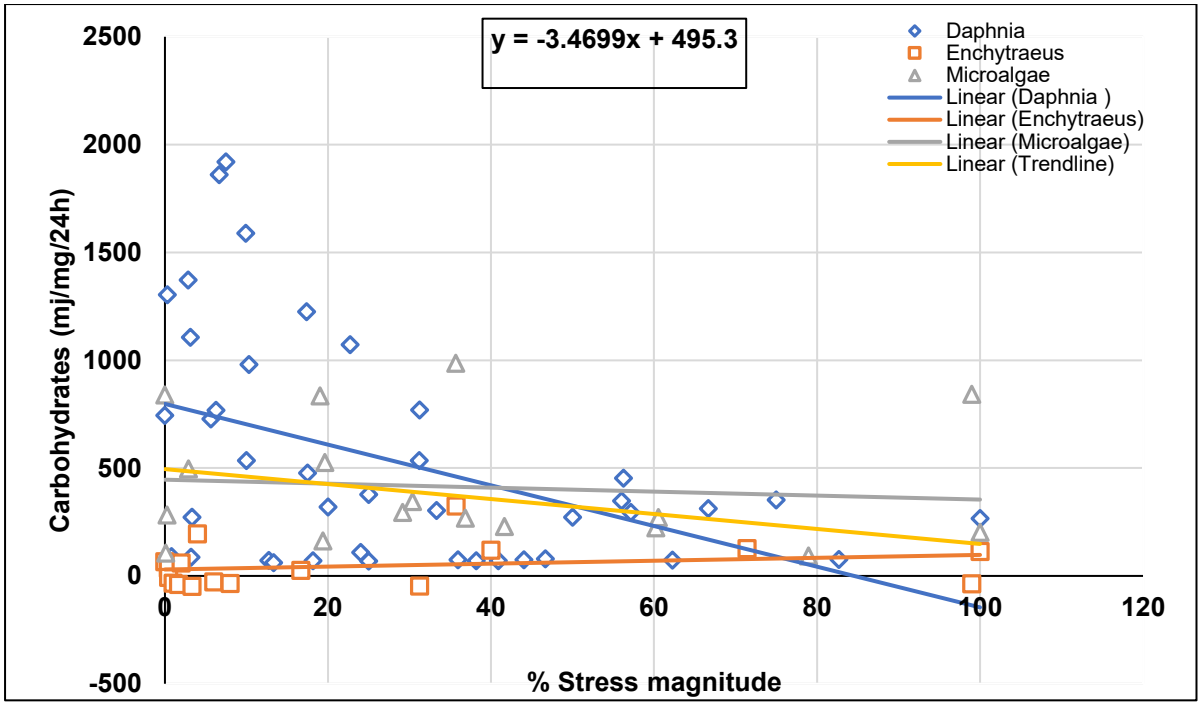

Fig. 5.4: Values of carbohydrates expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

5.3.2.4 Sensitivity of energy components to stress

Comparing the overall (all taxa) sensitivity of different energy components (proteins, lipids, and carbohydrates) to stress, proteins seemed to be the most significantly affected fraction followed by carbohydrates, while lipids showed no overall change. Regarding proteins, the reduction in total protein may be due to proteolysis, which produces free amino acids that are used to supply energy for detoxification processes during toxic stressful conditions. To fulfil energy requirements during exposure to toxicants, animals increase their energy demands, and this may cause catabolism of protein reserves (Sancho et al., 2009, Villarroel et al., 2013, Silva et al., 2020). This was the case in both daphnids and enchytraeids, which shows the importance of proteins as an energy source under toxic stress. For carbohydrates, the exposure to toxicants may increase the glycolytic activity, and this makes carbohydrates an easy resource for energy acquisition because they can easily be incorporated in pathways to produce ATP for mechanisms that could be involved in metal detoxification (De Coen et al., 2001; Sancho et al., 2009, Vandenbrouck et al., 2009; Novais and Amorim, 2013; Gomes et al., 2016). This was

the case for both daphnids and enchytraeids. For lipid, the overall sensitivity to stress was driven by the results of the enchytraeids and the microalgae, which showed no change with increasing the stress magnitude. Daphnids have been found to use the lipids as an energy resource under toxic stress, as lipid content was significantly decreased under higher stress magnitude. These results indicate that all the three energy components were affected by stress conditions in daphnids. Similar effects of toxicant exposure have been reported for bivalve species (*Saccostrea glomerata* and *Ostrea angasi*), where there was a depletion of protein, lipid and glycogen stores (Bartlett et al., 2020). However, the lipids were less affected by stress compared to proteins and carbohydrates, and this may be because animals turn to lipid catabolism to produce lipoproteins which are utilised to repair the cellular and tissue damages (Novais et al., 2011; Villarroel et al., 2013).

. This change to lipid utilization may come as complementary mechanisms after the primary mechanisms of metal detoxifications had been initially supplied by energy sourced from proteins and carbohydrates. Similar observations were made in *Mytilus edulis* exposed to tetrabromodiphenyl ether, where the energetic strategy has been adopted according to variation in the stress level, suggesting that the preference switched from protein utilization to lipid utilization in increasing stress magnitude (Jiang et al., 2021). A very important reason for differences in responses are due to differences between taxa and differences in toxicant types (Huang et al., 2013).

5.3.2.5 Available energy

The available energy (Ea) as total energy was measured in 29 datasets out of 33: 15 on daphnia, 7 on enchytraeids, and 7 on microalgae. The overall (all taxa grouped) Ea-total was not changed in increasing stress magnitude ($p = 0.15$). However, for daphnia, the Ea-total was significantly decreased in increasing stress magnitude ($p < 0.01$). In the case of enchytraeids ($p = 0.16$) and microalgae ($p = 0.19$), there was no significant change in Ea-total levels. Comparing Ea-total

levels between the three included taxa, daphnia had the highest Ea-total levels followed by the enchytraeids and then microalgae. The rate of Ea-total depletion under increasing stress magnitude was daphnia > enchytraeids, while Ea-total for microalgae was observed to increase with increasing stress magnitude, driven by the increase in some lipids classes under stress conditions as previously described (Figure 5.5).

In daphnia, Ea-total for control groups ranged from 261 mj/mg/ 24h (Smolders et al., 2005) to 6890 mj/mg/ 24h (De Coen and Janssen, 2003) and from 76 mj/mg/ 24h (Li et al., 2011) to 2514 mj/mg/ 24h (De Coen and Janssen, 2003) at 100% stress. For the enchytraeids, Ea-total ranged from 166 mj/mg/ 24h (Gomes et al., 2016) to 1283 mj/mg/ 24h (Novais and Amorim, 2013) at the control group and from 146 mj/mg/ 24h (Gomes et al., 2016) to 1185 mj/mg/ 24h (Novais and Amorim, 2013) under 100% stress. For the microalgae, Ea-total ranged from 248 mj/mg/ 24h (Feijão et al., 2020) to 6270 mj/mg/ 24h (Aderemi et al., 2018) at the control groups and from 782 mj/mg/ 24h (Duarte et al., 2020) to 5449 mj/mg/ 24h (Aderemi et al., 2018) at 100% stress.

The decrease in all energy reserve parameters under toxicant stress, as discussed above, indicated that the elevated energy demands under stress conditions are higher than energy supplied via food, which may be due to reduction in feeding rates or increase in consumed energy. This was observed as an overall decrease in the available energy (Ea-total) calculated when increasing the stress magnitude (Figure 5.5). It was observed in 26 datasets out of the 33 used in this review. These results show that exposure to chemical contaminants can impact the organism's energy budget, and this change in energy budget can be very useful when incorporated into organismal and ecological endpoints such as survival, reproduction, and growth (Goodchild et al., 2019). The energy reallocation to stress resistance mechanisms minimizes the energy available for other biological processes and, thus, gives a good indication of the overall condition of the stressed organisms when quantified. Comparing Ea-total values

across the three included taxa, daphnia had the highest Ea-total values followed by microalgae and then the enchytraeids. The rate of its depletion in increasing stress magnitude was daphnia > enchytraeids, while in Ea-total for microalgae increased with increasing stress (Figure 5. 5). This observation of Ea-total reflects the lipid content, since lipids have the highest caloric content compared to proteins and carbohydrates. The overall regression equation showed that the total available energy reserve under 0% stress was about 2740 mj/mg, this was reduced to around 1719 mj/mg under the highest stress levels. The overall results showed that Ea-total was affected and depleted under the stress of contaminants exposure.

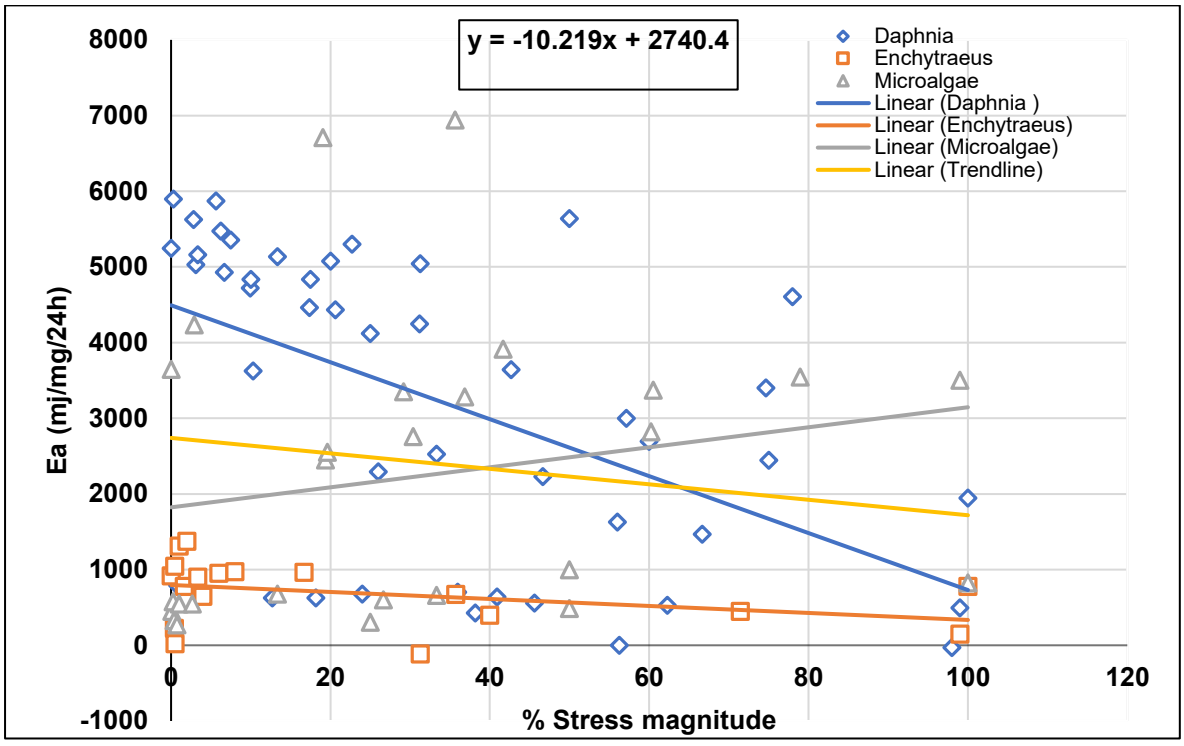

Fig. 5.5: Values of available energy (Ea) expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

Comparing the Ea-total data under PCP (Figure 4.9) and 3,4-DCA exposures (Figure 4.12) with data obtained from literature (Figure 5.5), Ea-total in rotifers exposed to PCP and 3,4-DCA showed decreased under increasing stress magnitude similar to the overall results of the collated data of the three taxa (Figure 5.5). The amount of Ea-total in rotifers under both PCP and 3,4-DCA exposures was higher than the value of the overall (all taxa grouped) Ea-total in

this chapter. The overall results from regression equation of the collated data (Figure 5.4) showed that the amount of Ea-total under 0% stress was about 2740 mj/mg, which was reduced to around 1719 mj/mg under the highest stress levels. While under PCP and 3,4-DCA exposures, the amount of total energy available (Ea-total) was 11279 and 8200 mj/mg under 0% stress and reduced to 8474 and 4954 mj/mg at the highest toxicant concentration in rotifers under PCP (Figure 4.9) and 3,4-DCA exposure (Figure 4.12) respectively. These differences in Ea-total amounts may be due to taxa-specific responses, as there were differences between the three taxa as well as with rotifers. Furthermore, difference in Ea-total values is expected to be due to using different methodologies to calculate the Ea content, which have been chosen according to the method used for calculating the CEA. Authors either calculate the difference in Ea content during the exposure time only or they calculate the total Ea content at the end of the exposure time only.

5.3.3 Consumed energy (Ec)

The consumed energy was measured in 24 datasets out of the 33 used: 10 in daphnia, 7 in the enchytraeids, and 7 in microalgae. The energy consumption was estimated by measuring the electron transport system activity (ETS) at the mitochondrial level which gives a cellular indication of the energy cost of basal maintenance and any other extra stress-related energy consumption. The change in Ec that happens after toxicant exposure is possibly due to the changes in energy associated with toxicant detoxification (Novais et al., 2013). Therefore, an increased respiration rate due to stress exposure can be linked to increased energy demand for homeostasis maintenance mechanisms and metal detoxification. The increase in Ec, together with the mobilization of Ea reserves, can decrease the CEA, which would have led to less energy being allocated for other biological processes, e.g., reproduction.

Exposure to toxicant stress is generally expected (Sokolova, 2013) and has been reported (e.g., Van Dievel et al., 2019; Verslycke et al., 2004) to increase the consumed energy in the stressed organisms (Ec). However, comparing between the three included taxa, daphnia and microalgae had the highest Ec levels followed by the enchytraeids; however, the rate of Ec change was constant under all stress magnitude, as the overall ($p = 0.34$) and all the taxa specific trend lines were not significantly different from a horizontal regression line (Figure 5.6). Thus, the increase in energy consumption as a result of toxicant stress cannot be generalized. For example, toxicant exposure was reported to decrease the ETS activity in the mosquito *Culex pipiens* (Delnat et al., 2019). Toxicant effects on Ec are also concentration-dependent, where under high concentrations, organisms may undergo metabolic depression (Rodrigues et al., 2017; Storey, 2015). Moreover, similar lack of effects on Ec have been reported for chemical toxicants on the polychaete *Hediste diversicolor* (Stomperudhaugen et al., 2009) and the damselfly larvae (Verheyen and Stoks, 2020).

The overall (all taxa grouped) observation for the collective data set analysis showed that under 0% stress, the Ec was around 5.87 mj/mg/h but increased to around 7 mj/mg/h (Figure 5.6) at 100% stress. However, given the small magnitude of these changes, they are unlikely to be of real consequence for the CEA values (reported in the section below), as the CEA value is dominated by the Ea contribution.

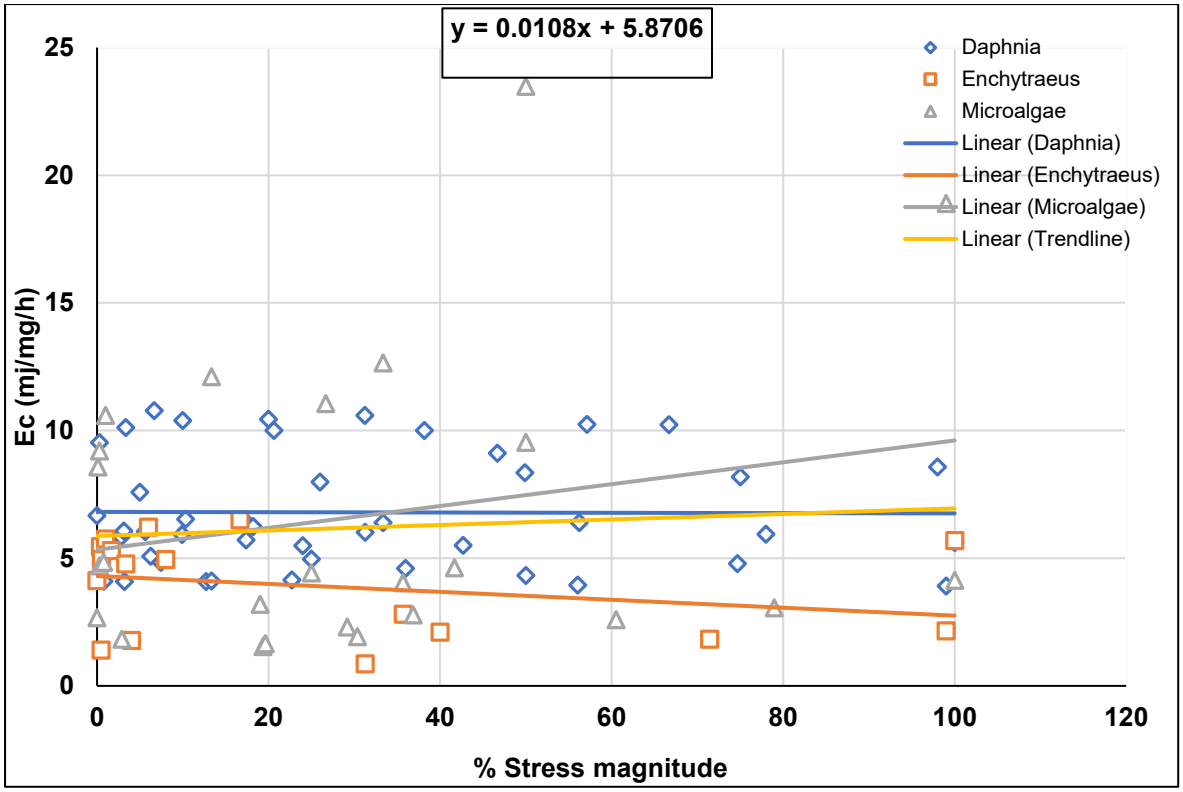

Fig. 5.6: Values of consumed energy (Ec) expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

5.3.4 The cellular energy allocation (CEA)

The CEA was measured in 26 datasets out of 33 used, 12 on daphnia, 7 on enchytraeids, and 7 on microalgae. For all daphnia, the CEA was calculated according to (De Coen and Janssen 1997), while for enchytraeids and microalgae the CEA was calculated as Ea / Ec. The cellular energy allocation (CEA) is a widely used biomarker that assesses the energy cost of exposure to contaminants, by calculating both the available energy (Ea) (as lipid, carbohydrate, and protein contents) and the consumed energy (Ec) (as ETS) on the cellular level (De Coen and Janssen, 1997). It is highlighted that the CEA is mainly determined by the amount of energy reserves (this being true regardless of it being calculated by subtracting Ec from Ea or dividing Ea by Ec).

The overall trend in the acquired data was no change in CEA with an increased magnitude of stress for all taxa ($p = 0.19$) (Figure 5.7). However, there was a decrease in CEA when increasing the stress magnitude in daphnids ($p < 0.001$). Comparing the three included taxa with each other, daphnia had the highest CEA values followed by microalgae and then enchytraeids. The trend of CEA with increasing stress magnitude was highest decreasing for daphnia > enchytraeids and microalgae. Moreover, the control and the other multipoint values of CEA greatly varied between taxa, where daphnia showed the highest values, microalgae showed medium values, and the enchytraeids have the lowest values. To overcome this problem, the Ea, Ec, and the CEA were expressed as % of control for every within-study control and then plotted against Ea % to illustrate the relationship between Ea and both Ec, and CEA (Figures 5.8 and 5.9).

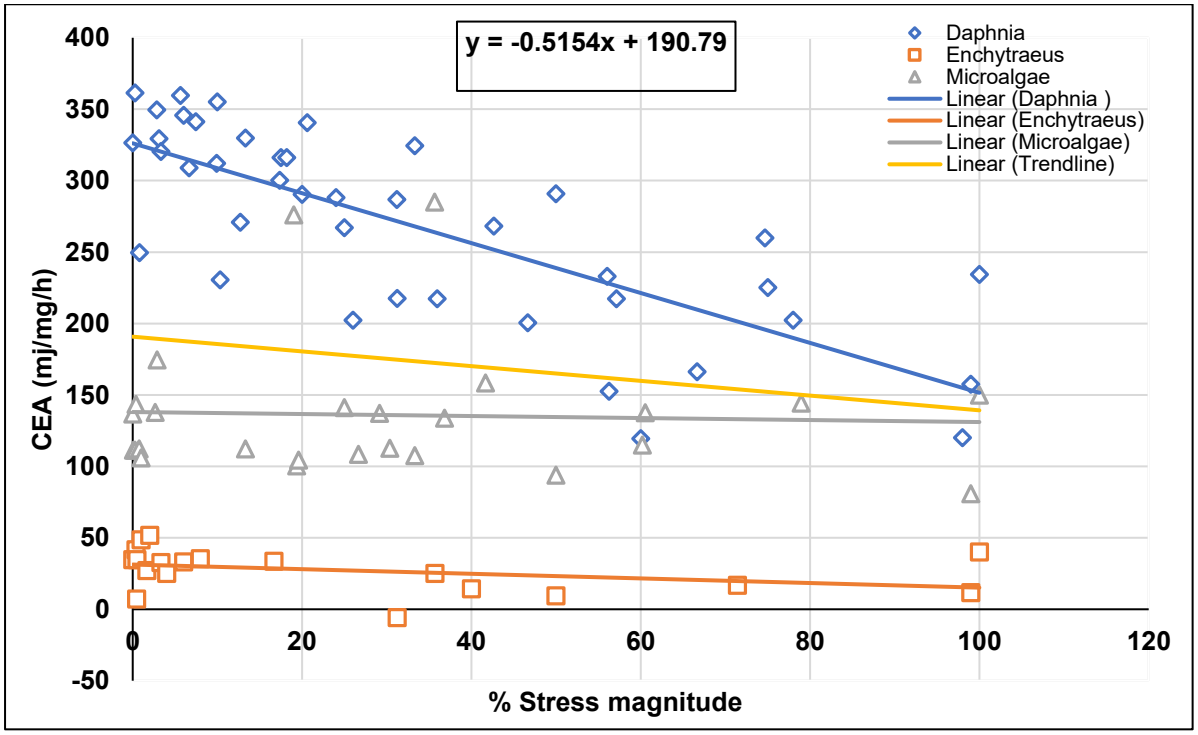

Fig. 5.7: Values of cellular energy allocation (CEA) expressed as (mj/mg) for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline

5.3.5 Relationship between CEA and Ea

The relationship between CEA and Ea was a positive linear relationship, as when Ea increased the CEA increased ($p < 0.01$) (Figure 5.8). By knowing the CEA % of control, the CEA at different magnitudes of stress can be calculated when the Ea of this stress level is known at the respective treatment, using the following Equation:

CEA % of control =0.9435 x (Ea % of control) + 0.6988.

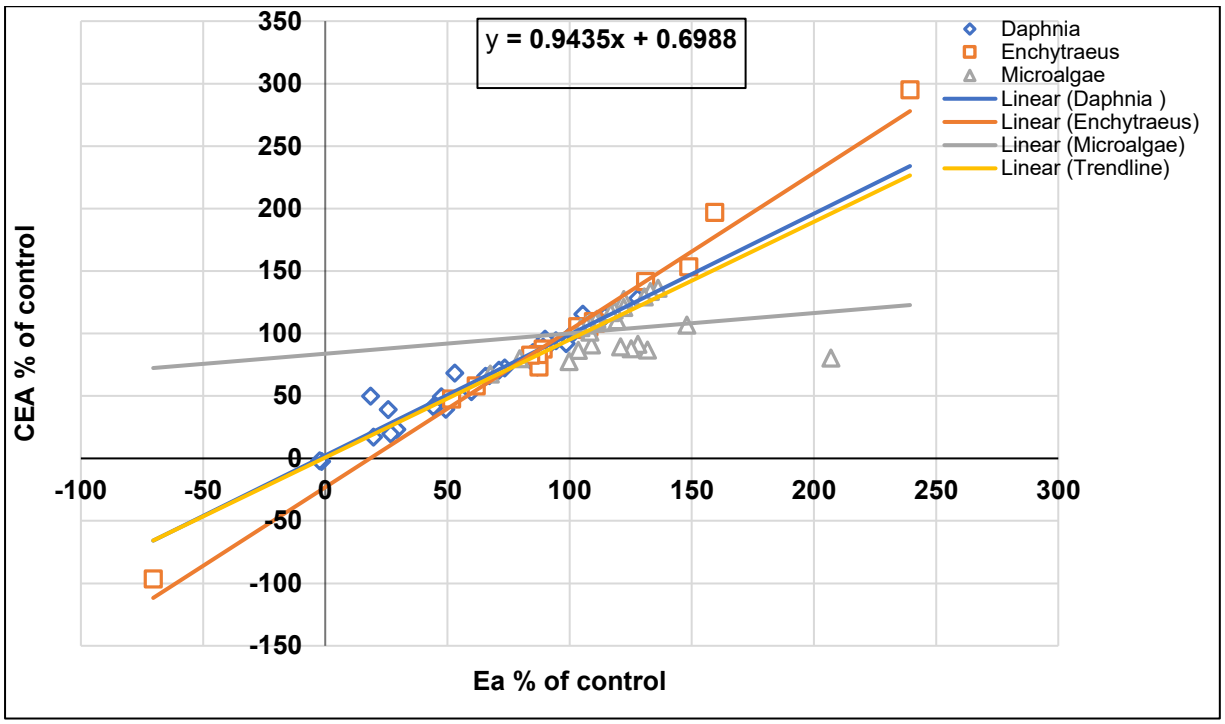

Fig. 5.8: Relationship of consumed energy (CEA) and (Ea) expressed as a percentage of control for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline.

5.3.6 The relationship between Ec and Ea

The relationship between Ec and Ea was constant, as when Ea% of control increased, the Ec% of control did not change (Figure 5.9). This relationship was only positive linear in the case of microalgae ($p = 0.019$). By knowing the Ec % of control, the CEA at different magnitudes of

stress can be calculated when the Ea of these stress levels is known at the respective treatment, using the following Equation:

Ec % of control = 0.3978 x (Ea % of control) + 101.4.

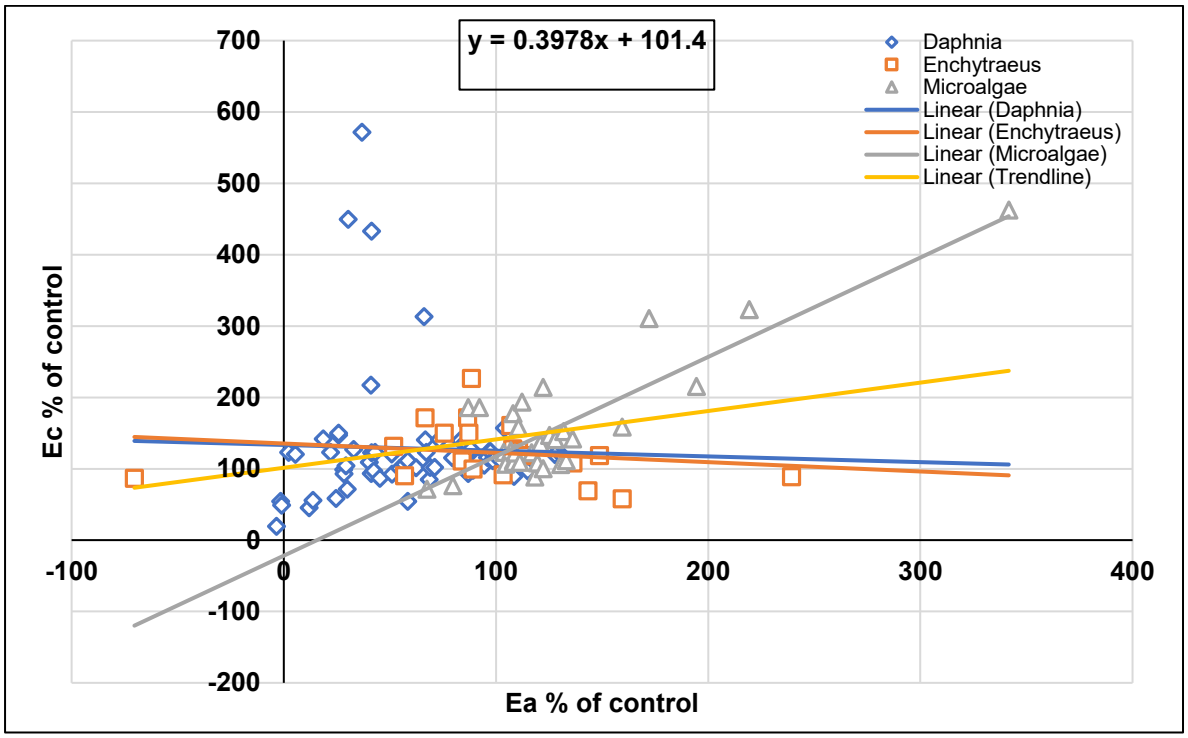

Fig. 5.9: Relationship of consumed energy (Ec) and (Ea) expressed as percentage of control for different aquatic taxa under different magnitudes of chemical stress during 24h of exposure. The equation refers to the overall trendline.

5.4 Conclusion

From the overall analysis, we can conclude that all the three energy components (proteins, lipids, and carbohydrates) have been affected by chemical stress in daphnids. Lipids were less significantly affected by stress compared to proteins and carbohydrates, and this may be because animals tend to use the lipids in later complementary mechanisms after the primary mechanisms of metal detoxifications have been initially supported by energy from proteins and

carbohydrates. In enchytraeids, only the proteins were affected by toxic stress, which indicates its importance as an energy resource for metal detoxification mechanisms for this taxon. In microalgae, there was no effects on energy components. This may be due to using pharmaceutical chemical toxicants in the microalgae studies, which may have different effects compared with metal and organic toxicants that have been used in studies based on daphnids and enchytraeids. Thus, there were no overall stress response in Ea-total in microalgae and enchytraeids while the Ea-total was significantly decreased under higher stress magnitude in daphnids. The difference between taxa Ea-total and individual energy components may be due to taxa-specific responses to toxic stress which cause differential pathways of energy re-allocations. As in enchytraeids, proteins seemed to be the main source of energy supply under toxic stress. While in daphnids, all the three components are utilized, but probably with different preference according to the stress level.

The overall Ec for every taxon did not change with increasing the stress magnitude. This might indicate that why the Ec contributes much less to the CEA compared with the Ea-total. This obtained numerical relationship between the Ec% of control and the Ea% of control are used in the next chapter (Chapter 6) to estimate the values of Ec in rotifers under different chemical exposure. This will be achieved using data of Ea in rotifers and data of Ec at control of rotifers. Integrating the results of Ec and Ea, the CEA could be easily calculated for rotifers under different chemical exposure.

CHAPTER SIX

Chapter 6: Calculation and changes in the consumed energy (Ec) and cellular energy allocation (CEA) in the freshwater rotifers *Brachinous Calyciflorus* exposed to toxicant stress

6.1 Introduction

In ecotoxicological studies, there are many methods used for assessment of the impacts of chemical toxicants. Cellular energy allocation (CEA) is one of those approaches that is used to assess the energy cost associated with exposure to chemical toxicants. The calculation of CEA is on the cellular level; it represents the balance between the available energy (Ea) (lipid, carbohydrate, and protein contents) and the consumed energy (Ec) (as ETS) (De Coen and Janssen, 1997). The use of energy related measures as indicators of environmental stress have been previously proposed by many authors (e.g., Bayne et al., 1979; Goldberg and Bertine, 2000; Kooijman and Bedaux, 1996; Widdows and Donkin, 1992). The rationale behind using energy related measurements in ecotoxicological studies is that the stress responses of organisms are costly for the organism in terms of metabolic demands. Bayne et al. (1979) proposed that a general assessment of physiological status and bioenergetics is required for the evaluation and interpretation of the impacts of pollution on organisms. This approach was given much attention under the method of scope for growth; however, linking the cellular energy reserves and energy consumption to higher levels of biological organization is still not well investigated.

The use of CEA to evaluate the energy budget of an organism in response to stressors is well established and has been applied to several invertebrates to test the impacts of toxicants on organisms and populations (De Coen and Janssen 2003; Smolders et al., 2004; Silva et al., 2019). To measure the CEA, the available energy and the consumed energy in the studied organisms must be calculated. Available energy under different chemical toxicants has been measured and reported in Chapter 4. In compliment to this, consumed energy was assessed and

reported in Chapter 5 as based upon numerical linkage between available energy and consumed energy in a range of taxa under chemical stress.

Thus, this Chapter aims to combine the two components, Ea and Ec to calculate the CEA. This was achieved through the following objectives:

1- Calculate the Ec of rotifers under control conditions
2- Convert the Ea of rotifers under each toxicant concentration to Ea % of control of each toxicant
3- Use the numerical linkage from Chapter 5 to calculate the Ec of the corresponding toxicant stress level
4- Calculate the CEA of each toxicant concentrations.

6.2 Materials and methods

6.2.1 Data extraction and calculation

It was not possible to measure the Ec values in the laboratory, due to Covid 19 restrictions on laboratory work from March 2020 to April 2021. Thus, data of Ec, Ea, and CEA of different taxa have been assembled from the literature and then used to quantitatively establish numerical linkages between Ec, Ea and CEA, as presented in Chapter 5. The outcomes of the literature-based approach were used to justify Ec values using the linkages established between the Ec% of control and the Ea % of control. These Ec values were then used to enable CEA assessment for rotifers to be completed using Ea values measured (Chapter 4) and Ec values justified (this Chapter) through the literature review presented in Chapter 5.

To be able to use the linkage between the Ec% of control and the Ea% of control obtained in Chapter 5 to calculate values of Ec of rotifers under our different toxicant concentrations used

in Chapter 4 (Table 4.1), we had to have two things: a) the Ea values of each toxicant concentration and its control to be used to calculate the Ea% of control and b) the Ec of rotifers under control conditions. Then these data were used to obtain the Ec values in rotifers using the equation of Ec% of control and Ea% of control that was obtained from data synthesis in Chapter 5:

Ec % of control = 0.3978 x (Ea % of control) + 101.4 (1)

Once the Ec values were calculated, these data were used to calculate the CEA values in rotifers under different toxicant concentrations (Table 4.1). CEA was calculated according to Verslycke et al. (2004a, b). The Ea, Ec and CEA values were calculated as follows:

Ea (available energy) = Ea-protein + Ea-lipid + Ea-carbohydrates (mj /mg)

Ec (energy consumption) = ETS activity (mj /mg/ h)

CEA (cellular energy allocation) = Ea/Ec (2)

Ec in rotifers was firstly calculated under conditions of starvation. The average oxygen consumption by rotifers was obtained from the literature, taking care to match experimental conditions herein with those available in the literature, i.e., under starvation. Collated data on oxygen consumption in *Brachionus sp.* from literature were expressed as µmol O_2 /mg DW/h.

All results were then standardized to be expressed as µmol O_2 /mg/h (Table 6.1). After that, oxygen consumption was transformed into energetic equivalents using the oxygenthalpic equivalents for an average lipid, protein, and sugar mixture (484 kJ /mol O_2) Gnaiger (1983), where 1µmol O_2/h = 484 mj/h. The energy consumption was then expressed as mj/mg/h. The quantified oxygen consumption under starvation was considered as the energy consumed (Ec) by rotifers under control conditions.

Given the Ec values under control conditions and the Ea values under the different toxicant concentrations and their control (those reported in Chapter 4), it was possible to assess the CEA using Equation (2). Ea values were converted to Ea% of control. This was possible by dividing the Ea value for every toxicant concentration by the Ea value of its control. To calculate the Ec value of each toxicant concentration, the Ea% of control was put in the Equation 1. The result of this equation was multiplied by the Ec value under the control and then divided by 100 to obtain the Ec values of the corresponding toxicant concentrations. Ec values were expressed as mj/mg/h.

To calculate the CEA, the Ea and Ec values of each toxicant concentration were applied into the Equation number 2. The CEA values were expressed as mj/mg.

6.2.2 Data analysis

The obtained results of both Ec and CEA were statistically analysed using the SPSS software package (version 25). Significant differences between the observations in the treatments and the control were analysed using one-way analysis of variance (ANOVA) after testing data for homogeneity of variance (Levene's test). Two-way ANOVA was used to compare the effects of the two heavy metals with each other under different stress levels as well as to compare the effects of the two organic toxicants to each other under different stress levels, where the toxicant type and the stress levels were the input two factors while the Ec and the CEA were used as dependent variables. Differences were considered significant at $P < 0.05$.

6.3 Results

6.3 Estimation of the consumed energy (Ec) values for rotifers

6.3.1 Ec in rotifers under control conditions

The overall average of the Ec obtained for the *Brachionus sp.* under starvation for 18h was 14.19 mj/mg/h. This quantified Ec value was considered as the Ec of rotifers under control

conditions in our experiments, which was used to estimate the Ec values under the stress of different toxicant concentrations.

Table 6.1: Respiration rates (Oxygen consumption) of *Brachionus sp* extracted from the literature that has been measured under two conditions: 1- (no food) where animals were not fed at the time of measuring the respiration, but they were previously fed, and 2- the animals have been starved for 18 h before measurements.

Oxygen consumption of *Brachionus* sp.		**Energy consumption (Ec)**		
(µmol O_2 /mg DW/ h)		**(mj/mg/h)**		**References**
No food	**Fasting for 18h**	**No food**	**Fasting for 18h**	
0.067	-	32.4	-	Doohan (1973)
0.047	-	22.74	-	(Galkovskaya, 1995)
0.052	0.020	25.16	9.68	(Kirk et al., 1999)
0.055	0.04	26.62	19.36	(Jensen et al., 2006)
0.055	0.028	26.62	13.55	Hirata and Yamasaki (1987)
	Average		**14.19**	

6.3.2 Ec of rotifers under toxicant concentrations

The following equation was used to calculate the Ec under each toxicant concentration:

Ec % of control = 0.3978 x (Ea % of control) + 101.4

This was possible after converting the Ea values to Ea% of control and considering that the Ec under control conditions is 14.19 mj/mg/h for all toxicant concentration.

6.3.2.1 Ec of rotifers under Cu

Under Cu exposure, there was a significant effect of Cu on the Ec values ($F_{4,40}$, $P < 0.05$). The Ec increased under all Cu exposures compared to control conditions (Figure 6.1). Comparing between the Cu exposures, the highest Ec value was observed under the highest Cu concentration (6 µg/L), where Ec was 20.49 ± 0.38 mj/mg/h, while the lowest Ec value was observed at 0.06 µg/L, which has Ec of 19.69 ± 0.56 mj/mg/h.

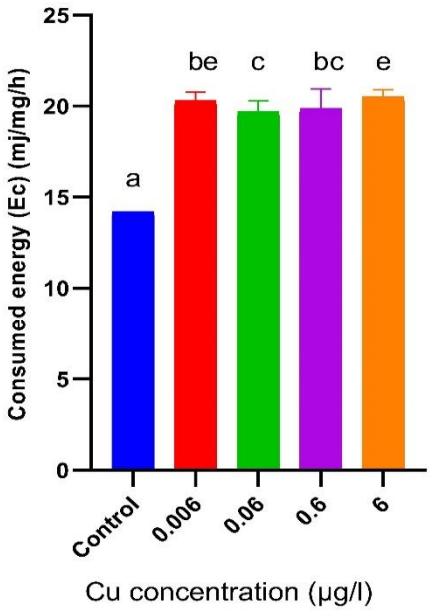

Fig. 6.1: The averages of the Ec (mj/mg/h) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of Copper (Cu). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cu concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.2.2 Ec of rotifers under Cd

There was a significant effect of Cd on the Ec values ($F_{4,40}$, $P < 0.05$). The Ec increased under all Cd exposures compared with control conditions (Figure 6.2). Comparing between the Cd treatments, the highest Ec value was observed under the Cd concentration of 0.06 µg/L, where Ec was 20.03 ± 0.34 mj/mg/h, while the lowest Ec value was observed at 0.6 µg/L, which has Ec of 19.48 ± 0.63 mj/mg/h.

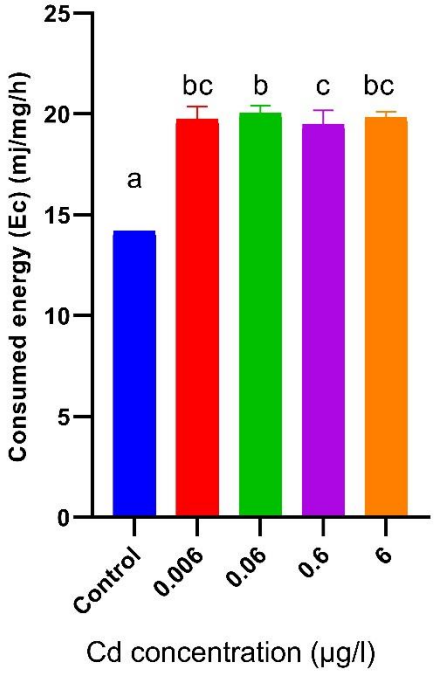

Fig. 6.2: The averages of the Ec (mj/mg/h) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of Cadmium (Cd). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between Cd concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.2.3 Ec of rotifers under PCP

Under PCP exposure, there was a significant effect of PCP on the Ec values ($F_{4,40}$, $P < 0.05$). The Ec increased under all PCP exposures compared with control conditions (Figure 6.3). Comparing between the PCP exposures, the highest Ec value was observed under the lowest PCP concentration (0.1 mg/L), where Ec was 19.13 ± 0.72 mj/mg/h, while the lowest Ec value was observed at 0.3 mg/L which has Ec of 18.33 ± 0.20 mj/mg/h**.**

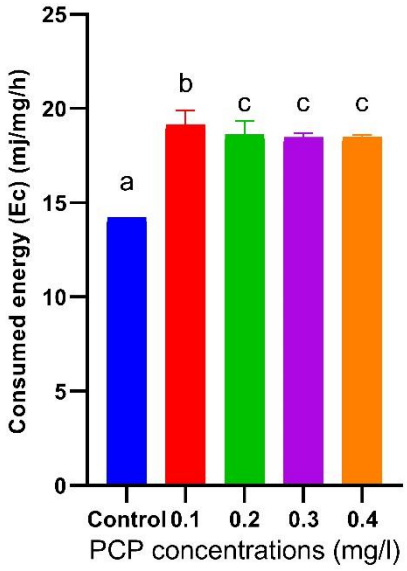

Fig. 6.3: The averages of the Ec (mj/mg/h) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of pentachlorophenol (PCP). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between PCP concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.2.4 Ec of rotifers under 3,4-DCA

There was a significant effect of 3,4-DCA on the Ec values ($F_{4,40}$, $P < 0.05$), as Ec increased under 3,4-DCA exposures compared with control conditions (Figure 6.4). Comparing between the 3,4-DCA treatments, the highest Ec value was observed under the lowest 3,4-DCA concentration (0.2 mg/L), where Ec was 20.02 ± 0.63 mj/mg/h, while the lowest Ec value was observed at the highest 3,4-DCA concentration (0.8 mg/L) which has Ec of 18.21 ± 0.63 mj/mg/h.

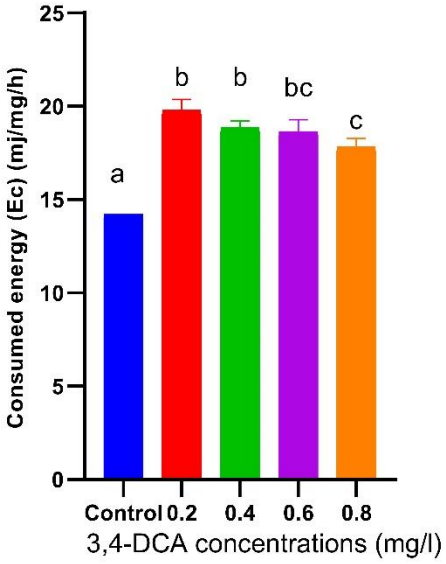

Fig. 6.4: The averages of the Ec (mj/mg/h) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of 3,4 dichloroaniline (3,4-DCA). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between 3,4-DCA concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.3 CEA of rotifers under toxicant stress

6.3.3.1 CEA under Cu

There was a significant effect of Cu on the CEA values ($F_{4,40}$, $P < 0.05$). The CEA decreased under all Cu exposures compared with control conditions where the CEA was 260.56 ± 21.2 mj/mg (Figure 6.5). Comparing between the Cu treatments, the lowest CEA value was observed under the Cu concentration of 0.06 µg/L, where CEA was 176.05 ± 14.20 mj/mg, while the highest CEA value was observed at the highest Cu concentration (6 µg/L), which has a CEA of 195.04 ± 8.5 mj/mg.

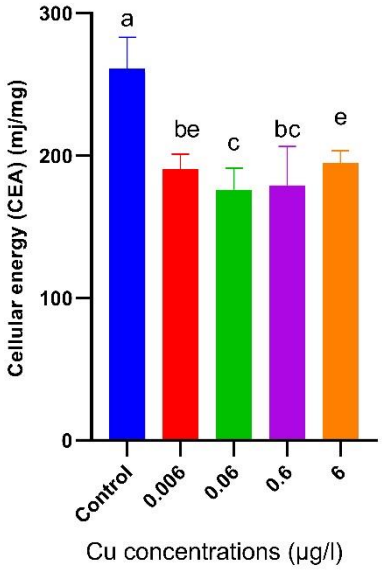

Fig. 6.5: The averages of the CEA (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of Copper (Cu). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cu concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.3.2 CEA under Cd

There was significant effect of Cd on the CEA values ($F_{4,40}$, $P < 0.05$). The CEA decreased under all Cd exposures compared with control conditions where the CEA was 274.67 ± 29.48 mj/mg (Figure 6.6). Comparing between the Cd treatments, the lowest CEA value was observed under the Cd concentration of 0.06 µg/L, where CEA was 185.21 ± 22.89 mj/mg, while the highest CEA value was observed at the lowest Cd concentration (0.006 µg/L), which has CEA of 208.34 ± 14.78 mj/mg.

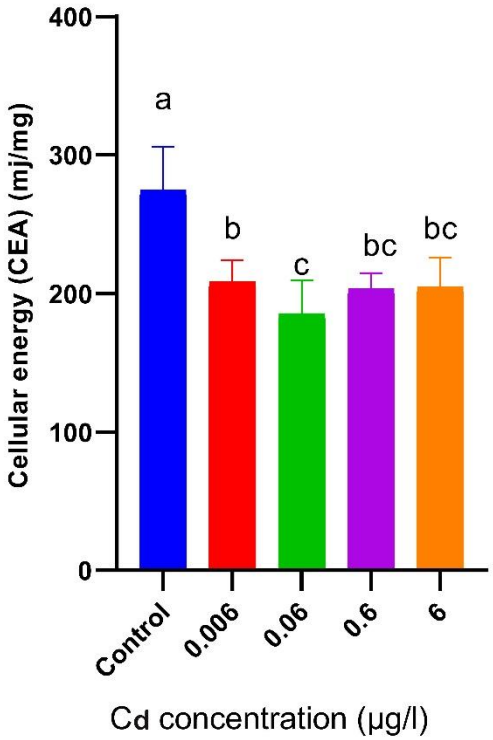

Fig. 6.6: The averages of the CEA (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of Cadmium (Cd). Differences were considered significant at P ≤ 0.05, lower case letters indicate the significant differences between Cd concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.3.3 CEA under PCP

There was a significant effect of PCP on the CEA values ($F_{4,40}$, $P < 0.05$). The CEA decreased under all PCP exposures compared with control conditions where the CEA was 794.88 ± 54.17 mj/mg (Figure 6.7). Comparing between the PCP treatments, the lowest CEA value was observed under the highest PCP concentration (0.4 mg/L), where CEA was 440.95 ± 10.87 mj/mg, while the highest value was observed at the lowest PCP concentration (0.1 mg/L), which has CEA of 493.05 ± 58.28 mj/mg.

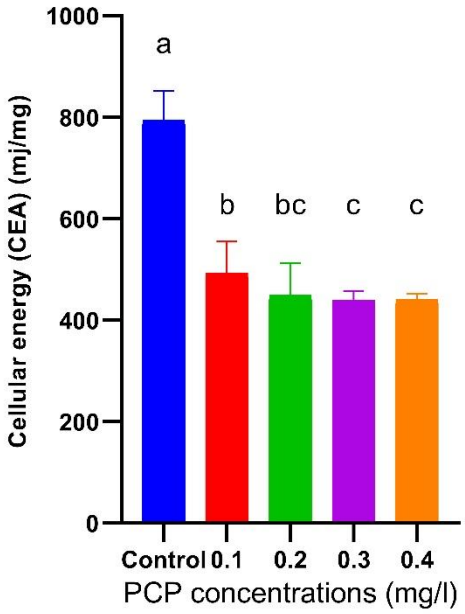

Fig. 6.7: The averages of the CEA (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of pentachlorophenol (PCP). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between PCP concentrations. Results are expressed as average ± standard deviations (N=3).

6.3.3.4 CEA under 3,4-DCA

There was significant effect of 3,4-DCA on the CEA values ($F_{4,40}$, $P < 0.05$). The CEA decreased under all the 3,4-DCA exposures compared with control conditions where the CEA was 577.82 ± 92.87 mj/mg (Figure 6.8). Comparing between the 3,4-DCA treatments, the lowest CEA value was observed under the highest 3,4-DCA concentration (0.8 mg/L), where CEA was 277.64 ± 29.04 mj/mg, while the highest value was observed at the lowest 3,4-DCA concentration (0.2 mg/L), which has Ec of 407.59 ± 32.05 mj/mg. The CEA values were inversely related with the Ec values under 3,4-DCA exposure.

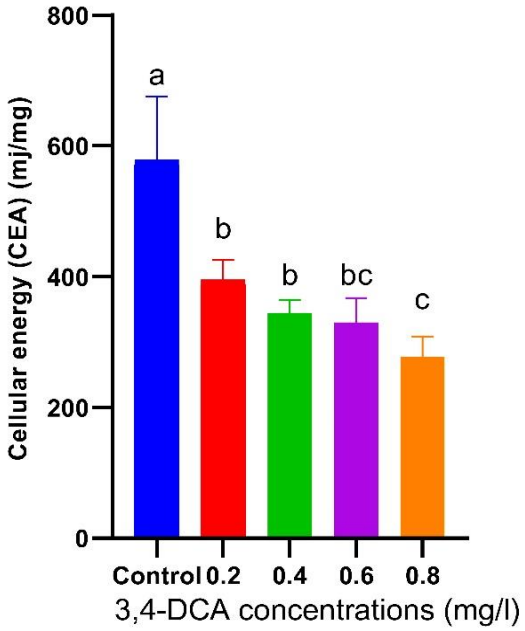

Fig. 6.8: The averages of the CEA (mj/mg) measured in the rotifer *B. calyciflorus* after 24h exposure to different concentrations of 3,4 dichloroaniline (3,4-DCA). Differences were considered significant at $P \leq 0.05$, lower case letters indicate the significant differences between 3,4-DCA concentrations. Results are expressed as average ± standard deviations (N=3).

6.4 Discussion

The CEA is a widely used approach to evaluate the energy budget of an organism in response to stressors and has been applied to several invertebrates to test the impacts of toxicants on organisms and populations (De Coen and Janssen 2003; Smolders et al. 2004; Silva et al. 2019). Under stress conditions depending on the toxicity level and its mode of action, the CEA can be reduced, leading to less energy being allocated for other biological processes, e.g., reproduction. This occurrence is the importance of using the CEA and how linking the cellular changes with higher biological organization levels could help better toxicant impact assessments.

One of the main factors that allow using the CEA with certain model organisms is the sensitivity of CEA to detect the impact of sublethal toxicant concentrations. So, this Chapter aimed to test the suitability and sensitivity of CEA to detect impacts of different chemical toxicants in the freshwater rotifer *Brachinous calyciflorus*. Our results elucidated the sensitivity of CEA to detect effects of sub-lethal concentrations of different chemical toxicants in rotifers. The CEA varied under different toxicant stress levels compared to the control conditions. Furthermore, the Ec and the CEA values differed between the different stress magnitudes, broadly speaking, with decreasing values of CEA under higher stress levels. These results suggest the usefulness of CEA in the ecotoxicological studies of rotifer *B. calyciflorus*, since it demonstrated the CEA to be a reliable tool for studying the impacts of chemical toxicants, changing in different exposure levels.

The values of consumed energy (Ec) were different between the control group and treatments, increasing under all toxicant exposure compared to control. Ec values under heavy metals did not show a marked trend but, in general, decreased under middle stress levels while it was higher under higher and lower concentrations. For Cu, the highest Ec was observed under the

highest Cu concentration (Figure 6.1). While under the Cd, the highest Ec value was observed under the second lowest concentration (Figure 6.2). Furthermore, under highest stress level, Cu seemed to have lower Ec values compared to Cd. For organic toxicants, there was a clear trend of decreasing Ec value in increasing stress for both PCP and 3,4-DCA.

The increased ETS activity and expenditure of energy under toxic conditions was reported to be associated with an up-regulation of genes involved in the mitochondrial oxidative phosphorylation under toxicant exposures, suggesting that the extra energy demand was directed to control metal toxicity in other species (Novais et al., 2012). This was the case when comparing the Ec value of exposed versus control rotifers. Conversely, in increasing concentration of the two organic compounds (PCP and 3,4-DCA), there was a slight, but significant, decrease in Ec.

This result of Ec under different organic toxicant concentrations did not agree with the overall (all taxa grouped) Ec observation for the collective dataset analysed in Chapter 5, which showed that the Ec did not changed when the magnitude of stress increased.

On the other hand, other studies on daphnia reported increase of the Ec under increased toxic stress (Tourinho et al., 2022). In this case, the variation in Ec values after toxicant exposure may be due to establishing toxicant detoxification mechanisms which are associated with changes in energy budgets (Novais et al., 2013). Thus, an increased respiration rate due to stress exposure can be linked to increased energy demand for homeostasis and metal detoxification (Villarroel et al., 2009; Novais et al., 2012; Novais et al., 2013). If the increase in Ec was accompanied with mobilization of Ea reserves, this can cause a decrease in the net energy budget, indicating that less energy was available, e.g. to cope with other physiological and biological functions such as growth and reproduction (Villarroel et al., 2009; Novais et al., 2012).

The decrease in Ec under higher stress levels may be related to more severe effects on stressed organisms, so animals tend to decrease maintenance costs, and this will be evident when it is accompanied with reduction in CEA due to high depletion of the energy budget (De Coen and Janssen, 2003), as has been reported for enchytraeids exposed to chemical toxicants (Gomes et al., 2015). This was clearly observed in rotifers exposed to high 3,4-DCA concentrations, where Ec decreased steadily in increasing stress, suggesting the higher toxicity of 3,4-DCA relative to PCP.

The Ec values identified for rotifers in this chapter are higher than the overall values of all taxa in Chapter 5, which ranged between 5.8 and 7 mj/mg/h. The higher Ec values for rotifers may be related to higher basal metabolic rates of rotifers as reported by Jensen et al. (2006). Our Ec values were more similar to those reported for microalgae, which ranged from 1.57 to 23 mj/mg/h. The differences in Ec values between the different studies may be due to taxa-specific responses, as there were differences between the three taxa when compared as well as when comparing daphnia and enchytraeids with rotifers. Furthermore, differences in Ec values are expected to be due to using different methodologies to calculate the Ec. Authors calculate the change in Ec from the zero time point to the end of exposure only or they calculate the total Ec at the end of the exposure period. Those explanations are also applied for differences in CEA associated to energy reserves in this Chapter.

Regarding the CEA, whenever the CEA and its components (Ec and Ea) were not sensitive to the changes in toxicity stress across a gradient stress magnitudes, it was concluded that it is not a good indicator for toxicity assessment (e.g Frreirae et al., 2015). On the contrary, our results indicated that the CEA method is sensitive to assess the impacts of both heavy metals and organic compounds in rotifer species, which suggest that it is a good indicator for toxicity stress in rotifers. Both heavy metals and organic compounds decreased the CEA in rotifers compared to the control (Figures 6.5 and 6.6). The trend under organic compounds was that CEA

decreased by increasing the stress magnitude with the lowest value under highest toxicant concentration.

The decrease in CEA, whenever it occurred, was referred to either as a depletion in Ea and/ or an increase in Ec, both resulting in a lower energy budget available for biological processes (De Coen and Janssen, 1997). In our results, under organic compounds, the decrease in CEA was driven by both an increase in Ec values and a decrease in Ea under toxic conditions compared to the control conditions. Saying this, results cannot confirm this conclusion, as we did not directly measure the Ec values due to the aforementioned causes. Furthermore, De Coen and Janssen (1997, 2003) and Muyssen et al., (2001) reported a significant CEA decrease in *D. magna* exposed to toxic stress due to a reduction in Ea contents, concluding that the Ea content, especially lipids, was the main driver of the CEA, meanwhile Ec had only a minor contribution to CEA total amount. Verheyen and Stoks (2020) reported also that the impact of the insecticide, chlorpyrifos, on the CEA of damselfly larvae was only driven by reductions in Ea, while Ec had no or little contribution. However, other studies on a different taxa (*Enchytraeus crypticus*) showed an increased Ec accompanied with a decrease in CEA under lower (EC_{10}) metal (Cu) exposures, with no changes in energy assimilation (Gomes et al., 2015).

Furthermore, the decrease in CEA accompanied with an increase in Ec and decrease in Ea was previously reported in different invertebrate taxa under exposure to organic compounds (Novais et al., 2013; Gomes et al., 2015). However, this result was different from Smolders et al. (2004) who reported that the decrease in CEA was mainly due to a rapid depletion of the available energy reserves and not by an increased Ec for zebra mussels exposed to a pollution gradient.

Results of data collated in Chapter 5 indicate that the CEA pattern reported for the overall data (all taxa grouped) was different from what we found in rotifers in Chapter 6. CEA in rotifers

decreased with increasing stress, while the overall data of chapter 5 showed no change in CEA. This overall response of the collated data was driven by data collected from microalgae and enchytraeids, which showed no significant decrease in CEA. The individual papers on microalgae and enchytraeids reported decrease in CEA under chemical stress but analyzing these as single dataset seemed to neutralize these differences in CEA. Nevertheless, our rotifer results were in agreement with the overall CEA data for daphnids. The amount of decrease in CEA under the highest level of stress in organic compounds for rotifers in our study was comparable with the overall amount of decrease in CEA for daphnids in Chapter 5. While the organic compounds caused the CEA to decrease to 44.5 - 51.5 % of control, the overall results for daphnids showed that the CEA decreased to 46.6% of control.

6.5 Conclusion

The CEA method was used to test the toxic impact of four toxicants on the rotifer *B. calyciflorus.* The changes in the total bioenergetics indicated that the CEA is an appropriate tool to test toxic impacts in rotifer species under exposure to the assessed heavy metals (Cu and Cd) and organic compounds (PCP and 3,4 DCA). Given the low concentration of the two heavy metals used, they seemed to have less toxic effects on rotifers compared to the organic compounds. However, we think that if the concentrations of heavy metals were closer to the thresholds, it would result in a higher toxic effect than was obtained. This needs further investigation to be confirmed. The two organic compounds (PCP and 3,4 DCA) seemed to have toxic effects on rotifers similar to the overall trend of the pooled data of different chemical toxicants (heavy metals and organic compounds) on daphnids identified in Chapter 5. Therefore, these results indicate that rotifers could be suitable and resourceful species for testing the ecotoxicological effects of other pollutants in aquatic ecosystems using the CEA approach.

CHAPTER SEVEN

Chapter 7: General Discussion

7.1 Introduction

From cells to populations and ecosystems, chemical contaminants in aquatic environments impact organisms at different levels (Schwarzenbach et al., 2006). Ecological impacts should therefore be evaluated in the context of interactions of pollutants within different ecosystems as well as their effects at different levels of biological organisation (Moiseenko, 2008).

To this end, the Cellular Energy Allocation (CEA) approach has been established in the literature as a reliable method to evaluate the energy budget of an organism in response to chemical stressors (De Coen and Janssen, 1997; 2003; Gomes et al., 2016a ; Silva et al., 2019; Tourinho et al., 2022). The method is based on the determination that the increased energy expenditure as a result of exposure to toxic stress compromises an organism's ability for growth, reproduction, and other non-basal metabolic processes (De Coen and Janssen, 2003). Although the CEA was originally developed and validated for *Daphnia magna,* it has been extended to other invertebrates to test the impacts of toxicants on individual organisms and populations (De Coen and Janssen 2003; Smolders et al., 2004; Silva et al., 2019). However, for a method that holds so much potential, the application of the CEA is still limited to very few aquatic species. There is, therefore, a need to increase the number of model aquatic species to facilitate toxicity impact screening in these ecosystems. In this regard, rotifers are an important species in the food chain of freshwater, coastal, and marine ecosystems. As they represent the principal food for many invertebrates and fish larvae (Gharaei et al., 2020), rotifers can facilitate the biotransfer of toxicants in these food chains and are, therefore, excellent candidate model species to test the CEA methodology.

This thesis was an attempt to extend the CEA approach to evaluate the toxic effects of chemical pollutants on rotifers. The CEA approach was optimized and applied to assess the toxicity of

four pervasive aquatic pollutants, copper (Cu), cadmium (Cd), pentachlorophenol (PCP), and 3,4-dichloroaniline (3,4-DCA), to the freshwater rotifer *Brachinous calyciflorus*. Briefly, the median lethal concentrations (LC_{50}) of the toxicants were determined in an acute toxicity test relying on concentration ranges reported in the literature. Results from acute toxicity tests were used to design a second experiment, which consisted of a range of lethal concentrations, to determine the LOEC and NOEC of each of the tested toxicants. Subsequently, these results informed tests to assess the effect of a range of sub-lethal concentrations on rotifer population density and growth rates. All of these experiments are reported in Chapter 2 of the thesis.

The optimisation of protocols for the measurement of the available energy components (i.e. proteins, lipids, and carbohydrates) is reported in Chapter 3. In Chapter 4, the effect of a gradient of sub-lethal concentrations on the available energy components was evaluated. To calculate the consumed energy, a literature review was conducted on studies that evaluated the effect of chemical pollutants on CEA in different marine taxa. The collected data was then used to consider/establish numerical linkages between consumed energy and the available energy under different magnitudes of stress. This review and analysis were reported in Chapter 5.

In Chapter 6, based on linkages and relationships established in Chapter 5, Energy consumption (Ec) was calculated in rotifers under different exposure scenarios and then the relevant CEA was calculated for each toxicant. The optimisation of the CEA procedure for the freshwater rotifers was discussed by summarising and discussing results from Chapter 2. Then the use of *B. calyciflorus* as a model organism for toxicity assessment under the CEA approach was validated by summarising and discussing results in Chapters 4 to 6. Chapter 7 ended with some recommendations and the future applications of CEA studies with rotifers.

7.2 Optimisation of the CEA procedure for freshwater rotifers

7.2.1 Acute toxicity of Cu, Cd, PCP, and 3, 4 - DCA to rotifers

The acute toxicity of metals and organic contaminants to both freshwater and marine rotifers has been the subject of extensive research (see review by Li et al., 2020; also see: Snell et al., 1991a; Ferrando and Andreu-Moliner, 1991; Ferrando et al., 1992; Hernandez-Flores et al., 2020; Rebolledo et al., 2020, 2021; Zhang et al., 2021). In this study, Cu, Cd, and PCP were acutely toxic to *B. calyciflorus* within the concentrations tested, with LC_{50} ranging from 0.012 mg/L for Cu, 1.46 mg/L for Cd, to 1.95 mg/L for PCP. These threshold concentrations were generally within the range of those previously reported in *B. calyciflorus* (Table 2.7) but also in other related species. For example in the marine rotifer *B. plicatilis*, Luna-Andrade et al. (2002) reported a 24 h LC_{50} of 0.014 – 0.022 mg Cu/L depending on feeding conditions; Arnold et al. (2011) reported a 48 h LC_{50} of 0.021 mg Cu/L, and Tait et al. (2016) reported a 48 h LC_{50} of 0.021 mg/L at low dissolved organic carbon (DOC). This was also the case for Cd and PCP (see Table 2.7 and review by Li et al., 2020). For metals, toxicity on rotifers has been found to be significantly influenced by food supply (e.g., Luna-Andrade et al., 2002; Arnold et al., 2011), presence/concentration of DOC (e.g., Cooper et al., 2014; Tait et al., 2016), salinity (Rebolledo et al., 2020, 2021), temperature (Rebolledo et al., 2020), and rotifer size or species (Kang et al., 2019).

In contrast with other tested toxicants, observed LC_{50} for 3,4-DCA in this study (124 mg/L) was higher than the concentrations assessed, indicating potentially low toxicity. The value was also double the concentrations previously reported by Snell et al. (1991a), Ferrando and Andreu-Moliner (1991), and Ferrando et al., (1992) for *B. calyciflorus*. Although in conformity with the current thesis, 3,4- DCA was observed to be less toxic than Cu, Cd, and PCP across these studies, the disparity in actual LC_{50} values with the current study may be explained by potential differences in rotifer strains or in the methodologies employed, as have been observed

for other organic toxicants (e.g. Liber and Solomon, 1994). For example, toxic effects also vary with the age of rotifers, as newly hatched neonates may have different sensitivities to toxicants than adult rotifers (Liber and Solomon, 1994; Dahms et al., 2011). Overall, deduced LC_{50} for the four toxicants allowed for the extrapolation of the range of concentrations for use in chronic toxicity tests.

7.2.2 Effect of sub-lethal Cd and Cu concentrations on the population density (PD) and the intrinsic growth rate(r) of rotifers

In *Brachionus* species, adverse effects on population density and/or reproduction have been used as sensitive endpoints to assess the impacts of several chemical stressors (e.g., Luna-Andrade et al., 2002; Gama-Flores et al., 2007; Sarma et al., 2008, 2010). Here, sub-lethal concentrations at both high and low-range values were tested on rotifer population density and growth rates. At lower chronic exposures for both metals, toxicity appeared to be more dependent on the duration of exposure rather than concentrations. The concentrations used for each toxicant had similar responses compared to the control after 24h of exposure and, where observed, significant differences were observed after 48 and/or 72h of exposure. A lack of significant effects in this range can be explained by the low concentrations, relative to those reported in previous studies (Sarma et al., 2008, 2010; Li et al., 2020). However, these concentrations at 24h exposure were useful in establishing the test range for the CEA experiment to preclude effects on population density and growth rates.

Even at higher sub-lethal concentrations, toxicants tested in this study had varying effects on *B. calyciflorus*. For metals, Cu appeared to show a hormetic effect on PD, which increased with exposure time, but no effect on r throughout the experiment, in direct contrast to the acute toxicity tests. Conversely, Cd significantly affected PD and r with increasing concentration and exposure time. These conflicting results have been previously reported as an artefact of hormesis, where two or more toxicants may have similar LC_{50}, but the responses at lower

concentration levels may differ (Sarma et al., 2008, 2010). For example, Cu is regarded as an essential metal and may increase reproduction rates in *Brachionus* species due to hormesis (Gama-Flores et al., 2007; Sarma et al., 2008). The hormetic responses are thought to be a kind of homeostatic overcompensation in which the stressed animals optimise their ability to face the stress conditions beyond their normal adaptation limits (Calabrese and Baldwin, 2003; Gama-Flores et al., 2007). However, the sub-lethal toxicity of Cd on *B. calyciflorus* has been previously reported to be greater than that of Cu (Gama-Flores et al., 2007). In general, both population density and growth rate are expected to be sensitive to chemical stress (Charoy, 1995; Forbes and Calow, 1999; Snell and Marcial, 2017; Xu et al., 2021a), especially in metals which can potentially interfere with cellular functions by reacting with proteins and phospholipids, among other molecules (Frausto da Silva and Williams, 1993; Jan et al., 2015; Abdelsattar et al., 2021). Previous studies indicate that these chronic effects are dependent on the metal (e.g., Sarma et al., 2008) and/or rotifer species (e.g., Huang et al., 2013; Han et al., 2021). Thus, the chronic toxicity of rotifers to stressors cannot be generalised.

Overall, the sub-lethal threshold concentrations for metals in this study agree with some of the values previously reported. The NOEC value of Cu was 0.0024 mg/L after 72h (Table 2.8), which agrees with results from Janssen et al. (1994) who reported that at 0.0025 mg Cu/L, there were no effects on r for *B. calyciflorus.* However, the values in this study were lower than those reported by Snell and Moffat (1992), who reported an NOEC of 0.020 mg Cu/L and a LOEC of 0.030 mg Cu/L after 48h of exposure, in agreement with Gama-Flores et al. (2007). Their findings may explain why a LOEC was not determined in this study, as it was out of the concentration ranges that were used. For Cd, the LOEC observed in the current study (0.07 mg Cd/L) agrees with that reported by Snell and Moffat (1992), but it is higher than those reported by Sarma et al. (2006) and Gama-Flores et al. (2007), both for *B.calyciflorus*. As the LOEC for

Cd in the present study was 0.07 mg/L, it may be concluded that the NOEC is lower than 0.07 mg/L, which was in accordance with 0.04 mg/L reported by Snell and Moffat (1992).

7.2.3 Effect of sub-lethal PCP and 3,4- DCA concentrations on the population density (PD) and the intrinsic growth rate(r) of rotifers

Rotifers are sensitive to several organic compounds, including carbamates, organochlorines, and organophosphates (Havens and Hanazato, 1993; Hanazato, 2001; Thorp and Covich, 2009; Kim et al., 2016; Lee et al., 2018). Like the metals, the two organic toxicants used in this study showed varying effects on PD and r at low and high-range sub-lethal exposures. Both PCP and 3,4- DCA did not significantly affect PD or r across the lower range concentration until after 48 and/or 72 h of exposure, further demonstrating the influence of the exposure period. Even at higher-range concentrations, PCP showed no effect on PD and r, but 3,4 DCA significantly affected both endpoints under three out of four of the used concentrations. Consequently, the highest concentrations here were diluted for use in the energy budget experiments (Chapter 4).

The lack of sub-lethal toxicity for PCP in this study is remarkable. PCP has been extensively demonstrated to be both acutely and chronically toxic to rotifers and other zooplankton species in previous studies (e.g. Liber and Solomon, 1994; Gomez et al., 1997; also see review by Li et al., 2020). For example, Gomez et al. (1997) reported a dose-dependent effect of PCP on predator-prey relationships of two rotifer species, *Asplanchna girodi* and *B. calyciflorus*, reporting a NOEC value of 0.11 mg/L, much less than the NOEC of 0.39 mg/L observed in this study. Likewise, Juchelka and Snell (1994) reported an NOEC value of 0.13 mg/L for 24h exposures, and Snell and Carmona (1995) reported 0.2 mg/L for asexual reproduction, both in *B. Calyciflorus*. Given the similar concentration ranges investigated across these studies and that feeding and reproduction are intermediate endpoints linked to growth rates and population density in both zooplanktons (Charoy, 1995; Nandini and Rao, 1998; Li et al., 2020) , it was expected that PCP will show significant sub-lethal toxicity in the current study. Consequently,

an LOEC value for PCP could not be determined (Table 2.8). The observations, however, partly agree with the findings of Janssen et al. (1994), with an LOEC of 0.8 mg/L for a 72h exposure period.

For 3,4-DCA, adverse effects on PD and r observed in this study were more severe than for PCP and both metals tested. These effects are consistent with observations in previous studies (e.g., Janssen et al., 1994; Juchelka and Snell, 1994; Girling et al., 2000) and may be due to effects on food consumption and assimilation, as the feeding rates of the rotifers can be reduced under the stress of organic compounds (Ferrando and Andreu, 1993). Additionally, organic toxicants may affect the number of offspring produced by delaying the age of the first reproduction and thus increasing the birth interval (Snell and Moffat, 1992; Girling et al., 2000). Enzymes of acetylcholine metabolism, such as choline acetyltransferase and acetylcholinesterase, can also be disrupted in rotifers due to organic stressors (Ferrando et al., 1993a). The NOEC value for the 3,4-DCA found in the current study (Table 2.8) was higher than previously reported results by Ferrando et al. (1993a) and Janssen et al. (1994), while the LOEC value was within the range of the two values reported by Ferrando et al. (1993a) and Janssen et al. (1994).

7.2.4 Optimal conditions for sub-lethal toxicity assessments in rotifers

Whereas PCP and Cu did not appear to affect PD and r across the range of sub-lethal concentrations assessed in this study, 3,4- DCA had the most severe chronic toxicity on *B. calyciflorus* in this study for the concentrations tested, followed by Cd. This order of least to most toxic was reversed relative to the acute tests, as both 3,4- DCA and Cd did not appear to be acutely toxic across the assessed concentrations. It demonstrates the need for chronic toxicity testing, which has been shown in several studies (e.g. Snell and Moffat, 1992; Marcial et al., 2005) to be more sensitive than acute tests. Another remarkable observation in this was the importance of exposure duration, alongside dosage, in evaluating toxic effects on rotifers,

with similar concentrations only resulting in adverse effects after prolonged exposure periods. This phenomenon has been observed across a range of organic (e.g., Ferrando et al., 1996; Mihaich et al., 2009) and inorganic (e.g., Lee et al., 2016; Xu et al., 2021b), highlighting the need for varying exposure durations. A possible explanation is the late onset of toxicity, especially for metals (Xu et al., 2021b), resulting from the ability of invertebrates for regulation or detoxification using a range of mechanisms (Rainbow, 2007; Rainbow and Luoma, 2011).

A third factor assessed by this study was the impact of the presence or absence of food during toxicity testing, which did not appear to affect test conclusions. Determinations of CEA throughout the literature have been split between studies choosing to feed (e.g., De Coen and Janssen, 2003) or not to feed (e.g., Tourinho et al., 2022) test organisms during exposure. Proponents argue that the reduction in the feeding rate of stress organisms can increase test sensitivity since feeding inhibition is reflected in net energy budget estimations (De Coen and Janssen, 2003). However, feeding can introduce organic matter, which is laborious to handle (e.g., requiring renewal systems) or may interfere with the bioavailability of assessed toxicants (Li et al., 2020). In this study, except for 3,4- DCA, the absence of food had no effect on PD and r at all sub-lethal concentrations assessed. However, the slope of increase in r concerning PD was higher for all toxicants under no food conditions compared with the food conditions. This observation may be explained according to the strategy of energy allocation, according to which energy may be more allocated to reproduction under food-limited stress conditions (Kirk, 1997; Kirk, 1999). The tolerance for starvation observed in this study aligns with observations by Kirk (1997) who noted a starvation survival time range of 10 – 120h in several rotifer species. And some rotifers have been observed to grow better under nutrient-limited conditions (Ramos-Rodriguez and Conde-Porcuna, 2003).

Overall, the evidence in this study supports a no-food regime to improve sensitivity, with tests lasting for 24h to ensure adequate toxicant exposure within the average starvation survival time

range. We corroborate the recent findings of Gomes et al. (2016a), where unfed *Enchytraeus albidus* populations assessed for CEA were better able to discriminate the effects of Cd across different exposure durations. The authors cautioned against direct comparison of food versus no-food exposures without considering exposure duration and recommended the exposure of test species without food.

7.3 Rotifers as model organisms in CEA toxicity assessment assays

7.3.1 Effect of sub-lethal metal concentrations on the energy budgets of rotifers

Limitations in calculating CEA in this study are highlighted in Section 7.4, especially regarding the reliance on modelled relationships in estimating energy consumption, and these apply to all four toxicants assessed in this study. For the two metals assessed (Cu and Cd), Cu only significantly decreased carbohydrate content at concentrations ≥ 0.06 µg/L but did not show any significant impact on protein and lipid stores across the concentrations tested. Similarly, Cd decreased protein content at concentrations ≥ 0.06 µg/L but did not show any significant impact on carbohydrate and lipid stores across the concentrations tested. These results did not, however, translate to changes in energy content, indicating that there was no impact on overall available energy (Ea) under stress relative to the control. A possible explanation for the lack of effect on energy budget is that metal concentrations used were below the suitable toxicity threshold in rotifers (e.g., Erk et al., 2008). For example, Soetaert et al. (2007) reported effects on energy budget in *D. magna* at exposures > 10 µg/L for 48h, whereas the maximum concentration used in the current study was 6 µg/L. The concentration range had been established and diluted based on thresholds obtained in Chapter 2 but should be closer to the thresholds in future studies.

Conversely, significant increases were observed in modelled Ec relative to control values at all Cu and Cd concentrations assessed. This also significantly impacted the overall CEA values,

with CEA reducing in increasing stress relative to the controls. This phenomenon is common in the literature. In their recent study, Tourinho et al. (2022) observed in populations of *D. magna* that exposure to silver nanoparticles and silver nitrate for 48h did not affect available energy or any of the individual energy stores (i.e. lipid, carbohydrate, and protein content) but led to a significant increase in energy consumption, thus decreasing CEA by up to 75% of control values. Gomes et al. (2015) observed in the oligochaete, *Enchytraeu crypticus*, that exposure to Cu increased protein budget but led to an increase in Ec, which ultimately reduced CEA relative to control values. The lack of effect on or possible increase in energy reserves at these low exposures has been attributed as a possible adaptation for detoxification and damage repair (Sokolova et al., 2012) or in preparation for more stress (Wilder and Jeyasingh, 2016). Heavy metals have been reported to decrease the CEA in several other invertebrate species, including freshwater gastropods (Moolman et al., 2007), the estuarine mysid shrimp *Neomysis integer* (Verslycke et al., 2004), and the zebra mussel (Smolders et al., 2004). In *Enchytraeus albidus*, exposure to Cd and Zn reduced the CEA with transcriptional, enzymatic, and reproductive alteration (Novais et al., 2013).

7.3.2 Effect of sub-lethal concentrations of PCP and 3,4- DCA on the energy budgets of rotifers

Unlike metals, the organic toxicants assessed in this study showed significant effects on both available energy and energy consumption, leading to a decrease in CEA values. Exposure to PCP induced a significant decrease, relative to control values, in protein and lipid energy reserves at all concentrations assessed, while carbohydrates only decreased significantly at concentrations ≥ 0.3 mg/L. When all three energy stores were combined as Ea, the effect of PCP was significant from 0.1 mg/L. Similarly, exposure to 3,4- DCA at concentrations ≥ 0.4 mg/L significantly reduced the individual energy fractions as well as in combination, as Ea. Both toxicants increased Ec, with the resulting CEA estimations, across all concentrations

assessed. Together, the results suggest increased sensitivity of the CEA methodology, especially in the Ea component, to organic contaminants when compared with metals at the concentrations assessed. Similar trends in both Ea and Ec for invertebrates exposed to organic contaminants have been previously reported (e.g., De Coen and Janssen, 2003; Smolders et al., 2004; Erk et al., 2011; Aderemi et al., 2018). Outside organic contaminants, Shang et al. (2021,2023) recently reported a decrease in both Ea and Ec in thick-shell mussels *Mytilus coruscus* exposed to acidified seawater and microplastics.

The depletion in energy reserves seen for organic toxicants in this study is consistent with the thesis of an increased energy expenditure in the adaptation to a toxic stressor (De Coen and Janssen, 2003). Reduction in total protein reserves may be related to the important role of proteins as a vital source of energy under stress, as drawing upon these reserves enables animals to support the increased energy demands necessary for survival (Sancho et al., 2009; Villarroel et al., 2013, Silva et al., 2020). As lipids are a highly efficient energy reserve, they might be consumed first by stressed organisms (De Coen and Janssen, 1997; Muyssen and Janssen, 2001; De Coen and Janssen, 2003; Soetaert et al., 2007; Amorim et al., 2012). Furthermore, lipid consumption can be very useful to support mechanisms that repair cellular and tissue damage under chemical exposure (Villarroel et al., 2013). In some cases, carbohydrate consumption by stressed organisms represented the main resource for energy used in metal detoxification mechanisms (De Coen et al., 2001). The depletion of carbohydrates may also highlight their important role as rapidly mobilisable metabolic fuels used to cover a rapid increase in energy demand under stress. Overall, the results show that exposure to organic toxicants influences the energy budget in rotifers. Consequently, energy reallocation to stress resistance mechanisms may limit the energy available for other biological processes. These changes in Ea, particularly under the highest concentrations, may be because these concentrations were closer to the threshold values defined in Chapter 2.

Although both organic and inorganic toxicants assessed in this study significantly increased Ec relative to control values, effects on energy consumption as a result of toxic stress cannot be generalised. For example, studies on the polychaete *Hediste diversicolor* (Stomperudhaugen et al., 2009) and the damselfly larvae (Verheyen and Stoks, 2020) have reported no significant effects for exposure to chemical toxicants on Ec even when the CEA was observed to reduce. On the other hand, chemical exposure was reported to decrease the ETS activity in the mosquito *Culex pipiens* (Delnat et al., 2019) and several other studies (e.g., Van Dievel et al., 2019; Verslycke et al., 2004; Tourinho et al., 2022). Toxic effects on Ec are stress level (concentration)-dependent, where animals may undergo metabolic depression under high concentrations (Rodrigues et al., 2017; Storey, 2015). As a consequence, a decrease in Ec under higher stress levels is related to more severe effects, so animals tend to decrease maintenance costs, and this is evident in a decrease in ETS activity (De Coen and Janssen, 2003; Gomes et al., 2015). An increased ETS activity due to stress, in general, can be linked to increased energy demand for homeostatic mechanisms and detoxification (Villarroel et al., 2009; Novais et al., 2012, 2013). The decline in CEA values would then be related to a combination of a decrease in catabolic activity and a decrease in metabolic rates with inhibition of the ETS activity, leading to the loss of mitochondrial integrity (Verslycke et al., 2003).

Overall, these results validate the suitability of the CEA in toxicity assessments for both organic toxicants in rotifers. The reduction of CEA in rotifers under sub-lethal concentrations can explain the reduction in PD and r under higher concentrations that were observed in Chapter 2. These toxicants can impact rotifer populations in natural environments and possibly lead to alterations in the food web and ecosystem performance.

7.4 Thesis limitations

As highlighted in the relevant chapters, the results reported in this thesis and summarised in Sections 7.1 and 7.2 above are subject to certain limitations. Firstly, the rotifer life stages used in the different toxicity assays were not identified, which could potentially affect the comparability of results between the different assays in this study and/or with previously published figures. *B. calcyflorus*, like other rotifers of the subclass Monogononta, reproduces both sexually and by asexual parthenogenesis (Dodds and Whiles, 2020), resulting in the occurrence of several types of females and eggs or haploid males following parthenogenesis. Typically, sexual reproduction only occurs as a result of undesirable environmental conditions, such as increased population density or changes in the photoperiod (Wallace, 2002; Kostopoulou et al., 2012; Dodds and Whiles, 2020; Gilbert, 2020). However, such conditions have been observed under laboratory culturing, prompting a shift in reproductive strategy, which may affect population composition and fitness. (see review by Gilbert, 2020). Moreover, neonate rotifers may reach adulthood within hours (Snell, 2014). It is, therefore, important to monitor laboratory cultures of rotifers and ascertain life stages before and after toxicity assays.

Similarly, the life stages of the microalgae, *Chlorella vulgaris*, harvested for the rotifer culture were not definitively determined before use. Microalgal species in a batch culture system grow in five phases: a lag phase in which cells adapt to the new environment; an exponential (or log) phase which is marked by rapid growth and cell division; a decline phase where the rate of division is limited by the availability of nutrients in the system; a stationary phase where cell division slows due to the consumption of available nutrients; and a death phase where cells starved of nutrients begin to die (Yousuf, 2020). Given the availability of healthy cells, algal cultures are ideal as feedstock in the log phase, and this is typically determined by cytometry or spectrophotometry (Sun et al., 2018; Gama-Flores et al., 2020; Pulgarin et al., 2022). However, the algal life stage in this thesis was determined by visual observation of the green

colouration, considering the increase in chlorophyll concentration characteristic of the log-phase Chlorella cultures (He et al., 2022). This method may not be accurate.

A second major limitation of this thesis is the reliability of analytical concentrations reported throughout the work. Stock toxicant solutions were prepared in a range of 10 – 1000 mg/L, but unfortunately could not be analysed or standardised due to time and logistics constraints. Furthermore, these stock solutions were diluted heavily to achieve relevant concentrations for the acute and chronic tests. For example, Cu concentrations were in a range of 0.0006 – 0.0024 mg/L in the high-range chronic test (Table 2.5**)** and 0.0000002 – 0.0002 mg/L in the low-range test (Table 2.6). However, concentrations in the high-range test are within the detection limits of some of the most developed analytical tools currently available (Elkhatat et al., 2021), including the ICP-MS-QQQ available at the UEA School of Environmental Sciences (Udochi, 2020), while the lower concentrations in the low-range test might be not detectable by any analytical technique. Given that these concentrations were achieved by diluting the stock solutions in synthetic fresh water, toxicity thresholds for the acute and, especially, chronic tests in this study should be interpreted with caution.

In reporting effect-level concentrations for the chronic and acute tests in this thesis, a third limitation is the use of NOEC/LOEC values rather than modelled Effective Concentrations ($EC_{10 \text{ or } 20}$). This has been a subject of debate in the recent literature. While proponents of ECx concentrations (e.g. Landis and Chapman, 2011; Fox 2011; Jager, 2012) argue that ANOVA-based toxicity metrics such as NOEC and LOEC are biased by exposure concentrations as well as the exposure-response relationship used in the individual studies, proponents of the NOEC/LOEC (e.g. Green et al., 2013) argue that ECx modelling is also flawed and not applicable to all datasets. As a result, new methods such as the NSEC (No Significant Effect Concentration) have been devised to address some of these shortcomings (Fisher and Fox, 2023).

A fourth limitation of this work is the the absence of the Quality Assurance (QA) and Quality Control (QC) procedures. Depending on this, the extraction step, and consequently the Ea values (i.e., protein, carbohydrate, and lipid content in test rotifers) reported in this thesis may be limited in reliability. Moreover, method development for the biochemical assay procedures described in Chapter 3 suffered from time and logistics constraints which affected quality analysis and control. Although calibration lines with strong linear relationships ($R^2 \geq 0.99$) were used, the lack of use multi-points (only 5-7 points were used for this study), certified reference materials and replication of known samples means that the accuracy and precision of analysis could not be determined (Olson and Markwell, 2007). This limitation may affect the comparability of reported Ea values with those reported in the literature. However, given the strength of the calibration, the limitation is not expected to affect the proportional change in Ea across the different toxicant concentrations.

A fifth limitation of this work is the reliance on modelled relationships to determine Ec values used in the calculation of CEA. This was necessitated by the suspension of laboratory research following mandatory lockdowns during the COVID-19 pandemic. Consequently, 21 peer-reviewed research articles were selected across a range of invertebrates (20 of which were focussed on daphnia) to establish the relationship between Ec versus Ea, both as a percentage of control values. The data was extensive, covering 18 datasets for daphnia and 7 datasets, each, for enchytraeids and microalgae. To standardise against methodological differences, concentrations were normalised across the studies relative to the maximum concentration used in each dataset. Despite efforts to standardise the datasets, the Ea and Ec values used in the CEA calculations were not independent observations, given that the Ec values were modelled from observed Ea. Ultimately, CEA values deduced in this study are only an estimate and not reflective of true values. The most significant advantage of this methodology is, however, that

it allows for a rapid and fairly accurate comparison of CEA values across a range of toxicant concentrations without the often laborious process of determining Ec experimentally.

7.5 Thesis recommendations

In conclusion, results from this thesis suggest that rotifers are suitable and resourceful species for testing the ecotoxicological effects of pollutants in aquatic ecosystems using the CEA approach. The results confirm the sensitivity of CEA in detecting the sub-lethal effects of organic and inorganic toxicants on rotifers. CEA was observed to vary under different toxicant stress levels compared to control conditions, which suggests the usefulness of CEA in ecotoxicological studies involving the freshwater rotifer *B. calyciflorus*. CEA seems to be a reliable endpoint for the impacts of chemical toxicants, as it was sensitive to the changes in the exposure gradient of the used toxicants. Therefore, I recommend the use of the CEA in rotifers to evaluate the effect of pentachlorophenol (PCP) and 3,4-dichloroaniline (3,4-DCA) toxicity. However, responses of CEA in rotifers under other toxicants should be evaluated before generalising our conclusions.

Our results also indicate that organic compounds more negatively impacted energy reserves than the heavy metals used. We attributed this to the lower sensitivity of *B. calyciflorus* to the range of heavy metal concentrations used in our study. Therefore, the main recommendation here is to re-evaluate the impacts of the two heavy metals on energy reserves using a higher concentration range which should be closer to the thresholds identified in Chapter 2. In contrast, the concentrations used for the two organic compounds are high enough to impact the CEA in rotifers. In general, much consideration should be given to the selection of the concentration range of the tested toxicant. For example, more robust preliminary range-finding trials should be conducted before the actual experiments. As our results for the two heavy

metals indicated, concentrations could have been set higher than those used to demonstrate impacts on CEA.

Exposure to toxicants is generally expected and was reported to increase energy consumption in stressed organisms (Ec). Our results showed that Ec seemed to be less sensitive to the degree of toxicant stress compared to Ea. Therefore, I propose the use of the *in vivo* ETS oxygen consumption approach (Osma et al. 2016b) to estimate the Ec when calculating the CEA. This approach is described in detail in the following section. It is expected that applying the *in vivo* ETS approach will improve the sensitivity and precision, thus increasing the overall sensitivity of calculated CEA.

7.6 Future research

7.6.1 Interaction between toxicants and other environmental stressors

The routinely used standard tests in environmental impact assessment studies can provide rapid data for decision and regulation policy making. However, the ecological relevance of these tests remains low due to their lower ability to evaluate any interactive effects that happen between coexistent multi-stressors. Moreover, they fail to test some specific biological effects (e.g., behavioural changes) that take more time (maybe multiple generations) to influence the tested organisms, and they lack long-term exposure to evaluate chronic stress (Thoré et al., 2021). This typically leads to an under- or over-estimation of the impact of pollution on aquatic animals (Liess et al., 2016; Philippe et al., 2019).

Incorporating these pollutant interactions into the CEA could help better predict toxic impacts. For example, Williams et al. (2016) reported that the combined effects of Corexit EC9500A (which is a chemical dispersant of the Corexit product line produced by Nalco Environmental Solutions LLC of Sugarland Texas) with temperature and salinity increased the negative outcomes on populations of the rotifer *B. plicatilis*. The synergistic interactions increased the

ability of the toxicant to disrupt lipid-based structures in the rotifers (e.g., cell membranes). The study highlights the importance of evaluating interactions to improve the ecological risk assessment, especially interactions with other environmental factors.

7.6.2 Possibility of transgenerational adaptation to chronic toxic exposures and its implications on ecotoxicological assessment assays

One important factor that determines the suitability of a toxicity assay is the sensitivity of the model animal to the used toxicant. Therefore, the possibility of transgenerational adaptation to toxicants may result in an overestimation of the relevant threshold for CEA assessments compared to non-adapted populations. For example, the pesticide toxicity on the mosquito larvae was higher under acute warming than under transgenerational warming (Meng et al., 2022). This result accounted for the negative effect of acute warming on the net energy budget since the depletion of Ea was higher to supply energy demands for detoxification. But under transgenerational warming, larvae had been acclimatised and, thus, consumed less energy to deal with toxic stress. Therefore, the use of multigenerational toxicity tests may better enhance the ability of the CEA approach to more accurately assess the impacts of long-term chemical exposures, especially if this is linked with other physiological endpoints.

7.6.3 *In vivo* calculation of the consumed energy (Ec)

Although the ETS activity was well correlated with the rate of oxygen consumption (RO_2) (Packard, 1985; Aristegui and Montero, 1995; Maldonado et al., 2012), the relationship between respiration and *in vitro* enzymatic measures of potential respiration (ETS= Φ) can be varied. This has prevented the use of ETS as a standard method of *in vivo* respiration. By studying the effect of starvation on marine zooplankton, Herrera et al. (2011) found that the RO_2 was decreased as a response to starvation while the *in vitro* ETS respiration (Φ) remained steady, consequently decreasing the RO_2/Φ ratio across the experiment. These observations

inspired more recent models in assessing the effect of starvation on respiratory metabolism. Osma et al. (2016 b) succeeded in predicting the *in vivo* RO_2 from the modelled oxygen consumption (VO_2), even though the values of RO_2 were relatively higher than predicted. They detected a higher correlation of the measured RO_2 with predicted VO_2 than with ETS (Φ) alone. The authors attributed the underestimation of the *in vivo* RO_2 in the used model to the unquantified role of ADP in the VO_2 calculations and recommended future quantification and insertion of ADP in the model equation. It is known that ATP synthesis is promoted by increasing concentrations of ADP, resulting in the increased respiration rate and utilisation of pyridine substrates.

Further research to optimize and integrate the use of *in vivo* oxygen consumption as a proxy for the consumed energy within the CEA method is expected to result in more reliable and robust assays. The results of this optimisation on different model organisms can be used for the correction, calibration, or error standardisation of the currently used ETS or traditional respirometry protocols. This can overcome the challenge of underestimating oxygen consumption under toxic stress when using conventional methods.

7.7 Thesis outcomes

1. Highlighting the importance of the cellular energy allocation (CEA) protocol to study the effects of chemical stress on energy metabolism in the freshwater rotifer *Brachinous calyciflorus*. These insights are useful as they support further opportunities to use CEA approaches to provide an early alarm for alterations that may happen in population density and/or population growth rates under chronic toxic exposure. Such eventualities have the potential to impact food webs and ecosystem performance.

2. This thesis succeeded in optimising the methods required to apply the CEA assay to rotifers. This was accomplished by identifying the LC_{50} of each toxicant used under our

experimental conditions, followed by identifying the NOEC and LOEC concentrations of each toxicant by testing gradients of toxicant concentrations on the population growth rate and population density. Ultimately, this work identifies specific concentration gradients of each toxicant expected to cause changes in the CEA.

3. Our results have indicated that the CEA is a sensitive assay to study the environmental impacts of pentachlorophenol (PCP) and 3,4-dichloroaniline (3,4-DCA). Future work is required to discover the response of CEA in rotifers exposed to other toxicants.

4. The two organic compounds used in our experiments negatively affected energy reserves (CEA) more than the heavy metals used in the present study. We attributed this to the lower potency of the range of heavy metal concentrations used. Therefore, the main recommendation here is to use a different range of heavy metal concentrations – closer to the thresholds that were identified in Chapter 2.

References

Abdelsattar, A. S., Dawoud, A., & Helal, M. A. (2021). Interaction of nanoparticles with biological macromolecules: A review of molecular docking studies. *Nanotoxicology*, *15*(1), 66-95. https://doi.org/10.1080/17435390.2020.1842537

Adam, N., Vergauwen, L., Blust, R., and Knapen, D. (2015). Gene transcription patterns and energy reserves in Daphnia magna show no nanoparticle specific toxicity when exposed to ZnO and CuO nanoparticles. *Environmental Research*, *138*(2015), 82–92. https://doi.org/10.1016/j.envres.2015.02.014

Aderemi, A. O., Novais, S. C., Lemos, M. F., Alves, L. M., Hunter, C., & Pahl, O. (2018). Oxidative stress responses and cellular energy allocation changes in microalgae following exposure to widely used human antibiotics. *Aquatic Toxicology, 203*, 130-139. https://doi.org/10.1016/j.aquatox.2018.08.008

Aguiar-González, B., Packard, T. T., Berdalet, E., Roy, S., & Gómez, M. (2012). Respiration predicted from an enzyme kinetic model and the metabolic theory of ecology in two species of marine bacteria. *Journal of Experimental Marine Biology and Ecology*, *412*, 1-12. https://doi.org/10.1016/j.jembe.2011.09.018

Aitken, A., & Learmonth, M. P. (2009). Protein determination by UV absorption. In: Walker, J.M. (Ed), *The protein protocols handbook* (pp. 3-6). Totowa, NJ: Humana Press. doi:10.1007/978-1-59745-198-7_1

Alonzo, F., & Mayzaud, P. (1999). Spectrofluorometric quantification of neutral and polar lipids in zooplankton using Nile red. *Marine chemistry*, *67*(3-4), 289-301. https://doi.org/10.1016/S0304-4203(99)00075-4

Amer, A.A.A. (2014). *The effect of cadmium chloride on the biology of gammarus pulex (Crustacea: Amphipoda).* PhD thesis, University of Exeter, Cornwall, UK.

Amoatey, P., & Baawain, M. S. (2019). Effects of pollution on freshwater aquatic organisms. *Water Environment Research, 91*(10), 1272-1287. https://doi.org/10.1002/wer.1221

Amorim, M. J. B., Gomes, S. I. L., Soares, A. M. V. M., & Scott-Fordsmand, J. J. (2012). Energy Basal Levels and Allocation among Lipids, Proteins, and Carbohydrates in *Enchytraeus albidus*: Changes Related to Exposure to Cu Salt and Cu Nanoparticles. *Water, Air, and Soil Pollution, 223*(1), 477-482. https://doi.org/10.1007/s11270-011-0867-9

Anderson, D. (1995). Society responds to contamination: Changes in pest control practices reduce toll on wildlife. *California Agriculture, 49*(6), 65-72. https://doi.org/10.3733/ca.v049n06p65

Arambašić, M. B., Bjelić, S., and Subakov, G. (1995). Acute toxicity of heavy metals (copper, lead, zinc), phenol and sodium on Allium cepa L., Lepidium sativum L. and Daphnia magna St.: Comparative investigations and the practical applications. *Water Research*, *29*(2), 497–503. https://doi.org/10.1016/0043-1354(94)00178-A

Argese, E., Marcomini, A., Bettiol, C., Perin, G., & Miana, P. (1994). Submitochondrial particle response to linear alkylbenzene sulfonates, nonylphenol polyethoxylates and their biodegradation derivatives. *Environmental Toxicology and Chemistry: An International Journal, 13*(5), 737-742. https://doi.org/10.1002/etc.5620130507

Arias-Almeida, J. C., & Rico-Martínez, R. (2011). Toxicity of cadmium, lead, mercury and methyl parathion on *Euchlanis dilatata* Ehrenberg 1832 (Rotifera: Monogononta). *Bulletin of environmental contamination and toxicology*, *87*(2), 138-142. https://doi.org/10.1007/s00128-011-0308-x

Arístegui, J., & Montero, M. F. (1995a). Plankton community respiration in Bransfield Strait (Antarctic Ocean) during austral spring. *Journal of plankton research*, *17*(8), 1647-1659. https://doi.org/10.1093/plankt/17.8.1647

Arístegui, J., & Montero, M. F. (1995b). The relationship between community respiration and ETS activity in the ocean. *Journal of plankton research, 17*(7), 1563-1571. https://doi.org/10.1093/plankt/17.7.1563

Arnold, W. R., Diamond, R. L., & Smith, D. S. (2011). Acute and chronic toxicity of copper to the euryhaline rotifer, Brachionus plicatilis ("L" strain). *Archives of environmental contamination and toxicology*, *60*, 250-260. https://doi.org/10.1007/s00244-010-9556-8

Arnould, J. P., Boyd, I. L., & Clarke, A. (1995). A simplified method for determining the gross chemical composition of pinniped milk samples. *Canadian journal of zoology*, *73*(2), 404-410. https://doi.org/10.1139/z95-045

Arzate-Cárdenas, M. A., and Martínez-Jerónimo, F. (2012a). Energy reserve modification in different age groups of Daphnia schoedleri (Anomopoda: Daphniidae) exposed to hexavalent chromium. *Environmental Toxicology and Pharmacology*, *34*(1), 106–116. https://doi.org/10.1016/j.etap.2012.03.003

Arzate-Cárdenas, M. A., and Martínez-Jerónimo, F. (2012b). Energy resource reallocation in Daphnia schodleri (Anomopoda: Daphniidae) reproduction induced by exposure to hexavalent chromium. *Chemosphere*, *87*(4), 326–332. https://doi.org/10.1016/j.chemosphere.2011.12.014

Azuara-Garcia, R., Sarma, S. S. S., & Nandini, S. (2006). The combined effects of zinc and alga on the life table demography of *Anuraeopsis fissa* and *Brachionus rubens* (Rotifera). *Journal of Environmental Science and Health, Part A*, *41*(4), 559-572. https://doi.org/10.1080/10934520600564394

Baillieul, M., Smolders, R., and Blust, R. (2005). The effect of environmental stress on absolute and mass-specific scope for growth in Daphnia magna Strauss. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *140*(3–4), 364–373. https://doi.org/10.1016/j.cca.2005.03.007

Baird, D.J., Douben, P.E.T., Greig-Smith, P., & Maltby, L. (eds.). (1996). *ECOtoxicology: Ecological Dimensions.* London, UK: Chapman & Hall.

Baliarsingh, S. K., Srichandan, S., Padmavati, T., Naik, S., & Sahu, K. C. (2010, November). Accumulation of heavy metals by freshwater zooplankton - a toxicological study. *Recent Advances in Space Technology Services and Climate Change* (RSTS & CC-2010), 7-14. IEEE conference, Chennai, India. https://doi.org/10.1109/RSTSCC.2010.5712789

Barnes, R.S.K., Calow, P.P., Olive, P.J.W., Golding, D.W., & Spicer, J.I. (2001). *The Invertebrates: A Synthesis*. Oxford, UK: Blackwell Science Ltd.

Bartlett, J., Maher, W., Ubrihien, R., Krikowa, F., Edge, K., Potts, J., and Taylor, A. (2020). Fitness of two bivalves Saccostrea glomerata and Ostrea angasi exposed to a metal contamination gradient in Lake Macquarie, NSW Australia: Integrating subcellular, energy metabolism and embryo development responses. *Ecological Indicators*, *110*(October 2019), 105869. https://doi.org/10.1016/j.ecolind.2019.105869

Bartlett, P. L., Long, P. M., Lugosi, G., & Tsigler, A. (2020). Benign overfitting in linear regression. *Proceedings of the National Academy of Sciences*, *117*(48), 30063-30070. https://doi.org/10.1073/pnas.1907378117

Başalan Över, S., Guven, C., Taskin, E., Çakmak, A., Piner Benli, P., and Sevgiler, Y. (2021). Effects of Different Ammonia Levels on Tribenuron Methyl Toxicity in Daphnia magna. *Archives of Environmental Contamination and Toxicology*, *81*(1), 46–57. https://doi.org/10.1007/s00244-021-00841-3

Bayne BL, Moore MN, Widdows J, Livingstone DR, Salkeld P. 1979. Measurement of the responses of individuals to environ- mental stress and pollution: Studies with bivalve molluscs. Philos Trans R Soc Lond B Biol Sci 286:563–581.

Bayne, B. L., Holland, D. L., Moore, M. N., Lowe, D. M., & Widdows, J. (1978). Further studies on the effects of stress in the adult on the eggs of *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom, 58*(4), 825-841. http://doi.org/10.1017/S0025315400056794

Beaumelle, L., Lamy, I., Cheviron, N., & Hedde, M. (2014). Is there a relationship between earthworm energy reserves and metal availability after exposure to field-contaminated

soils? *Environmental pollution, 191*, 182-189. http://dx.doi.org/10.1016/j.envpol.2014.04.021

BeMiller, J. N. (2017). Carbohydrate Analysis. In: Nielsen, S. S. (Ed), *Food Analysis* (5th ed., 333–360). Cham, Denmark: Springer. https://doi:10.1007/978-3-319-45776-5_19

Ben-Amotz, A., Fishler, R., & Schneller, A. (1987). Chemical composition of dietary species of marine unicellular algae and rotifers with emphasis on fatty acids. *Marine biology*, *95*(1), 31-36. https://doi.org/10.1007/BF00447482

Bertoldi, F. C., Sant'Anna, E., & Oliveira, J. L. B. (2008). Chlorophyll content and minerals profile in the microalgae *Chlorella vulgaris* cultivated in hydroponic wastewater. *Ciência Rural*, *38*(1), 54-58. https://doi.org/10.1590/S0103-84782008000100009

Blackmore, G. (1998). An overview of trace metal pollution in the coastal waters of Hong Kong. *Science of the Total Environment, 214*(1-3), 21-48. https://doi.org/10.1016/S0048-9697(98)00052-7

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. http://doi:10.1139/o59-099

Bloom, W. L., & Willcox, M. L. (1951). Determination of dextran in blood and urine. *Proceedings of the Society for Experimental Biology and Medicine*, *76*(1), 3-4. https://doi.org/10.3181/00379727-76-18372

Bodar, C. W. M., Van der Sluis, I., Van Montfort, J. C. P., Voogt, P. A., & Zandee, D. I. (1990). Cadmium resistance in *Daphnia magna. Aquatic Toxicology, 16*(1), 33-39. https://doi.org/10.1016/0166-445X(90)90075-Z

Bojadzija Savic, G., Bormans, M., Edwards, C., Lawton, L., Briand, E., and Wiegand, C. (2020). Cross talk: Two way allelopathic interactions between toxic Microcystis and Daphnia. *Harmful Algae*, *94*(November 2019), 101803. https://doi.org/10.1016/j.hal.2020.101803

Borowitzka, M. A., & Borowitzka, L. J. (1988). *Micro-algal biotechnology*. Cambridge University Press. United Kingdom.

Bossuyt, B., Escobar, Y., & Janssen, C. (2005). Multigeneration acclimation of *Daphnia magna* Straus to different bioavailable copper concentrations. *Ecotoxicology And Environmental Safety*, *61*(3), 327-336. doi: 10.1016/j.ecoenv.2005.03.004

Bouchnak, R., and Steinberg, C. E. W. (2013). Algal diets and natural xenobiotics impact energy allocation in cladocerans. I. Daphnia magna. *Limnologica*, *43*(6), 434–440. https://doi.org/10.1016/j.limno.2013.01.007

Bowen, J. L., & Valiela, I. (2001). The ecological effects of urbanization of coastal watersheds: historical increases in nitrogen loads and eutrophication of Waquoit Bay estuaries. *Canadian Journal of Fisheries and Aquatic Sciences, 58*(8), 1489-1500. https://doi.org/10.1139/f01-094

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Brodin, T., Fick, J., Jonsson, M., & Klaminder, J. (2013). Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science*, *339*(6121), 814-815. doi: 10.1126/science.1226850

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., & Jonsson, M. (2014). Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. *Philosophical Transactions of The Royal Society B: Biological Sciences*, *369*(1656), 20130580. doi: 10.1098/rstb.2013.0580

Brown, R. J. (2001). *Development of culture and toxicity testing methods for the freshwater copepod* Bryocamptus zschokkei. PhD thesis, University of Plymouth. https://pearl.plymouth.ac.uk/bitstream/handle/10026.1/2085/REBECCA%20JAYNE%20BROWN.PDF?sequence=1&isAllowed=y

Brummer, Y. and Cui, S. (2005). Understanding carbohydrate analysis. In: S.W. Cui (Ed.), *Food carbohydrates: Chemistry, physical properties, and applications* (pp. 67-104). Boca Raton, FL: CRC Press.

Brun, N. R., Fields, P. D., Horsfield, S., Mirbahai, L., Ebert, D., Colbourne, J. K., and Fent, K. (2019). Mixtures of Aluminum and Indium Induce More than Additive Phenotypic and Toxicogenomic Responses in Daphnia magna. *Environmental Science and Technology*, *53*(3), 1639–1649. https://doi.org/10.1021/acs.est.8b05457

Burba, A. (1999). The design of an experimental system of estimation methods for effects of heavy metals and their mixtures on daphnia magna. *Acta Zoologica Lituanica*, *9*(2), 21–29. https://doi.org/10.1080/13921657.1999.10512283

Burbank, S. E., & Snell, T. W. (1994). Rapid toxicity assessment using esterase biomarkers in *Brachionus calyciflorus* (Rotifera). *Environmental Toxicology and Water Quality*, *9*(3), 171-178. https://doi.org/10.1002/tox.2530090303

Byeon, E., Yoon, C., Lee, J. S., Lee, Y. H., Jeong, C. B., Lee, J. S., and Kang, H. M. (2020). Interspecific biotransformation and detoxification of arsenic compounds in marine rotifer and copepod. *Journal of Hazardous Materials*, *391*(January). https://doi.org/10.1016/j.jhazmat.2020.122196

Calabrese, E. J., & Baldwin, L. A. (2003). Inorganics and hormesis. *Critical reviews in Toxicology*, *33*(3-4), 215-304. https://doi.org/10.1080/713611040

Calow, P. (1991). Physiological costs of combating chemical toxicants: ecological implications. *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology*, *100*(1-2), 3-6. https://doi.org/10.1016/0742-8413(91)90110-f

Calow, P., & Sibly, R. M. (1990). A Physiological Basis of Population Processes: Ecotoxicological Implications. *Functional Ecology, 4*(3), 283-288. https://doi.org/10.2307/2389587

Cañizares-Villanueva, R. O., Martínez-Jerónimo, F., and Espinosa-Chávez, F. (2000). Acute toxicity to Daphnia magna of effluents containing Cd, Zn, and a mixture Cd-Zn, after metal removal by Chlorella vulgaris. *Environmental Toxicology*, *15*(3), 160–164. https://doi.org/10.1002/1522-7278(2000)15:3<160::AID-TOX2>3.0.CO;2-7

Canli, M. (2005). Dietary and water-borne Zn exposures affect energy reserves and subsequent Zn tolerance of Daphnia magna. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *141*(1), 110–116. https://doi.org/10.1016/j.cca.2005.05.007

Canli, M. (2006). Effects of Copper Pre-exposure Routes on the Energy Reserves and Subsequent Copper Toxicity in Daphnia magna. *Far Eastern Entomologist*, *165*(April), 16. https://doi.org/10.1002/tox

Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., & Levi, Y. (2004). Assessment of river contamination by estrogenic compounds in Paris area (France). *The Science of the Total Environment, 324*: 55–66. https://doi.org/10.1016/j.scitotenv.2003.10.035

Carić, M., Sanko-Njire, J., & Skaramuca, B. (1993). Dietary effects of different feeds on the biochemical composition of the rotifer (*Brachionus plicatilis* Müller). *Aquaculture*, *110*(2), 141-150. https://doi.org/10.1016/0044-8486(93)90268-4

Castell, L. L., & Mann, R. (1994). Optimal staining of lipids in bivalve larvae with Nile Red. *Aquaculture*, *119*(1), 89-100. https://doi.org/10.1016/0044-8486(94)90445-6

Cecchine, G., and Snell, T. W. (1999). Toxicant exposure increases threshold food levels in freshwater rotifer populations. *Environmental Toxicology*, *14*(5), 523–530. https://doi.org/10.1002/(SICI)1522-7278(199912)14:5<523::AID-TOX6>3.0.CO;2-2

Chabrol, E., & Charonnat, R. (1937). Determination of total lipids. *Press Medical, 45***,** 1713 – 1720.

Chapman, P. M. (1995). Ecotoxicology and pollution—key issues. *Marine Pollution Bulletin, 31*(4-12), 167-177. https://doi.org/10.1016/0025-326X(95)00101-R

Chapman, P. M., Wang, F., Janssen, C. R., Goulet, R. R., & Kamunde, C. N. (2003). Conducting ecological risk assessments of inorganic metals and metalloids: current

status. *Human and ecological risk assessment, 9*(4), 641-697. https://doi.org/10.1080/713610004

Charoy, C. (1995). Modification of the swimming behaviour of *Brachionus calyciflorus* (Pallas) according to food environment and individual nutritive state. *Hydrobiologia, 313/314*(1), 197-204. https://doi.org/10.1007/BF00025951

Chen, J., and Guo, R. (2015). The process-dependent impacts of dimethoate on the feeding behavior of rotifer. *Chemosphere*, *119*, 318–325. https://doi.org/10.1016/j.chemosphere.2014.06.073

Chen, J., Wang, Z., Li, G., & Guo, R. (2014). The swimming speed alteration of two freshwater rotifers *Brachionus calyciflorus* and *Asplanchna brightwelli* under dimethoate stress. *Chemosphere*, *95*, 256-260. https://doi.org/10.1016/j.chemosphere.2013.08.086

Chen, W., Sommerfeld, M., & Hu, Q. (2011). Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. *Bioresource technology*, *102*(1), 135-141. https://doi.org/10.1016/j.biortech.2010.06.076

Chen, W., Zhang, C., Song, L., Sommerfeld, M., & Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of microbiological methods*, *77*(1), 41-47. https://doi.org/10.1016/j.mimet.2009.01.001

Cheng, Y. S., Zheng, Y., & VanderGheynst, J. S. (2011). Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. *Lipids*, *46*(1), 95-103. http://doi.org/10.1007/s11745-010-3494-0

Chia, M. A., Lombardi, A. T., & Melão, M. D. (2013). Growth and biochemical composition of *Chlorella vulgaris* in different growth media. *Anais da Academia Brasileira de Ciências*, *85*(4), 1427-1438. https://doi.org/10.1590/0001-3765201393312

Clark Jr, L. C. (1956). Monitor and control of blood and tissue oxygen tensions. *ASAIO Journal*, *2*(1): 41–48.

Cole, T. A., Fok, A. K., Ueno, M. S., & Allen, R. D. (1990). Use of nile red as a rapid measure of lipid content in ciliates. *European journal of protistology*, *25*(4), 361-368. https://doi.org/10.1016/S0932-4739(11)80129-X

Compton, S. J., & Jones, C. G. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical biochemistry*, *151*(2), 369-374. https://doi.org/10.1016/0003-2697(85)90190-3

Congdon, R. W., Muth, G. W., & Splittgerber, A. G. (1993). The binding interaction of Coomassie blue with proteins. *Analytical biochemistry*, *213*(2), 407-413. https://doi.org/10.1006/abio.1993.1439

Connon, R., Hooper, H. L., Sibly, R. M., Lim, F. L., Heckmann, L. H., Moore, D. J., Watanabe, H., Soetaert, A., Cook, K., Maund, S. J., Hutchinson, T. H., Moggs, J., De Coen, W.,

Iguchi, T., and Callaghan, A. (2008). Linking molecular and population stress responses in Daphnia magna exposed to cadmium. *Environmental Science and Technology*, *42*(6), 2181–2188. https://doi.org/10.1021/es702469b

Connon, R.E., Geist, J., & Werner, I. (2012). Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensors*, *12*: 12741–12771. https://doi:10.3390/s120912741

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Del Borghi, M. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification*, *48*(6), 1146-1151. https://doi.org/10.1016/j.cep.2009.03.006

Cooksey, K. E., Guckert, J. B., Williams, S. A., & Callis, P. R. (1987). Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *Journal of microbiological methods*, *6*(6), 333-345. https://doi:10.1016/0167-7012(87)90019-4

Cooper, C. A., Tait, T., Gray, H., Cimprich, G., Santore, R. C., McGeer, J. C., ... & Smith, D. S. (2014). Influence of salinity and dissolved organic carbon on acute Cu toxicity to the rotifer Brachionus plicatilis. *Environmental science & technology*, *48*(2), 1213-1221. https://doi.org/10.1021/es402186w

Cortés, E. (2016). Perspectives on the intrinsic rate of population growth. *Methods in Ecology and Evolution*, *7*(10), 1136-1145. http://doi: 10.1111/2041-210X.12592

Crossland, N. O., & Hillaby, J. M. (1985). Fate and effects of 3, 4-dichloroaniline in the laboratory and in outdoor ponds: II. chronic toxicity to Daphnia SPP. and other invertebrates. *Environmental Toxicology and Chemistry: An International Journal*, *4*(4), 489-499. https://doi.org/10.1002/etc.5620040409

Cui, R., Kwak, J. Il, and An, Y. J. (2018). Comparative study of the sensitivity of Daphnia galeata and Daphnia magna to heavy metals. *Ecotoxicology and Environmental Safety*, *162*(June), 63–70. https://doi.org/10.1016/j.ecoenv.2018.06.054

Dahms, H. U., Hagiwara, A., & Lee, J. S. (2011). Ecotoxicology, ecophysiology, and mechanistic studies with rotifers. *Aquatic toxicology, 101*(1), 1-12. https://doi.org/10.1016/j.aquatox.2010.09.006

De Coen, W. M., & Janssen, C. R. (1997). The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *Journal of Aquatic Ecosystem Stress and Recovery*, *6*(1), 43-55. https://doi.org/10.1023/A:1008228517955

De Coen, W. M., & Janssen, C. R. (2003). The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental*

Toxicology and Chemistry: An International Journal, *22*(7), 1632-1641. https://doi.org/10.1002/etc.5620220727

De Coen, W. M., Janssen, C. R., & Persoone, G. (1995). Biochemical assessment of Cellular Energy Allocation in *Daphnia magna* exposed to toxic stress as an alternative to the conventional 'Scope for Growth 'methodology. In *Proceedings of the International Symposium on Biological Markers of Pollution* (pp. 163-170).

De Coen, W. M., Janssen, C. R., & Segner, H. (2001a). The use of biomarkers in *Daphnia magna* toxicity testing V. In vivo alterations in the carbohydrate metabolism of *Daphnia magna* exposed to sublethal concentrations of mercury and lindane. *Ecotoxicology and Environmental Safety, 48*(3), 223-234. https://doi.org/10.1006/eesa.2000.2009

De Coen, W.M., Janssen, C.R., & Giesy, J.P. (2001b). Biomarker applications in ecotoxicology: Bridging the gap between toxicology and ecology. In Persoone, G., Janssen, C., & De Coen, W. (eds.). *New Microbiotests for Routine Toxicity Screening and Biomonitoring*. Dordrecht: Kluwer Academic Publishers.

de Lima, D. P., dos Santos, E. D. A., Marques, M. R., Giannesi, G. C., Beatriz, A., Yonekawa, M. K., & Montanholi, A. D. S. (2018). Fungal bioremediation of pollutant aromatic amines. *Current Opinion in Green and Sustainable Chemistry, 11*, 34-44. https://doi.org/10.1016/j.cogsc.2018.03.012

DEFRA [Department for Environment Food & Rural Affairs]. (2014). *Water Framework Directive implementation in England and Wales: new and updated standards to protect the water environment.* Retrieved from https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/307788/river-basin-planning-standards.pdf

Delnat, V., Tran, T. T., Janssens, L., & Stoks, R. (2019). Daily temperature variation magnifies the toxicity of a mixture consisting of a chemical pesticide and a biopesticide in a vector mosquito. *Science of the Total Environment*, *659*, 33-40. https://doi.org/10.1016/j.scitotenv.2018.12.332

Depledge, M.H. & Fossi, M.C. (1994). The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology, 3*: 161–172.

Dietz, T.H. (1974). Body fluid composition and aerial oxygen consumption in the freshwater mussel, ligumia subrostrata (SAY): Effects of dehydration and anoxic stress. *The Biological Bulletin, 147*(3): 560–572. https://doi.org/10.2307/1540740

Dobsikova, R. (2004). Daphnia magna in ecotoxicology–a review. *Bull VURH Vodnany*, *40*, 58-81.

Dodds, W.K., & Whiles, M.R. (2020). *Freshwater Ecology: Concepts and Environmental Applications of Limnology*, *3rd ed*. London, UK: Academic Press. https://doi.org/10.1016/B978-0-12-813255-5.00027-2

Donker, M. H. (1992). Energy reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal-contaminated sites. *Functional Ecology*, *6* (4), 445–454. https://doi.org/10.2307/2389282

Doohan, M. (1973). International Association for Ecology An Energy Budget for Adult *Brachionus plicatilis* Muller (Rotatoria). In M. Doohan (Ed.), *Springer in cooperation with International Association for Ecology Stable*. URL: http://www.jstor.org/s. *13*(4), 351–362.

Drago, C., and Weithoff, G. (2021). Variable fitness response of two rotifer species exposed to microplastics particles: The role of food quantity and quality. *Toxics*, *9*(11), 1–13. https://doi.org/10.3390/toxics9110305

Duarte, B., Feijão, E., de Carvalho, R. C., Duarte, I. A., Silva, M., Matos, A. R., Cabrita, M. T., Novais, S. C., Lemos, M. F. L., Marques, J. C., Caçador, I., Reis-Santos, P., and Fonseca, V. F. (2020). Effects of propranolol on growth, lipids and energy metabolism and oxidative stress response of phaeodactylum tricornutum. *Biology*, *9*(12), 1–21. https://doi.org/10.3390/biology9120478

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. A. J. N. (1951). A colorimetric method for the determination of sugars. *Nature*, *168*(4265), 167-167. https://doi.org/10.1038/168167a0

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical chemistry*, *28*(3), 350-356. doi:10.1021/ac60111a017

Duffus, J. H. (2002). " Heavy metals" a meaningless term? (IUPAC Technical Report). *Pure and Applied Chemistry, 74*(5), 793-807. https://doi.org/10.1351/pac200274050793

Durou, C., Smith, B. D., Roméo, M., Rainbow, P. S., Mouneyrac, C., Mouloud, M., ... & Amiard-Triquet, C. (2007). From biomarkers to population responses in *Nereis diversicolor*: assessment of stress in estuarine ecosystems. *Ecotoxicology and Environmental Safety, 66*(3), 402-411. https://doi.org/10.1016/j.ecoenv.2006.02.016

Ebele, A. J., Abdallah, M. A. E., & Harrad, S. (2017). Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging contaminants, 3*(1), 1-16. https://doi.org/10.1016/j.emcon.2016.12.004

ECETOC [European Centre for Ecotoxicology and Toxicology of Chemicals]. (2003). *Aquatic Hazard Assessment II Databases*, Technical Report, no. 91. Brussels, Belgium: ECETOC. https://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-091.pd

Elkhatat, A. M., Soliman, M., Ismail, R., Ahmed, S., Abounahia, N., Mubashir, S., ... & Khraisheh, M. (2021). Recent trends of copper detection in water samples. *Bulletin of the National Research Centre*, *45*(1), 1-18. https://doi.org/10.1186/s42269-021-00677-w

Elnabarawy, M. T., Welter, A. N., & Robideau, R. R. (1986). Relative sensitivity of three daphnid species to selected organic and inorganic chemicals. *Environmental Toxicology and Chemistry, 5(4),* 393-398. https://doi.org/10.1002/etc.5620050409

El-Sayed, H. S., and El-Khodary, G. M. (2019). Impact of copper and cadmium on the nutritional value of the rotifer brachionus plicatilis and their effect on dicentrarchus labrax fish larvae. *Egyptian Journal of Aquatic Biology and Fisheries*, *23*(2), 491–503. https://doi.org/10.21608/ejabf.2019.33919

Elsey, D., Jameson, D., Raleigh, B., & Cooney, M. J. (2007). Fluorescent measurement of microalgal neutral lipids. *Journal of microbiological methods*, *68*(3), 639-642. http://10.1016/j.mimet.2006.11.008

Erk, M., Ivanković, D., & Strižak, Ž. (2011). Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker. *Marine pollution bulletin, 62*(5), 1124-1129. https://doi.org/10.1016/j.marpolbul.2011.02.056

Erk, M., Muyssen, B. T. A., Ghekiere, A., & Janssen, C. R. (2008). Metallothionein and cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* exposed to cadmium at different salinities. *Journal of Experimental Marine Biology and Ecology*, *357*(2), 172–180. https://doi.org/10.1016/j.jembe.2008.01.015

Eryalcin, K. (2018). Effects of Different Commercial Feeds and Enrichments on Biochemical Composition and Fatty Acid Profile of Rotifer (*Brachionus plicatilis*, Muller 1786) and *Artemia franciscana*. *Turkish Journal of Fisheries and Aquatic Sciences*, *18*, 81-90. https://doi.org/10.4194/1303-2712-v18_1_09

European Chemicals Bureau [ECB]. (2006). *3,4-DICHLOROANILINE (3,4-DCA): Summary Risk Assessment Report, CAS No: 95-76-1*. Retrieved from https://echa.europa.eu/documents/10162/29d15fce-5e74-4f9f-9c4b-befbf3ec5428

Fakhri, Y., Mohseni-Bandpei, A., Conti, G. O., Ferrante, M., Cristaldi, A., Jeihooni, A. K., ... & Baninameh, Z. (2018). Systematic review and health risk assessment of arsenic and lead in the fished shrimps from the Persian Gulf. *Food and chemical toxicology, 113*, 278-286. https://doi.org/10.1016/j.fct.2018.01.046

Feder, M. E. (1981). Effect of body size, trophic state, time of day, and experimental stress on oxygen consumption of anuran larvae: an experimental assessment and evaluation of the literature. *Comparative Biochemistry and Physiology Part A: Physiology, 70*(4), 497-508. https://doi.org/10.1016/0300-9629(81)92561-5

Feijão, E., Cruz de Carvalho, R., Duarte, I. A., Matos, A. R., Cabrita, M. T., Novais, S. C., Lemos, M. F. L., Caçador, I., Marques, J. C., Reis-Santos, P., Fonseca, V. F., and Duarte, B. (2020). Fluoxetine Arrests Growth of the Model Diatom Phaeodactylum tricornutum by Increasing Oxidative Stress and Altering Energetic and Lipid Metabolism. *Frontiers in Microbiology*, *11*(July), 1–17. https://doi.org/10.3389/fmicb.2020.01803

Feng, G. D., Zhang, F., Cheng, L. H., Xu, X. H., Zhang, L., & Chen, H. L. (2013). Evaluation of FT-IR and Nile Red methods for microalgal lipid characterization and biomass composition determination. *Bioresource technology*, *128*, 107-112. https://doi.org/10.1016/j.biortech.2012.09.123

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. *Science*, *246*(4926), 64-71. https://doi.org/10.1126/science.2675315

Fent, K. (2001). Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicology in Vitro, 15*(4-5), 477-488. https://doi.org/10.1016/S0887-2333(01)00053-4

Fernández-Casalderry, A., Ferrando, M. D., & Andreu-Moliner, E. (1992). Acute toxicity of several pesticides to rotifer (*Brachionus calyciflorus*). *Bulletin of environmental contamination and toxicology*, *48*(1), 14-17. https://doi.org/10.1007/BF00197477

Ferrando, M. D., & Andreu, E. (1993). Feeding behavior as an index of copper stress in *Daphnia magna* and *Brachionus calyciflorus*. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *106*(2), 327-331. https://doi.org/10.1016/0742-8413(93)90141-7

Ferrando, M. D., & Andreu-Moliner, E. (1991). Acute lethal toxicity of some pesticides to *Brachionus calyciflorus* and *Brachionus plicatilis*. *Bulletin of Environmental Contamination and Toxicology*, 47(3), 479–484. https://doi.org/10.1007/BF01702214

Ferrando, M. D., & Andreu-Moliner, E. (1992). Acute toxicity of toluene, hexane, xylene, and benzene to the rotifers *Brachionus calyciflorus* and *Brachionus plicatilis*. *Bulletin of Environmental Contamination and Toxicology*, *49*(2). 266-271.https://doi:10.1007/BF00191765.

Ferrando, M. D., Andreu-Moliner, E., & Fernández-Casalderrey, A. (1992). Relative sensitivity of *Daphnia magna* and *Brachionus calyciflorus* to five pesticides. *Journal of Environmental Science & Health Part B*, *27*(5), 511-522. DOI: 10.1080/03601239209372798

Ferrando, M. D., Janssen, C. R., Andreu, E., & Persoone, G. (1993a). Ecotoxicological studies with the freshwater rotifer *Brachionus calyciflorus* II. An assessment of the chronic toxicity of lindane and 3, 4-dichloroaniline using life tables. *Hydrobiologia*, *255*(1), 33-40.

Ferrando, M. D., Janssen, C. R., Andreu, E., & Persoone, G. (1993b). Ecotoxicological Studies with the Freshwater Rotifer *Brachionus calyciflorus* III. The Effects of Chemicals on the Feeding-Behavior. *Ecotoxicology and Environmental Safety26*(1), 1-9. https://doi.org/10.1006/eesa.1993.1035.

Ferrando, M. D., Sancho, E., & Andreu-Moliner, E. (1996). Chronic toxicity of fenitrothion to an algae (Nannochloris oculata), a rotifer (Brachionus calyciflorus), and the cladoceran (Daphnia magna). *Ecotoxicology and Environmental Safety*, *35*(2), 112-120. https://doi.org/10.1006/eesa.1996.0090

Ferreira, N. G. C., Cardoso, D. N., Morgado, R., Soares, A. M. V. M., and Loureiro, S. (2015). Long-term exposure of the isopod Porcellionides pruinosus to nickel: Costs in the energy budget and detoxification enzymes. *Chemosphere*, *135*, 354–362. https://doi.org/10.1016/j.chemosphere.2015.04.025

Ferreira, N. G. C., Morgado, R. G., Amaro, A., Machado, A. L., Soares, A. M. V. M., and Loureiro, S. (2016). The effects of temperature, soil moisture and UV radiation on biomarkers and energy reserves of the isopod Porcellionides pruinosus. *Applied Soil Ecology*, *107*, 224–236. https://doi.org/10.1016/j.apsoil.2016.06.007

Ferreira, N. G. C., Morgado, R., Santos, M. J. G., Soares, A. M. V. M., and Loureiro, S. (2015). Biomarkers and energy reserves in the isopod Porcellionides pruinosus: The effects of long-term exposure to dimethoate. *Science of the Total Environment*, *502*, 91–102. https://doi.org/10.1016/j.scitotenv.2014.08.062

Filenko, O. F., and Samoylova, T. A. (2008). Effect of heavy metals on samples and model population of rotifers Brachionus plicatilis muller at different salinity. *Moscow University Biological Sciences Bulletin*, *63*(2), 80–83. https://doi.org/10.3103/s0096392508020077

Finlay, B. J., Span, A., & Ochsenbein-Gattlen, C. (1983). Influence of physiological state on indices of respiration rate in protozoa. *Comparative Biochemistry and Physiology Part A: Physiology, 74*(2), 211-219. https://doi.org/10.1016/0300-9629(83)90590-X

Fisher, R., & Fox, D. R. (2023). Introducing the No Significant Effect Concentration (NSEC). *Environmental Toxicology and Chemistry*, *42*(9), 2019-2028. https://doi.org/10.1002/etc.5610

Forbes, V.E, & Calow, P. (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicology?. *Environmental Toxicology And Chemistry*, *18*(7), 1544-1556. doi: 10.1002/etc.5620180729

Fox, D. R. (2011). Response to Landis and Chapman (2011). *Integrated Environmental Assessment and Management, 8*(1), 4-4. https://doi.org/10.1002/ieam.1264

Fraser, D.L. (2017). *Evaluation of the neurotoxicity of pentachlorophenol and its active metabolites on SH-SY5Y neuroblastoma cells.* PhD thesis. University of Pretoria, South Africa.

Frausto daSilva, J. J. R., & Williams, R. J. P. (1993). *The biological chemistry of the elements: the inorganic chemistry of life*. Oxford, UK: Clarendon Press.

Friedenauer, S., & Berlet, H. H. (1989). Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Analytical biochemistry*, *178*(2), 263-268. https://doi.org/10.1016/0003-2697(89)90636-2

Frolov, A. V., & Pankov, S. L. (1992). The effect of starvation on the biochemical composition of the rotifer *Brachionus plicatilis*. *Journal of the marine biological association of the United Kingdom, 72*(2), 343-356. https://doi.org/10.1017/S0025315400037747

Frolov, A. V., Pankov, S. L., Geradze, K. N., Pankova, S. A., & Spektorova, L. V. (1991). Influence of the biochemical composition of food on the biochemical composition of the rotifer *Brachionus plicatilis*. *Aquaculture, 97*(2-3), 181-202. https://doi.org/10.1016/0044-8486(91)90264-8

Gad, S. C. & Pham, T. (2014). Sodium Pentachlorophenate. In: P. Wexler, M. Abdollahi, A. De Peyster, S. C. Gad, H. Greim, S. Harper, …& T. J. Wiegand (Eds). *Encyclopedia of Toxicology* (pp 338-340). London, UK: Elsevier.

Gaete, H., Olivares, Y., and Escobar, C. (2013). Assessment of the effect of a commercial formulation of methomyl on reproduction of Daphnia obtusa Kürz (1874). *Latin American Journal of Aquatic Research*, *41*(5), 979–984. https://doi.org/10.3856/vol41-issue5-fulltext-16

Galkovskaya, G. A. (1995). Oxygen consumption rate in rotifers. *Hydrobiologia*, *313–314*(1), 147–156. https://doi.org/10.1007/BF00025944

Gama-Flores, J. L., Huidobro-Salas, M. E., Sarma, S. S. S., & Nandini, S. (2020). Population responses and fatty acid profiles of Brachionus calyciflorus (Rotifera) in relation to different thermal regimes. *Journal of Thermal Biology*, *94*, 102752. https://doi.org/10.1016/j.jtherbio.2020.102752

Gama-Flores, J. L., Sarma, S. S. S., & Nandini, S. (2006). Effect of cadmium level and exposure time on the competition between zooplankton species *Moina macrocopa* (Cladocera) and *Brachionus calyciflorus* (Rotifera). *Journal of Environmental Science and Health, Part A*, *41*(6), 1057-1070. https://doi.org/10.1080/10934520600620287

Gama-Flores, J.L., Castellanos-Paez, M.E., Sarma, S.S.S. & Nandini, S. (2007b). Life table demography of *Ceriodaphnia dubia* (Cladocera) exposed to copper at different levels and periods. *Journal of Environmental Biology*, *28*(3), 691-696.

Gama-Flores, Jose Luis, Castellanos-Paez, M. E., Sarma, S. S. S., and Nandini, S. (2007a). Effect of pulsed exposure to heavy metals (copper and cadmium) on some population variables of Brachionus calyciflorus Pallas (Rotifera: Brachionidae: Monogononta). *Hydrobiologia*, *593*(1), 201–208. https://doi.org/10.1007/s10750-007-9042-0

Gama-Flores, José Luis, Sarma, S. S. S., and Nandini, S. (2005). Interaction among copper toxicity, temperature and salinity on the population dynamics of Brachionus rotundiformis (Rotifera). *Hydrobiologia*, *546*(1), 559–568. https://doi.org/10.1007/s10750-005-4300-5

Gao, M., Zhang, Z., Lv, M., Song, W., and Lv, Y. (2018). Toxic effects of nanomaterial-adsorbed cadmium on Daphnia magna. *Ecotoxicology and Environmental Safety*, *148*(399), 261–268. https://doi.org/10.1016/j.ecoenv.2017.10.038

García-García, G., Picazo-Paez, E. A., Nandini, S., & Sarma, S. S. S. (2007). Combined effects of sediment and lead (PbCl2) on the demography of *Brachionus patulus* (Rotifera: Brachionidae). *Hydrobiologia, 593*(1), 209-218. https://doi.org/10.1007/s10750-007-9039-8

Gates, R. E. (1991). Elimination of interfering substances in the presence of detergent in the bicinchoninic acid protein assay. *Analytical biochemistry*, *196*(2), 290-295. https://doi.org/10.1016/0003-2697(91)90468-9

Genicot, G., Leroy, J. L. M. R., Van Soom, A., & Donnay, I. (2005). The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. *Theriogenology*, *63*(4), 1181-1194. https://doi.org/10.1016/j.theriogenology.2004.06.006

Gentile, J. H., Gentile, S. M., Hairston, N. G., & Sullivan, B. K. (1982). The use of life-tables for evaluating the chronic toxicity of pollutants to *Mysidopsis bahia*. In: M.D. Morgan (Ed). *Ecology of Mysidacea* (pp. 179-187). Dordrecht, The Netherlands: Springer. https://doi.org/10.1007/978-94-009-8012-9_19

Gharaei, A., Karimi, M., Mirdar Harijani, J., Miri, M., & Faggio, C. (2020). Population growth of *Brachionus calyciflorus* affected by deltamethrin and imidacloprid insecticides. *Iranian Journal of Fisheries Sciences*, *19*(2), 588–601. https://doi.org/10.22092/ijfs.2018.117180

Giacomazzi, S., & Cochet, N. (2004). Environmental impact of diuron transformation: a review. *Chemosphere*, *56*(11), 1021-1032. https://doi.org/10.1016/j.chemosphere.2004.04.061

Giesy, J. P., & Graney, R. L. (1989). Recent developments in and intercomparisons of acute and chronic bioassays and bioindicators. *Environmental Bioassay Techniques and their Application,* 21-60. https://doi.org/10.1007/978-94-009-1896-2_3

Gilbert, J. J. (2020). Variation in the life cycle of monogonont rotifers: commitment to sex and emergence from diapause. *Freshwater biology*, *65*(4), 786-810. https://doi.org/10.1111/fwb.13440

Girling, A. E., Tattersfield, L., Mitchell, G. C., Crossland, N. O., Pascoe, D., Blockwell, S. J., ... & Jüttner, I. (2000). Derivation of predicted no-effect concentrations for lindane, 3, 4-dichloroaniline, atrazine, and copper. *Ecotoxicology and Environmental Safety*, *46*(2), 148-162. https://doi.org/10.1006/eesa.1999.1901

Glazier, D. S., and Calow, P. (1992). Energy allocation rules in Daphnia magna: clonal and age differences in the effects of food limitation. *Oecologia*, *90*(4), 540–549. https://doi.org/10.1007/BF01875448

Gnaiger, E. (1983). Appendix C. Calculation of energetic and biochemical equivalents of respiration oxygen consumption. In F. Ga (Ed.), *Polarographic Oxygen Sensores.* Springer Verlag, Berlin Heidelberg (pp. 337-345).

Goldberg, E.D., Bertine, K.K., 2000. Beyond the mussel watch—new directions for monitoring marine pollution. Science of the Total Environment 247, 165–174.

Goldstein, I. F. (1981). The use of biological markers in studies of health effects of pollutants: Foreword to the proceedings of the second workshop on health surveillance around Point Sources of Pollution. *Environmental Research, 25*(1), 236-240. https://doi.org/10.1016/0013-9351(81)90092-X

Gomes, S. I. L., Scott-Fordsmand, J. J., and Amorim, M. J. B. (2015a). Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): The effect of Cu and Ag. *International Journal of Environmental Research and Public Health*, *12*(6), 6858–6878. https://doi.org/10.3390/ijerph120606858

Gomes, S. I. L., Soares, A. M. V. M., and Amorim, M. J. B. (2015b). Changes in cellular energy allocation in Enchytraeus crypticus exposed to copper and silver—linkage to effects at higher level (reproduction). *Environmental Science and Pollution Research*, *22*(18), 14241–14247. https://doi.org/10.1007/s11356-015-4630-4

Gomes, S. I. L., Soares, A. M. V. M., and Amorim, M. J. B. (2016b). Effect of Cu and Ni on cellular energy allocation in Enchytraeus albidus. *Ecotoxicology*, *25*(8), 1523–1530. https://doi.org/10.1007/s10646-016-1706-y

Gomes, S. I., Soares, A. M., & Amorim, M. J. (2016a). Energy reserves and cellular energy allocation studies: Should food supply be provided?. *Geoderma*, *284*, 51-56. https://doi.org/10.1016/j.geoderma.2016.08.016

Gómez, A. (2005). Molecular ecology of rotifers: from population differentiation to speciation. In: A. Herzig, R.D. Gulati, C.D. Jersabek, & L. May. (Eds). *Rotifera X. Developments in Hydrobiology, vol 181*. Dordrecht, The Netherlands: Springer. https://doi.org/10.1007/1-4020-4408-9_7

Gómez, M., Torres, S., & Hernández-León, S. (1996). Modification of the electron transport system (ETS) method for routine measurements of respiratory rates of zooplankton. *South African Journal of Marine Science*, *17*(1), 15-20. https://doi.org/10.2989/025776196784158446

Goodchild, C. G., Simpson, A. M., Minghetti, M., and DuRant, S. E. (2019). Bioenergetics-adverse outcome pathway: Linking organismal and suborganismal energetic endpoints to adverse outcomes. *Environmental Toxicology and Chemistry*, *38*(1), 27–45. https://doi.org/10.1002/etc.4280

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry*, *177*, 751-766.

Granada, L., Lemos, M. F., Bossier, P., & Novais, S. C. (2023). Swimming behaviour as an alternative endpoint to assess differences in abiotic stress sensitivities between strains of Brachionus koreanus (Rotifera: Monogononta). *Environmental Science and Pollution Research*, *30*(19), 56137-56147. https://doi.org/10.1007/s11356-023-26190-3

Green, J. W., Springer, T. A., & Staveley, J. P. (2013). The drive to ban the NOEC/LOEC in favor of ECx is misguided and misinformed. *Integrated environmental assessment and management*, *9*(1), 12-16. https://doi.org/10.1002/ieam.1367

Greenspan, P., & Fowler, S. D. (1985). Spectrofluorometric studies of the lipid probe, nile red. *Journal of lipid research*, *26*(7), 781-789. https://doi.org/10.1016/S0022-2275(20)34307-8

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *The Journal of cell biology*, *100*(3), 965-973. https://doi.org/10.1083/jcb.100.3.965

Gribble, K. E., and Mark Welch, D. B. (2013). Life-span extension by caloric restriction is determined by type and level of food reduction and by reproductive mode in Brachionus manjavacas (Rotifera). *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *68*(4), 349–358. https://doi.org/10.1093/gerona/gls170

Guan, R., and Wang, W. X. (2006). Comparison between two clones of Daphnia magna: Effects of multigenerational cadmium exposure on toxicity, individual fitness, and biokinetics. *Aquatic Toxicology*, *76*(3–4), 217–229. https://doi.org/10.1016/j.aquatox.2005.10.003

Gunderson, A. R., Armstrong, E. J., & Stillman, J. H. (2016). Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. *Annual review of marine science*, *8*, 3

Guo, R., Ren, X., and Ren, H. (2012a). A new method for analysis of the toxicity of organophosphorus pesticide, dimethoate on rotifer based on response surface methodology. *Journal of Hazardous Materials*, *237–238*, 270–276. https://doi.org/10.1016/j.jhazmat.2012.08.041

Guo, R., Ren, X., and Ren, H. (2012b). Effects of dimethoate on rotifer Brachionus calyciflorus using multigeneration toxicity tests. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *47*(9), 883–890. https://doi.org/10.1080/03601234.2012.693869

Gust, K. A., Lotufo, G. R., Barker, N. D., Ji, Q., and May, L. K. (2021). Mode of action evaluation for reduced reproduction in Daphnia pulex exposed to the insensitive munition, 1-methyl-3-nitro-1-nitroguanidine (MeNQ). *Ecotoxicology*, *30*(6), 1203–1215. https://doi.org/10.1007/s10646-021-02447-w

Gutierrez, M. F., Gagneten, A. M., & Paggi, J. C. (2010). Copper and chromium alter life cycle variables and the equiproportional development of the freshwater copepod *Notodiaptomus conifer* (SARS). *Water, Air, & Soil Pollution, 213*(1), 275-286. https://doi.org/10.1007/s11270-010-0383-3

Guzmán-Agüero, J. E., Nieves-Soto, M., Hurtado, M. Á., Piña-Valdez, P., & Garza-Aguirre, M. D. C. (2013). Feeding physiology and scope for growth of the oyster Crassostrea corteziensis (Hertlein, 1951) acclimated to different conditions of temperature and salinity. *Aquaculture International*, *21*(2), 283-297. https://doi.org/10.1007/s10499-012-9550-4

Habib, M. A. B., Yusoff, F. M., Phang, S. M., & Mohamed, S. (2003). Growth and nutritional values of *Moina micrura* fed on *Chlorella vulgaris* grown in digested palm oil mill effluent. *Asian Fisheries Science*, *16*(2), 107-119. http://doi.org/10.33997/j.afs.2003.16.2.002

Hagger, J. A., Jones, M. B., Leonard, D. P., Owen, R., & Galloway, T. S. (2006). Biomarkers and integrated environmental risk assessment: are there more questions than answers?. *Integrated Environmental Assessment and Management: An International Journal, 2*(4), 312-329. https://doi.org/10.1002/ieam.5630020403

Hall, R. J., Driscoll, C. T., Likens, G. E., & Pratt, J. M. (1985). Physical, chemical, and biological consequences of episodic aluminum additions to a stream 1. *Limnology and Oceanography, 30*(1), 212-220. https://doi.org/10.4319/lo.1985.30.1.0212

Han, C., Kim, H. J., Lee, J. S., Sakakura, Y., and Hagiwara, A. (2021). Species-specific effects of iron on temperate and tropical marine rotifers in reproduction, lipid and ROS metabolisms. *Chemosphere*, *277*, 130317. https://doi.org/10.1016/j.chemosphere.2021.130317

Han, Y., Wen, Q., Chen, Z., & Li, P. (2011). Review of methods used for microalgal lipid-content analysis. *Energy Procedia*, *12*, 944-950. https://doi.org/10.1016/j.egypro.2011.10.124

Hanazato, T. (2001). Pesticide effects on freshwater zooplankton: an ecological perspective. *Environmental pollution*, *112*(1), 1-10. https://doi.org/10.1016/S0269-7491(00)00110-X

Hansul, S., Fettweis, A., Smolders, E., and De Schamphelaere, K. (2021). Interactive Metal Mixture Toxicity to Daphnia magna Populations as an Emergent Property in a Dynamic Energy Budget Individual-Based Model. *Environmental Toxicology and Chemistry*, *40*(11), 3034–3048. https://doi.org/10.1002/etc.5176

Hardie, D. G., & Hawley, S. A. (2001). AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays, 23*(12), 1112-1119. https://doi.org/10.1002/bies.10009

Harmon, S.M. (2009). Effects of pollution on freshwater organisms. *Water Environment Research*, *81*(10), 2030-2069. DOI:10.2175/106143009X12445568400692

Harmon, S.M., & Wiley, F.E. (2010). Effects of pollution on freshwater organisms. *Water Environment Research, 82*(10), 1945-2000. https://doi.org/10.2175/106143010X12756668802139

Harmon, S.M., & Wiley, F.E. (2012). Effects of pollution on freshwater organisms. *Water Environment Research, 84*(10), 1691-1736. https://doi.org/10.2175/106143012X13407275695715

Haskins, S. D., Kelly, D. G., & Weir, R. D. (2010). Novel pressurized solvent extraction vessels for the analysis of polychlorinated biphenyl congeners in avian whole blood. *Analytica chimica acta*, *677*(1), 19-23. https://doi.org/10.1016/j.aca.2009.12.036

Havens, K. E., & Hanazato, T. (1993). Zooplankton community responses to chemical stressors: a comparison of results from acidification and pesticide contamination research. *Environmental pollution*, *82*(3), 277-288. https://doi.org/10.1016/0269-7491(93)90130-G

He, Z., Qu, Y., Jin, W., Zhou, X., Han, W., Song, K., ... & Jiang, G. (2022). Effect of microwave on biomass growth and oxygen production of microalgae Chlorella pyrenoidosa cultured in real wastewater. *Process Safety and Environmental Protection*, *161*, 22-33. https://doi.org/10.1016/j.psep.2022.03.015

Herawati, N., Suzuki, S., Hayashi, K., Rivai, I. F., & Koyama, H. (2000). Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia, and China by soil type. *Bulletin of Environmental Contamination and Toxicology, 64*(1), 33-39. DOI : 10.1007/s001289910006

Hernández-Flores, S., Santos-Medrano, G. E., Rubio-Franchini, I., & Rico-Martínez, R. (2020). Evaluation of bioconcentration and toxicity of five metals in the freshwater rotifer *Euchlanis dilatata* Ehrenberg, 1832. *Environmental Science and Pollution Research*, *27*(12), 14058-14069. https://doi.org/10.1007/s11356-020-07958-3

Hernández-Ruiz, E., Alvarado-Flores, J., Rubio-Franchini, I., Ventura-Juárez, J., & Rico-Martínez, R. (2016). Adverse effects and bioconcentration of chromium in two

freshwater rotifer species. *Chemosphere*, *158*, 107-115. https://doi.org/10.1016/j.chemosphere.2016.05.067

Herrera, A., Packard, T., Santana, A., & Gómez, M. (2011). Effect of starvation and feeding on respiratory metabolism in *Leptomysis lingvura* (GO Sars, 1866). *Journal of Experimental Marine Biology and Ecology, 409*(1-2), 154-159. https://doi.org/10.1016/j.jembe.2011.08.016

Herzig, A. (1987). The analysis of planktonic rotifer populations: A plea for long-term investigations. In: L. May, R. Wallace, A. Herzig (Eds). *Rotifer Symposium IV* (pp. 163-180). Dordrecht, The Netherlands: Springer. https://doi.org/10.1007/978-94-009-4059-8_22

Hickey, C. W., & Vickers, M. L. (1992). Comparison of the sensitivity to heavy metals and pentachlorophenol of the mayflies *Deleatidium* spp. and the cladoceran *Daphnia magna*. *New Zealand Journal of Marine and Freshwater Research, 26*(1), 87-93. https://doi.org/10.1080/00288330.1992.9516504

Hirata H. & Yamasaki S. (1987) Effect of feeding on the respiration rate of the rotifer *Brachionus plicatilis*. In *Rotifer Symposium IV* (pp. 283-288). Springer, Dordrecht.

Howarth, R. W., & Marino, R. (2006). Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. *Limnology and oceanography, 51*(1-2), 364-376.

Huang, L., Xi, Y., Zha, C., and Wen, X. (2013). Responses in the population growth and reproduction of freshwater rotifer brachionus calyciflorus to four organochlorine pesticides. *Annales de Limnologie*, *49*(1), 79–85. https://doi.org/10.1051/limn/2013038

Illman, A. M., Scragg, A. H., & Shales, S. W. (2000). Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. *Enzyme and microbial technology*, *27*(8), 631-635. https://doi.org/10.1016/S0141-0229(00)00266-0

Isani, G., Serra, R., Cattani, O., Cortesi, P., & Carpene, E. (1997). Adenylate energy charge and metallothionein as stress indices in *Mytilus galloprovincialis* exposed to cadmium and anoxia. *Journal of the Marine Biological Association of the United Kingdom, 77*(4), 1187-1197. https://doi.org/10.1017/S0025315400038704

Jager, T. (2012). Bad habits die hard: the NOEC's persistence reflects poorly on ecotoxicology. *Environmental Toxicology and Chemistry*, *31*(2), 228-229. https://doi.org/10.1002/etc.746

James, C. M., & Abu-Rezeq, T. S. (1988). Effect of different cell densities of *Chlorella capsulata* and a marine Chlorella sp. for feeding the rotifer *Brachionus plicatilis*. *Aquaculture*, *69*(1-2), 43-56. https://doi.org/10.1016/0044-8486(88)90185-8

Jan, A. T., Azam, M., Siddiqui, K., Ali, A., Choi, I., & Haq, Q. M. R. (2015). Heavy metals and human health: mechanistic insight into toxicity and counter defense system of

antioxidants. *International journal of molecular sciences*, *16*(12), 29592-29630. https://doi.org/10.3390/ijms161226183

Janssen, C. R., Persoone, G., & Snell, T. W. (1994). Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus*. *Aquatic Toxicology, 28*(3-4), 243-258. https://doi.org/10.1016/0166-445X(94)90036-1

Jayaraj, R., Megha, P., & Sreedev, P. (2016). Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. *Interdiscip Toxicol, 9* (3–4): 90–100. https://doi.org/10.1515/intox-2016-0012

Jeeja, P. K., Imelda, J., & Paulraj, R. (2011). Nutritional composition of rotifer (*Brachionus plicatilis* Muller) cultured using selected natural diets. *Indian Journal of Fisheries*, *58*(2), 59-65.

Jensen, T. C., Anderson, T. R., Daufresne, M., & Hessen, D. O. (2006). Does excess carbon affect respiration of the rotifer *Brachionus calyciflorus* Pallas? *Freshwater Biology, 51*(12), 2320-2333. https://doi.org/10.1111/j.1365-2427.2006.01653.x

Jeon, J., Kretschmann, A., Escher, B. I., and Hollender, J. (2013). Characterization of acetylcholinesterase inhibition and energy allocation in Daphnia magna exposed to carbaryl. *Ecotoxicology and Environmental Safety*, *98*, 28–35. https://doi.org/10.1016/j.ecoenv.2013.09.033

Jeong, C. B., Kang, H. M., Lee, M. C., Byeon, E., Park, H. G., and Lee, J. S. (2019a). Effects of polluted seawater on oxidative stress, mortality, and reproductive parameters in the marine rotifer Brachionus koreanus and the marine copepod Tigriopus japonicus. *Aquatic Toxicology*, *208*(December 2018), 39–46. https://doi.org/10.1016/j.aquatox.2018.12.019

Jeong, C. B., Lee, Y. H., Park, J. C., Kang, H. M., Hagiwara, A., and Lee, J. S. (2019b). Effects of metal-polluted seawater on life parameters and the induction of oxidative stress in the marine rotifer Brachionus koreanus. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *225*(July), 108576. https://doi.org/10.1016/j.cbpc.2019.108576

Jeong, C. B., Won, E. J., Kang, H. M., Lee, M. C., Hwang, D. S., Hwang, U. K., Zhou, B., Souissi, S., Lee, S. J., and Lee, J. S. (2016). Microplastic Size-Dependent Toxicity, Oxidative Stress Induction, and p-JNK and p-p38 Activation in the Monogonont Rotifer (Brachionus koreanus). *Environmental Science and Technology*, *50*(16), 8849–8857. https://doi.org/10.1021/acs.est.6b01441

Jiang, Y., Wang, Y., Sun, T., Lu, K., Zhao, X., Zhang, Z., Lv, M., Liu, C., and Zhou, B. (2021). Depicting an energetic chain involved in physiological responses of blue mussel Mytilus edulis coping with BDE-47 exposure. *Chemosphere*, *269*, 128736. https://doi.org/10.1016/j.chemosphere.2020.128736

Johnson, K. R., Ellis, G., & Toothill, C. (1977). The sulfophosphovanillin reaction for serum lipids: a reappraisal. *Clinical Chemistry*, *23*(9), 1669-1678. https://doi.org/10.1093/clinchem/23.9.1669

Jones, J. I., Murphy, J. F., Anthony, S. G., Arnold, A., Blackburn, J. H., Duerdoth, C. P., ... & Skates, J. (2017). Do agri-environment schemes result in improved water quality?. *Journal of Applied Ecology*, *54*(2), 537-546. https://doi.org/10.1111/1365-2664.12780

Jones, J., Manning, S., Montoya, M., Keller, K., & Poenie, M. (2012). Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *Journal of the American Oil Chemists' Society*, *89*(8), 1371-1381. https://doi.org/10.1007/s11746-012-2044-8

Jones, K. C., & De Voogt, P. (1999). Persistent organic pollutants (POPs): state of the science. *Environmental Pollution, 100*(1-3), 209-221. https://doi.org/10.1016/S0269-7491(99)00098-6

Juárez-Franco, M. F., Sarma, S. S. S., & Nandini, S. (2007). Effect of cadmium and zinc on the population growth of *Brachionus havanaensis* (Rotifera: Brachionidae). *Journal of Environmental Science and Health, Part A*, *42*(10), 1489-1493. https://doi.org/10.1080/10934520701480938

Juchelka, C. M., & Snell, T. W. (1994). Rapid toxicity assessment using rotifer ingestion rate. *Archives of Environmental Contamination and Toxicology*, *26*(4), 549-554. https://doi.org/10.1007/BF00214160

Kaneko, G., Yoshinaga, T., Yanagawa, Y., Kinoshita, S., Tsukamoto, K., & Watabe, S. (2005). Molecular characterization of Mn-superoxide dismutase and gene expression studies in dietary restricted *Brachionus plicatilis* rotifers. In: A. Herzig, R.D. Gulati, C.D. Jersabek, & L. May (Eds). *Developments in Hydrobiology, vol 181, Rotifera X: Rotifer Research: Trends, New Tools and Recent Advances* (pp. 117 – 123). Dordrecht, Netherlands: Springer. https://doi.org/10.1007/1-4020-4408-9_10

Kang, H. M., Byeon, E., Jeong, H., Lee, Y., Hwang, U. K., Jeong, C. B., Yoon, C., and Lee, J. S. (2021). Arsenic exposure combined with nano- or microplastic induces different effects in the marine rotifer Brachionus plicatilis. *Aquatic Toxicology*, *233*(September 2020). https://doi.org/10.1016/j.aquatox.2021.105772

Kang, H. M., Lee, J. S., Lee, Y. H., Kim, M. S., Park, H. G., Jeong, C. B., & Lee, J. S. (2019). Body size-dependent interspecific tolerance to cadmium and their molecular responses in the marine rotifer Brachionus spp. *Aquatic Toxicology*, *206*, 195-202. https://doi.org/10.1016/j.aquatox.2018.10.020

Ke, L. X., Xi, Y. L., Zha, C. W., & Dong, L. L. (2009). Effects of three organophosphorus pesticides on population growth and sexual reproduction of rotifer *Brachionus calyciflorus* Pallas. *Acta Ecologica Sinica*, *29*(3), 182-185. https://doi.org/10.1016/j.chnaes.2009.07.008

Keilin, D. (1966). *The history of cell respiration and cytochrome.* Cambridge, U.K: Cambridge University Press.

Kiedrzyńska, E., Kiedrzyński, M., Urbaniak, M., Magnuszewski, A., Skłodowski, M., Wyrwicka, A., & Zalewski, M. (2014). Point sources of nutrient pollution in the lowland river catchment in the context of the Baltic Sea eutrophication. *Ecological engineering, 70*, 337-348. https://doi.org/10.1016/j.ecoleng.2014.06.010

Kim, H. Y., Yu, S., Jeong, T. Y., & Kim, S. D. (2014). Relationship between trans-generational effects of tetracycline on Daphnia magna at the physiological and whole organism level. *Environmental Pollution*, *191*, 111–118. https://doi.org/10.1016/j.envpol.2014.04.022

Kim, Haeyeon, Kim, J. S., Kim, P. J., Won, E. J., and Lee, Y. M. (2018). Response of antioxidant enzymes to Cd and Pb exposure in water flea Daphnia magna: Differential metal and age — Specific patterns. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *209*(March), 28–36. https://doi.org/10.1016/j.cbpc.2018.03.010

Kim, Haeyeon, Yim, B., Bae, C., and Lee, Y. M. (2017). Acute toxicity and antioxidant responses in the water flea Daphnia magna to xenobiotics (cadmium, lead, mercury, bisphenol A, and 4-nonylphenol). *Toxicology and Environmental Health Sciences*, *9*(1), 41–49. https://doi.org/10.1007/s13530-017-0302-8

Kim, Hokyun, Lim, B., Kim, B. Do, & Lee, Y. M. (2016). Effects of heavy metals on transcription and enzyme activity of Na+/K+-ATPase in the monogonont rotifer, Brachionus koreanus. *Toxicology and Environmental Health Sciences*, *8*(2), 128–134. https://doi.org/10.1007/s13530-016-0270-4

Kim, K. T., Lee, Y. G., and Kim, S. D. (2006). Combined toxicity of copper and phenol derivatives to Daphnia magna: Effect of complexation reaction. *Environment International*, *32*(4), 487–492. https://doi.org/10.1016/j.envint.2005.11.002

Kimura, K., Yamaoka, M., & Kamisaka, Y. (2004). Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *Journal of microbiological methods*, *56*(3), 331-338. https://doi.org/10.1016/j.mimet.2003.10.018

King, F. D., & Packard, T. T. (1975). Respiration and the activity of the respiratory electron transport system in marine zooplankton. *Limnology and Oceanography, 20*(5), 849-854. https://doi.org/10.4319/lo.1975.20.5.0849

Kirk, K. L. (1997). Life-History Responses to Variable Environments: Starvation and Reproduction in Planktonic Rotifers. *Ecology*, *78*(2), 434. https://doi.org/10.2307/2266019

Kirk, K. L., Ellis, J., & Taylor, J. (1999). Physiological responses to variable environments: storage and respiration in starving rotifers. *Freshwater Biology*, *42*(4), 637-644. https://doi.org/10.1046/j.1365-2427.1999.00502.x

Kiss, A., & Virág, D. (2009). Interpretation and modelling of environmental behaviour of diverse pesticides by revealing photodecomposition mechanisms. *Microchemical journal, 92*(2), 119-122. https://doi.org/10.1016/j.microc.2008.12.002

Klimek, B., Fiałkowska, E., Kocerba-Soroka, W., Fyda, J., Sobczyk, M., and Pajdak-Stós, A. (2013). The toxicity of selected trace metals to lecane inermis rotifers isolated from activated sludge. *Bulletin of Environmental Contamination and Toxicology*, *91*(3), 330–333. https://doi.org/10.1007/s00128-013-1062-z

Knops, M., Altenburger, R., and Segner, H. (2001). Alterations of physiological energetics, growth and reproduction of Daphnia magna under toxicant stress. *Aquatic Toxicology*, *53*(2), 79–90. https://doi.org/10.1016/S0166-445X(00)00170-3

Kolkmeier, M. A., and Brooks, B. W. (2013). Sublethal silver and NaCl toxicity in Daphnia magna: A comparative study of standardized chronic endpoints and progeny phototaxis. *Ecotoxicology*, *22*(4), 693–706. https://doi.org/10.1007/s10646-013-1061-1

Kooijman SALM, Bedaux JJM (1996) Analysis of toxicity tests on Daphnia survival and reproduction. Water Res 30:1711– 1723

Kooijman, S. A. L. M., & Metz, J. A. J. (1984). On the dynamics of chemically stressed populations: the deduction of population consequences from effects on individuals. *Ecotoxicology and Environmental Safety, 8*(3), 254-274. https://doi.org/10.1016/0147-6513(84)90029-0

Kooijman, S.A.L.M. (2010). *Dynamic energy and mass budgets in biological systems*. Cambridge, U.K: Cambridge University Press. https://doi.org/10.1017/CBO9780511565403

Kostopoulou, V., Carmona, M. J., & Divanach, P. (2012). The rotifer *Brachionus plicatilis*: an emerging bio-tool for numerous applications. *Journal of Biological Research, 17*, 97 - 112.

Kou, Z., Bei, S., Sun, J., & Pan, J. (2013). Fluorescent measurement of lipid content in the model organism *Chlamydomonas reinhardtii*. *Journal of applied phycology*, *25*(6), 1633-1641. https://doi.org/10.1007/s10811-013-0011-x

Kruger, N. J. (2009). The Bradford method for protein quantitation. In: Walker, J.M. (Ed), *The protein protocols handbook* (pp. 17-24). Totowa, NJ: Humana Press. http://doi.org/10.1007/978-1-59745-198-7_4

Lalande, M., & Pinel-Alloul, B. (1986). Acute toxicity of cadmium, copper, mercury and zinc to *Tropocyclops prasinus mexicanus* (cyclopoida, copepoda) from three quebec lakes. *Environmental Toxicology and Chemistry, 5*(1), 95-102. https://doi.org/10.1002/etc.5620050113

Landis, W. G., & Chapman, P. M. (2011). Well past time to stop using NOELs and LOELs. *Integrated Environmental Assessment and Management*, *7*(4), vi-viii. https://doi.org/10.1002/ieam.249

Lari, E., Gauthier, P., Mohaddes, E., and Pyle, G. G. (2017). Interactive toxicity of Ni, Zn, Cu, and Cd on Daphnia magna at lethal and sub-lethal concentrations. *Journal of Hazardous Materials*, *334*, 21–28. https://doi.org/10.1016/j.jhazmat.2017.03.060

Laurentin, A., & Edwards, C. A. (2003). A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Analytical biochemistry*, *315*(1), 143-145. http://doi.org/10.1016/s0003-2697(02)00704-2

Le Parc, A., Lee, H., Chen, K., & Barile, D. (2014). Rapid quantification of functional carbohydrates in food products. *Food and Nutrition Sciences*, *5*(1), 71-78. http://doi.org/10.4236/fns.2014.51010

Lee, M. C., Fonseca, E., Park, J. C., Yoon, D. S., Choi, H., Kim, M., Han, J., Cho, H. S., Shin, K. H., Santos, M. L., Jung, J. H., Castro, L. F. C., and Lee, J. S. (2019). Tributyltin affects retinoid x receptor-mediated lipid metabolism in the marine rotifer brachionus koreanus. *Environmental Science and Technology*, *53*(13), 7830–7839. https://doi.org/10.1021/acs.est.9b01359

Lee, S. J., Yoon, B. D., & Oh, H. M. (1998). Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology techniques*, *12*(7), 553-556. https://doi.org/10.1023/A:1008811716448

Lee, Y. C., Whang, L. M., Ngo, M. H., Chen, T. H., and Cheng, H. H. (2016). Acute toxicity assessment of TFT-LCD wastewater using Daphnia similis and Cyprinus carpio. *Process Safety and Environmental Protection*, *104*(1), 499–506. https://doi.org/10.1016/j.psep.2016.03.003

Lee, Y. H., Park, J. C., Hwang, U. K., Lee, J. S., & Han, J. (2018). Adverse effects of the insecticides chlordecone and fipronil on population growth and expression of the entire cytochrome P450 (CYP) genes in the freshwater rotifer *Brachionus calyciflorus* and the marine rotifer *Brachionus plicatilis*. *Aquatic Toxicology*, *202*, 181-187. https://doi.org/10.1016/j.aquatox.2018.07.014

Leung, K. M. Y., & Furness, R. W. (2001). Survival, growth, metallothionein and glycogen levels of *Nucella lapillus* (L.) exposed to sub-chronic cadmium stress: the influence of nutritional state and prey type. *Marine Environmental Research, 52*(2), 173-194. https://doi.org/10.1016/S0141-1136(00)00271-3

Li, A. J., Leung, P. T. Y., Bao, V. W. W., Yi, A. X. L., and Leung, K. M. Y. (2014). Temperature-dependent toxicities of four common chemical pollutants to the marine medaka fish, copepod and rotifer. *Ecotoxicology*, *23*(8), 1564–1573. https://doi.org/10.1007/s10646-014-1297-4

Li, L., Sillanpää, M., and Schultz, E. (2017). Influence of titanium dioxide nanoparticles on

cadmium and lead bioaccumulations and toxicities to Daphnia magna. *Journal of Nanoparticle Research*, *19*(6). https://doi.org/10.1007/s11051-017-3916-5

Li, M., Czymmek, K. J., and Huang, C. P. (2011). Responses of Ceriodaphnia dubia to TiO2 and Al2O3 nanoparticles: A dynamic nano-toxicity assessment of energy budget distribution. *Journal of Hazardous Materials*, *187*(1–3), 502–508. https://doi.org/10.1016/j.jhazmat.2011.01.061

Li, X. D., Wang, X. Y., Xu, M. E., Jiang, Y., Yan, T., & Wang, X. C. (2020). Progress on the usage of the rotifer Brachionus plicatilis in marine ecotoxicology: a review. *Aquatic Toxicology*, *229*, 105678. https://doi.org/10.1016/j.aquatox.2020.105678

Liang, Y., Chen, X., Lu, X., Jin, S., Min, Y., & Yang, J. (2017). Combined effects of microcystin and nitrite on the growth, lipid peroxidation, and antioxidant responses of the freshwater rotifer *Brachionus calyciflorus*. *Aquatic Toxicology*, *192*, 78–88. https://doi.org/10.1016/j.aquatox.2017.09.013.

Liang, Y., Shao, L., Jiang, Q., and Yang, J. (2020). Changes in the life-cycle parameters and glutathione-related antioxidant defense system of rotifer Brachionus calyciflorus under the combined stress of microcystin-LR and ammonia. *Aquatic Ecology*, *54*(1), 243–256. https://doi.org/10.1007/s10452-019-09739-8

Liang, Y., Zhou, Y., Wang, Y., Liu, R., Qi, J., Lin, Y., Zhang, T., and Jiang, Q. (2021). Use of physiological activities to estimate the population growth of rotifer (Brachionus calyciflorus) under the stress of toxic Microcystis and nitrite. *Chemosphere*, *285*(219), 131419. https://doi.org/10.1016/j.chemosphere.2021.131419

Liber, K., & Solomon, K. R. (1994). Acute and chronic toxicity of 2, 3, 4, 6-tetrachlorophenol and pentachlorophenol to *Daphnia* and rotifers. *Archives of Environmental Contamination and Toxicology, 26*(2), 212-221. https://doi.org/10.1007/BF00224807

Lie, O., Haaland, H., Hemre, G. I., Maage, A., Lied, E., Rosenlund, G., ... & Olsen, Y. (1997). Nutritional composition of rotifers following a change in diet from yeast and emulsified oil to microalgae. *Aquaculture International*, *5*(5), 427-438. https://doi.org/10.1023/A:1018384711958

Liess, M., Foit, K., Knillmann, S., Schäfer, R. B., & Liess, H. D. (2016). Predicting the synergy of multiple stress effects. *Scientific reports*, *6*(1), 1-8. https://doi.org/10.1038/srep32965.

Lim, E. S., Le, T. H., Lee, S. H., Lee, S. K., Kim, Y. H., and Min, J. (2009). Daphnia magna specific responses to As(III), As(V), and Cd. *Toxicology and Environmental Health Sciences*, *1*(3), 196–199. https://doi.org/10.1007/BF03216486

Linko, R. R., Kaitaranta, J. K., & Vuorela, R. (1985). Comparison of the fatty acids in Baltic herring and available plankton feed. *Comparative Biochemistry and physiology. B,*

Comparative Biochemistry, *82*(4), 699-705. https://doi.org/10.1016/0305-0491(85)90511-5

Liu, X. (2014). *Quantitative Determination of Lipid Analysis Using Nile Red Fluorometry.* PhD thesis, Université d'Ottawa/University of Ottawa, Canada. http://dx.doi.org/10.20381/ruor-3823

Liu, X., Wang, Z., Yu, J., Lü, B., Ma, L., & Chen, Z. (2013). Improved fluorescence spectrometric determination of lipid content in *Botryococcus braunii*. *Chinese Journal of Biotechnology*, *29*(3), 382-391.

Liu, Yanhua, Chen, M., Ma, Y., Guo, R., Yan, Z., and Chen, J. (2022). Reproductive stimulation and energy allocation variation of BDE-47 and its derivatives on Daphnia magna. *Chemosphere*, *288*(P1), 132492. https://doi.org/10.1016/j.chemosphere.2021.132492

Liu, Yingying, Fan, W., Xu, Z., Peng, W., and Luo, S. (2018). Comparative effects of graphene and graphene oxide on copper toxicity to Daphnia magna: Role of surface oxygenic functional groups. *Environmental Pollution*, *236*(2018), 962–970. https://doi.org/10.1016/j.envpol.2017.10.082

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry. 193*(1), 265–275. http://dx.doi.org/10.1016/S0021-9258(19)52451-6

Lubbers, D. W. & Opitz, N. (1985). *Method and arrangement for measuring the concentration of gases* (U.S. Patent No. RE31, 879). U.S. Patent and Trademark Office. https://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=%2Fnetahtml%2FPTO%2Fsrchnum.htm&r=1&f=G&l=50&s1=RE31879.PN.&OS=PN/RE31879&RS=PN/RE31879

Luna-Andrade, A., Aguilar-Duran, R., Nandini, S., & Sarma, S. S. S. (2002). Combined effects of copper and microalgal (Tetraselmis suecica) concentrations on the population growth of Brachionus plicatulids' Müller (Rotifera). *Water, Air, and Soil Pollution*, *141*, 143-153. https://doi.org/10.1023/A:1021346512560

Lürling, M. (2006). Investigation of a rotifer (Brachionus calyciflorus) - Green alga (Scenedesmus pectinatus) interaction under non- and nutrient-limited conditions. *Annales de Limnologie*, *42*(1), 9–17. https://doi.org/10.1051/limn/2006007

Lv, X., Huang, B., Zhu, X., Jiang, Y., Chen, B., Tao, Y., Zhou, J., and Cai, Z. (2017). Mechanisms underlying the acute toxicity of fullerene to Daphnia magna: Energy acquisition restriction and oxidative stress. *Water Research*, *123*, 696–703. https://doi.org/10.1016/j.watres.2017.07.023

Mahasneh, I. A. (1997). Production of single cell protein from five strains of the microalga *Chlorella spp*. (Chlorophyta). *Cytobios*, *90*(362-363), 153-161.

Maldonado, F., Packard, T.T., & Gómez, M. (2012). Understanding tetrazolium reduction and the importance of substrates in measuring respiratory electron transport activity. *Journal of Experimental Marine Biology and Ecology*, *434–435,*110 –118. https://doi.org/10.1016/j.jembe.2012.08.010

Marcial, H.S., Hagiwara, A. & Snell, T.W. (2005). Effect of Some Pesticides on Reproduction of Rotifer *Brachionus plicatilis* Müller. *Hydrobiologia 546(1)*, 569–575. https://doi-org.uea.idm.oclc.org/10.1007/s10750-005-4302-3

Martinez-Haro, M., Beiras, R., Bellas, J., Capela, R., Coelho, J. P., Lopes, I., ... & Marques, J. C. (2015). A review on the ecological quality status assessment in aquatic systems using community based indicators and ecotoxicological tools: what might be the added value of their combination?. *Ecological Indicators, 48*, 8-16. http://dx.doi.org/10.1016/j.ecolind.2014.07.024

Martínez-Jerónimo, F., Arzate-Cárdenas, M., and Ortiz-Butrón, R. (2013). Linking sub-individual and population level toxicity effects in Daphnia schoedleri (Cladocera: Anomopoda) exposed to sublethal concentrations of the pesticide α-cypermethrin. *Ecotoxicology*, *22*(6), 985–995. https://doi.org/10.1007/s10646-013-1077-6

Mashjoor, S., Yousefzadi, M., Zolgharnain, H., Kamrani, E., and Alishahi, M. (2018). Organic and inorganic nano-Fe3O4: Alga Ulva flexuosa-based synthesis, antimicrobial effects and acute toxicity to briny water rotifer Brachionus rotundiformis. *Environmental Pollution*, *237*, 50–64. https://doi.org/10.1016/j.envpol.2018.02.036

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical biochemistry*, *339*(1), 69-72. https://doi.org/10.1016/j.ab.2004.12.001

Melatunan, S., Calosi, P., Rundle, S. D., Moody, A. J., & Widdicombe, S. (2011). Exposure to elevated temperature and pCO2 reduces respiration rate and energy status in the periwinkle *Littorina littorea*. *Physiological and Biochemical Zoology, 84*(6), 583-594. https://doi.org/10.1086/662680

Meng, S., Tran, T. T., Van Dinh, K., Delnat, V., & Stoks, R. (2022). Acute warming increases pesticide toxicity more than transgenerational warming by reducing the energy budget. *Science of the Total Environment*, *805*, 150373. https://doi.org/10.1016/j.scitotenv.2021.150373

Mercurio, P. (2016). *Herbicide persistence and toxicity in the tropical marine environment.* PhD thesis, University of Queensland, Australia.

Mihaich, E. M., Friederich, U., Caspers, N., Hall, A. T., Klecka, G. M., Dimond, S. S., ... & Hentges, S. G. (2009). Acute and chronic toxicity testing of bisphenol A with aquatic invertebrates and plants. *Ecotoxicology and environmental safety*, *72*(5), 1392-1399. https://doi.org/10.1016/j.ecoenv.2009.02.005

Milam, C. D., Bouldin, J. L., Farris, J. L., Schulz, R. A. L. F., Moore, M. T., Bennett, E. R., ... & Smith Jr, S. (2004). Evaluating acute toxicity of methyl parathion application in constructed wetland mesocosms. *Environmental Toxicology: An International Journal*, *19*(5), 471-479. https://doi.org/10.1002/tox.20052

Minkoff, G., Lubzens, E., & Kahan, D. (1983). Environmental factors affecting hatching of rotifer (Brachionus plicatilis) resting eggs. *Hydrobiologia*, *104*, 61-69. https://doi.org/10.1007/BF00045953

Minnesota Pollution Control Agency [MPCA]. (2008). *Nutrients: Phosphorus, Nitrogen Sources, Impact on Water Quality: A General Overview.* Available at: https://www.pca.state.mn.us/sites/default/files/wq-iw3-22.pdf (Accessed:14 DEC 2021).

Moiseenko, T. I. (2008). Aquatic ecotoxicology: theoretical principles and practical application. *Water Resources, 35*(5), 530-541. https://doi.org/10.1134/S0097807808050047

Montúfar-Meléndez, A. I., Sánchez-Ortíz, J. R., Sarma, S. S. S., & Nandini, S. (2007). Combined effects of temperature and lead concentration on the competition between the rotifers *Brachionus havanaensis* and *Brachionus rubens* (Rotifera: Brachionidae). *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *42*(3), 393–398. https://doi.org/10.1080/10934520601144717

Moolman, L. J. H. J., Van Vuren, J. H. J., & Wepener, V. (2007). Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. *Ecotoxicology and Environmental Safety, 68*(3), 443-450. https://doi.org/10.1016/j.ecoenv.2006.12.017

Moreira, R. A., da Silva Mansano, A., Rocha, O., & Daam, M. A. (2016). The use of rotifers as test species in the aquatic effect assessment of pesticides in the tropics. *Hydrobiologia, 773*(1), 1-9. https://doi.org/10.1007/s10750-016-2675-0

Moreira, R. A., de Araujo, G. S., Silva, A. R. R. G., Daam, M. A., Rocha, O., Soares, A. M. V. M., and Loureiro, S. (2020). Effects of abamectin-based and difenoconazole-based formulations and their mixtures in Daphnia magna: a multiple endpoint approach. *Ecotoxicology*, *29*(9), 1486–1499. https://doi.org/10.1007/s10646-020-02218-z

Mount, D. I., & Norberg, U. T. (1984). A seven-day life cycle cladoceran toxicity test. *Environmental Toxicology and Chemistry, 3*(3), 425-434. https://doi.org/10.1002/etc.5620030307

Munari Versteeg, D. J., Stalmans, M., Dyer, S. D., & Janssen, C. (1997). *Ceriodaphnia* and *Daphnia*: A comparison of their sensitivity to xenobiotics and utility as a test species. *Chemosphere, 34*(4), 869-892. https://doi.org/10.1016/S0045-6535(97)00014-3

Murano, H., Matsuzaki, K., Shiraishi, H., and Wakabayashi, M. (2007). Effects of heavy metals in river waters in Japan on immobility and mortality of Daphnia magna and Oryzias latipes larvae. *Fisheries Science*, *73*(5), 1078–1086. https://doi.org/10.1111/j.1444-2906.2007.01439.x

Muyssen, B. T. A., & Janssen, C. R. (2001). Multigeneration zinc acclimation and tolerance in Daphnia magna: Implications for water-quality guidelines and ecological risk assessment. *Environmental Toxicology and Chemistry, 20*(9), 2053-2060. https://doi.org/10.1002/etc.5620200926

Muyssen, Brita T.A., Janssen, C. R., and Bossuyt, B. T. A. (2002). Tolerance and acclimation to zinc of field-collected Daphnia magna populations. *Aquatic Toxicology*, *56*(2), 69–79. https://doi.org/10.1016/S0166-445X(01)00206-5

Muyssen, Brita T.A., Messiaen, M., and Janssen, C. R. (2010). Combined cadmium and temperature acclimation in Daphnia magna: Physiological and sub-cellular effects. *Ecotoxicology and Environmental Safety*, *73*(5), 735–742. https://doi.org/10.1016/j.ecoenv.2009.12.018

Nakamatsu, Y., & Tanaka, T. (2004). Food resource use of hyperparasitoid *Trichomalopsis apanteloctena* (Hymenoptera: Pteromalidae), an idiobiotic ectoparasitoid. *Annals of the Entomological Society of America*, *97*(5), 994-999. https://doi.org/10.1603/0013-8746(2004)097

Nandini, S., & Rao, T. R. (1998). Somatic and population growth in selected cladoceran and rotifer species offered the cyanobacterium it *Microcystis aeruginosa* as food. *Aquatic Ecology*, *31*(3), 283-298. https://doi.org/10.1023/A:1009940127959

Nandini, S., Chaparro-Herrera, D. D. J., Cardenas-Arriola, S. L., & Sarma, S. S. S. (2007a). Population growth of *Brachionus macracanthus* (Rotifera) in relation to cadmium toxicity: Influence of algal (*Chlorella vulgaris*) density. *Journal of Environmental Science and Health, Part A*, *42*(10), 1467-1472. https://doi.org/10.1080/10934520701480896

Nandini, S., Sarma, S. S. S., Amador-López, R. J., and Bolaños-Muñioz, S. (2007b). Population growth and body size in five rotifer species in response to variable food concentration. *Journal of Freshwater Ecology*, *22*(1), 1–10. https://doi.org/10.1080/02705060.2007.9664139

Nelson, D.L. & Cox, M.M. (2008). *Lehninger principles of biochemistry*. New York: W.H, Freeman.

Nguyen, N. D., Matsuura, T., Kato, Y., and Watanabe, H. (2021). DNMT3.1 controls trade-offs between growth, reproduction, and life span under starved conditions in Daphnia magna. *Scientific Reports*, *11*(1), 1–12. https://doi.org/10.1038/s41598-021-86578-4

Nhu, C.V. (2004). A Comparison of yield and quality of the rotifer (*Brachionus plicatilis*-L. Strain) fed different diets under aquaculture conditions, Vietnam. *Asian Fisheries Science*, *17*, 357-363. https://doi.org/10.33997/j.afs.2004.17.4.008

Nielsen, S. S. (2017). Total Carbohydrate by Phenol-Sulfuric Acid Method. In: Nielsen, S. S. (Ed), *Food Analysis Laboratory Manual* (3rd ed.,137–141). Cham, Denmark: Springer. https://doi:10.1007/978-3-319-44127-6_14

Niemuth, N. J., Curtis, B. J., Laudadio, E. D., Sostare, E., Bennett, E. A., Neureuther, N. J., Mohaimani, A. A., Schmoldt, A., Ostovich, E. D., Viant, M. R., Hamers, R. J., and Klaper, R. D. (2021). Energy Starvation in Daphnia magna from Exposure to a Lithium Cobalt Oxide Nanomaterial. *Chemical Research in Toxicology*, *34*(11), 2287–2297. https://doi.org/10.1021/acs.chemrestox.1c00189

Nikinmaa, M. (2014). *An introduction to aquatic toxicology*. Waltham, MA: Academic Press.

Nisbet, R. M., Gurney, W. S. C., Murdoch, W. W., & McCauley, E. (1989). Structured population models: a tool for linking effects at individual and population level. *Biological Journal of the Linnean Society*, *37*(1-2), 79-99. https://doi.org/10.1111/j.1095-8312.1989.tb02006.x

Novais, S. C., & Amorim, M. J. (2013). Changes in cellular energy allocation in *Enchytraeus albidus* when exposed to dimethoate, atrazine, and carbendazim. *Environmental Toxicology and Chemistry, 32*(12), 2800-2807. http://dx.doi.org/10.1002/etc.2368

Novais, S. C., De Coen, W., & Amorim, M. J. (2012). Transcriptional responses in *Enchytraeus albidus* (Oligochaeta): comparison between cadmium and zinc exposure and linkage to reproduction effects. *Environmental toxicology and chemistry*, *31*(10), 2289-2299. https://doi.org/10.1002/etc.1946

Novais, S. C., Soares, A. M. V. M., De Coen, W., and Amorim, M. J. B. (2013). Exposure of Enchytraeus albidus to Cd and Zn - Changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic and reproductive effects. *Chemosphere*, *90*(3), 1305–1309. https://doi.org/10.1016/j.chemosphere.2012.09.030

Oetjen, G. W. (2000). DISTILLATION| Freeze-Drying. *Encyclopedia of Separation Science*, 1023-1034. https://doi.org/10.1016/B0-12-226770-2/05591-5.

Olsen, G. H., Carroll, J. L., Sva, E., and Camus, L. (2008). Cellular energy allocation in the Arctic sea ice amphipod Gammarus wilkitzkii exposed to the water soluble fractions of oil. *Marine Environmental Research*, *66*(1), 213–214. https://doi.org/10.1016/j.marenvres.2008.02.063

Olsen, G. H., Sva, E., Carroll, J. L., Camus, L., De Coen, W., Smolders, R., Øveraas, H., and Hylland, K. (2007). Alterations in the energy budget of Arctic benthic species exposed to oil-related compounds. *Aquatic Toxicology*, *83*(2), 85–92. https://doi.org/10.1016/j.aquatox.2007.03.012

Olsen, R. E., & Henderson, R. J. (1989). The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. *Journal of Experimental Marine Biology and Ecology*, *129*(2), 189-197. https://doi.org/10.1016/0022-0981(89)90056-7

Olson, B. J. S. C., & Markwell, J. (2007). Assays for Determination of Protein Concentration. *Current Protocols in Pharmacology*, *38*(1), A.3A.1–A.3A.29. http://doi:10.1002/0471141755.pha03as38

Opitz, N. (1986). O2 optodes for analyzing micro blood samples using thin sensor layers with small O2 capacities and special reflection properties for optical decoupling of sensor and sample. In: I. S. Longmuir (Ed.). *Advances in Experimental Medicine and Biology (AEMB, volume 200)*: *Oxygen Transport to Tissue VIII* (pp. 137–142). New York, USA: Springer. https://doi.org/10.1007/978-1-4684-5188-7

Osma, N., Aristizabal, M., Fernández-Urruzola, I., Packard, T. T., & Gómez, M. (2016a). Influence of starvation on respiratory metabolism and pyridine nucleotide levels in the marine dinoflagellate *Oxyrrhis marina*. *Protist*, *167*(2), 136-147. https://doi.org/10.1016/j.protis.2016.01.002

Osma, N., Fernández-Urruzola, I., Packard, T. T., Postel, L., Gómez, M., & Pollehne, F. (2014). Short-term patterns of vertical particle flux in northern Benguela: a comparison between sinking POC and respiratory carbon consumption. *Journal of Marine Systems, 140*, Part B, 150-162. https://doi.org/10.1016/j.jmarsys.2014.01.004

Osma, N., Maldonado, F., Fernández-Urruzola, I., Packard, T. T., & Gómez, M. (2016b). Variability of respiration and pyridine nucleotides concentration in oceanic zooplankton. *Journal of Plankton Research*, *38*(3), 537-550. https://doi.org/10.1093/plankt/fbw001

Owens, T. G., & King, F. D. (1975). The measurement of respiratory electron-transport-system activity in marine zooplankton. *Marine Biology, 30*(1), 27-36. https://doi.org/10.1007/BF00393750

Pablos, M. V., García-Hortigüela, P., and Fernández, C. (2015). Acute and chronic toxicity of emerging contaminants, alone or in combination, in Chlorella vulgaris and Daphnia magna. *Environmental Science and Pollution Research*, *22*(7), 5417–5424. https://doi.org/10.1007/s11356-015-4119-1

Pacheco, A., Martins, A., and Guilhermino, L. (2018). Toxicological interactions induced by chronic exposure to gold nanoparticles and microplastics mixtures in Daphnia magna. *Science of the Total Environment*, *628–629*, 474–483. https://doi.org/10.1016/j.scitotenv.2018.02.081

Packard, T. T. (1985). Measurement of electron transport activity of microplankton. *Advances in aquatic microbiology*, *3*, 207-261.

Packard, T. T., & Christensen, J. P. (2004). Respiration and vertical carbon flux in the Gulf of Maine water column. *Journal of Marine Research*, *62*(1), 93-115. https://doi.org/10.1357/00222400460744636

Packard, T. T., & Gómez, M. (2008). Exploring a first-principles-based model for zooplankton respiration. *ICES Journal of Marine Science*, *65*(3), 371-378. https://doi.org/10.1093/icesjms/fsn003

Packard, T. T., & Gómez, M. (2013). Modeling vertical carbon flux from zooplankton respiration. *Progress in Oceanography, 110*, 59-68. https://doi.org/10.1016/j.pocean.2013.01.003

Packard, T. T., Berdalet, E., Blasco, D., Roy, S. O., St-Amand, L., Lagacé, B., ... & Gagnó, J. P. (1996). Oxygen consumption in the marine bacterium *Pseudomonas nautica* predicted from ETS activity and bisubstrate enzyme kinetics. *Journal of Plankton Research*, *18*(10), 1819-1835. https://doi.org/10.1093/plankt/18.10.1819

Packard, T. T., Denis, M., Rodier, M., & Garfield, P. (1988). Deep-ocean metabolic CO2 production: calculations from ETS activity. *Deep Sea Research Part A. Oceanographic Research Papers, 35*(3), 371-382. https://doi.org/10.1016/0198-0149(88)90016-7

Packard, T. T., Osma, N., Fernández-Urruzola, I., Codispoti, L. A., Christensen, J. P., & Gómez, M. (2015). Peruvian upwelling plankton respiration: calculations of carbon flux, nutrient retention efficiency, and heterotrophic energy production. *Biogeosciences, 12*(9), 2641-2654. https://doi.org/10.5194/bg-12-2641-2015

Packard, T.T., Healy, M.L., & Richards, F.A. (1971). Vertical distribution of the activity of the respiratory electron transport system in marine plankton. *Limnology and Oceanography, 16(1), 60-70*. https://doi.org/10.4319/lo.1971.16.1.0060

Padrilah, S. N., Sabullah, M. K., Shukor, M. Y. A., Yasid, N. A., Shamaan, N. A., & Ahmad, S. A. (2018). Toxicity effects of fish histopathology on copper accumulation. *Pertanika Journal of Tropical Agricultural Science*, *41*(2), 519-540.

Pan, L., Xi, Y. L., Li, Z. C., Zhao, Q. Q., & Hu, Z. J. (2016). Effects of mercury on the life table demography of the rotifer *Brachionus calyciflorus* under different algal food (*Scenedesmus obliquus*) densities. *Acta Ecologica Sinica*, *36*(3), 218-223. https://doi.org/10.1016/j.chnaes.2016.04.009

Park, J. C., Han, J., Lee, M. C., Kang, H. M., Jeong, C. B., Hwang, D. S., Wang, M., and Lee, J. S. (2017). Adverse effects of BDE-47 on life cycle parameters, antioxidant system, and activation of MAPK signaling pathway in the rotifer Brachionus koreanus. *Aquatic Toxicology*, *186*, 105–112. https://doi.org/10.1016/j.aquatox.2017.02.025

Park, J. C., Lee, M. C., Yoon, D. S., Han, J., Kim, M., Hwang, U. K., ... & Lee, J. S. (2018). Effects of bisphenol A and its analogs bisphenol F and S on life parameters, antioxidant system, and response of defensome in the marine rotifer *Brachionus koreanus*. *Aquatic toxicology*, *199*, 21-29. https://doi.org/10.1016/j.aquatox.2018.03.024

Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., Fiumano, V., & Isidori, M. (2014). Acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. *Chemosphere*, *115*(1), 59-66. https://doi.org/10.1016/j.chemosphere.2014.01.013

Patrício Silva, A. L., Amorim, M. J. B., & Holmstrup, M. (2015). Adaptations of enchytraeids to single and combined effects of physical and chemical stressors. *Environmental Reviews*, *24*(1), 1–12. https://doi.org/10.1139/er-2015-0048.

Patrício-Silva, A. L., and Amorim, M. J. B. (2016). Effect of freeze-thaw cycles and 4-nonylphenol on cellular energy allocation in the freeze-tolerant enchytraeid Enchytraeus albidus. *Environmental Science and Pollution Research*, *23*(4), 3548–3555. https://doi.org/10.1007/s11356-015-5593-1

Payne, S. A., Johnson, B. A., & Otto, R. S. (1999). Proximate composition of some north-eastern Pacific forage fish species. *Fisheries Oceanography*, *8*(3), 159-177. https://doi.org/10.1046/j.1365-2419.1999.00097.x

Pedersen, F., Bjornestad, E., Vulpius, T., & Rasmussen, H.B. (1998). *Immobilisation test of aniline compounds with the crustacean* Daphnia magna, Project No. 303587. Copenhagen, Denmark: Danish Environmental Protection Agency.

Pedersen, S. A., Håkedal, O. J., Salaberria, I., Tagliati, A., Gustavson, L. M., Jenssen, B. M., ... & Altin, D. (2014). Multigenerational exposure to ocean acidification during food limitation reveals consequences for copepod scope for growth and vital rates. *Environmental Science & Technology, 48*(20), 12275-12284. http://pubs.acs.org/doi/abs/10.1021/es501581j

Peeters, F., Li, J., Straile, D., Rothhaupt, K. O., and Vijverberg, J. (2010). Influence of low and decreasing food levels on Daphnia-algal interactions: Numerical experiments with a new dynamic energy budget model. *Ecological Modelling*, *221*(22), 2642–2655. https://doi.org/10.1016/j.ecolmodel.2010.08.006

Pérez-Legaspi, I. A., & Rico-Martínez, R. (2001). Acute toxicity tests on three species of the genus Lecane (Rotifera: Monogononta). *Hydrobiologia*, *446*(1), 375-381. https://doi.org/10.1023/A:1017531712808

Pérez-Legaspi, I. A., Quintanar, J. L., & Rico-Martínez, R. (2012). Comparing toxicity endpoints on *Lecane quadridentata* (Rotifera: Monogononta) exposed to two

anticholinesterases pesticides. *Environmental Toxicology*, *27*(9), 518-525. https://doi.org/10.1002/tox.20668

Philippe, C., Hautekiet, P., Grégoir, A. F., Thoré, E. S., Brendonck, L., De Boeck, G., & Pinceel, T. (2019). Interactive effects of 3, 4-DCA and temperature on the annual killifish *Nothobranchius furzeri*. *Aquatic Toxicology*, *212*, 146-153. https://doi.org/10.1016/j.aquatox.2019.05.009

Picado, A., Bebianno, M. J., Costa, M. H., Ferreira, A., & Vale, C. (2007). Biomarkers: a strategic tool in the assessment of environmental quality of coastal waters. *Hydrobiologia, 587*(1), 79-87. https://doi.org/10.1007/s10750-007-0695-5

Pieters, B. J., Jager, T., Kraak, M. H. S., and Admiraal, W. (2006). Modeling responses of Daphnia magna to pesticide pulse exposure under varying food conditions: Intrinsic versus apparent sensitivity. *Ecotoxicology*, *15*(7), 601–608. https://doi.org/10.1007/s10646-006-0100-6

Pillai, B. R., & Diwan, A. D. (2002). Effects of acute salinity stress on oxygen consumption and ammonia excretion rates of the marine shrimp *Metapenaeus monoceros*. *Journal of Crustacean Biology, 22*(1), 45-52. https://doi.org/10.1163/20021975-99990207

Pinger, C., Copeman, L., Stowell, M., Cormack, B., Fugate, C., & Rogers, M. (2022). Rapid measurement of total lipids in zooplankton using the sulfo-phospho-vanillin reaction. *Analytical Methods*, *14*(27), 2665-2672. https://doi-org/10.1039/D2AY00665K

Postelnicu, T. (2011). Probit analysis. In *International encyclopedia of statistical science* (pp. 1128-1131). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-04898-2_461

Prashanth, L., Kattapagari, K. K., Chitturi, R. T., Baddam, V. R. R., & Prasad, L. K. (2015). A review on role of essential trace elements in health and disease. *Journal of dr. ntr university of health sciences, 4*(2), 75.

Preston, B. L., Snell, T. W., & Kneisel, R. (1999). UV-B exposure increases acute toxicity of pentachlorophenol and mercury to the rotifer *Brachionus calyciflorus*. *Environmental Pollution*, *106*(1), 23-31. https://doi.org/10.1016/S0269-7491(99)00065-2

Preston, B., & Snell, T. (2001). Full life-cycle toxicity assessment using rotifer resting egg production: implications for ecological risk assessment. *Environmental Pollution*, *114*(3), 399-406. doi: 10.1016/s0269-7491(00)00232-3

Pulgarin, A., Decker, J., Chen, J., Giannakis, S., Ludwig, C., Refardt, D., & Pick, H. (2022). Effective removal of the rotifer Brachionus calyciflorus from a Chlorella vulgaris microalgal culture by homogeneous solar photo-Fenton at neutral pH. *Water Research*, *226*, 119301. https://doi.org/10.1016/j.watres.2022.119301

Radix, P., Léonard, M., Papantoniou, C., Roman, G., Saouter, E., Gallotti-Schmitt, S., ... & Vasseur, P. (2000). Comparison of four chronic toxicity tests using algae, bacteria, and invertebrates assessed with sixteen chemicals. *Ecotoxicology and Environmental Safety*, *47*(2), 186-194. https://doi.org/10.1006/eesa.2000.1966

Radix, P., Severin, G., Schramm, K. W., & Kettrup, A. (2002). Reproduction disturbances of *Brachionus calyciflorus* (rotifer) for the screening of environmental endocrine disrupters. *Chemosphere*, *47*(10), 1097-1101. https://doi.org/10.1016/S0045-6535(01)00335-6

Rainbow, P. S. (2007). Trace metal bioaccumulation: models, metabolic availability and toxicity. *Environment international*, *33*(4), 576-582. https://doi.org/10.1016/j.envint.2006.05.007

Rainbow, P. S., & Luoma, S. N. (2011). Metal toxicity, uptake and bioaccumulation in aquatic invertebrates—modelling zinc in crustaceans. *Aquatic toxicology*, *105*(3-4), 455-465. https://doi.org/10.1016/j.aquatox.2011.08.001

Ramos-RodrÍguez, E., & Conde-Porcuna, J. M. (2003). Nutrient limitation on a planktonic rotifer: Life history consequences and starvation resistance. *Limnology and Oceanography*, *48*(2), 933-938. https://doi.org/10.4319/lo.2003.48.2.0933

Rand, G.M. & Petrocelli, S.R. (1985). *Fundamentals of Aquatic Toxicology: Methods and Applications*. New York, USA: Hemisphere Publishing.

Rao, P., & Pattabiraman, T. N. (1989). Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. *Analytical biochemistry*, *181*(1), 18-22. https://doi.org/10.1016/0003-2697(89)90387-4

Read, S. M., & Northcote, D. H. (1981). Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical biochemistry*, *116*(1), 53-64. https://doi.org/10.1016/0003-2697(81)90321-3

Rebolledo, U. A., Nandini, S., Sarma, S. S. S., & Escobar-Sánchez, O. (2020). Effect of salinity and temperature on the acute and chronic toxicity of arsenic to the marine rotifers Proales similis and Brachionus ibericus. *Marine Pollution Bulletin*, *157*, 111341. https://doi.org/10.1016/j.marpolbul.2020.111341

Rebolledo, U. A., Páez-Osuna, F., and Fernández, R. (2021a). Single and mixture toxicity of As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, and Zn to the rotifer Proales similis under different salinities. *Environmental Pollution*, *271*. https://doi.org/10.1016/j.envpol.2020.116357

Rebolledo, U. A., Rico-martínez, R., and Páez-osuna, F. (2021b). *Synergistic Effect of Chloroquine and Copper to the Euryhaline Rotifer Proales Similis*. 1–17.

Rehman, A., Shakoori, F. R., and Shakoori, A. R. (2008). Heavy metals resistant Rotifers from a chromium contaminated wastewater can help in environmental clean-up. *Pakistan Journal of Zoology*, *40*(5), 309–316.

Reid, A.J., Carlson, A.K., Hanna, D.E.L., Olden, J.D., Ormerod, S.J., & Cooke S.J. (2020). Conservation challenges to freshwater ecosystems. In: M.I. Goldstein & D.A.

Rico-Martinez, R., & Dodson, S. I. (1992). Culture of the rotifer *Brachionus calyciflorus* Pallas. *Aquaculture*, *105*(2), 191-199. https://doi.org/10.1016/0044-8486(92)90130-D

Rico-Martínez, R., Arzate-Cárdenas, M. A., Robles-Vargas, D., Pérez-Legaspi, I. A., Jesús, A. F., & Santos-Medrano, G. E. (2016). Rotifers as Models in Toxicity Screening of Chemicals and Environmental Samples. *INVERTEBRATES-EXPERIMENTAL MODELS IN TOXICITY SCREENING*, 57-99. http://dx.doi.org/10.5772/61771

Rico-Martínez, R., Pérez-Legaspi, I. A., Arias-Almeida, J. C., & Santos-Medrano, G. E. (2013). Rotifers in ecotoxicology. In: J.F. Férard, & C. Blaise (Eds.). *Encyclopedia of Aquatic Ecotoxicology*. Dordrecht, Netherlands: Springer. https://doi.org/10.1007/978-94-007-5704-2

Ríos-Arana, J. V., Walsh, E. J., and Ortiz, M. (2007). Interaction effects of multi-metal solutions (As, Cr, Cu, Ni, Pb and Zn) on life history traits in the rotifer Plationus patulus. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *42*(10), 1473–1481. https://doi.org/10.1080/10934520701480904

Rodrigues, A. C., Gravato, C., Quintaneiro, C., Bordalo, M. D., Golovko, O., Žlábek, V., ... & Pestana, J. L. (2017). Exposure to chlorantraniliprole affects the energy metabolism of the caddisfly *Sericostoma vittatum*. *Environmental toxicology and chemistry*, *36*(6), 1584-1591. https://doi.org/10.1002/etc.3684

Roe, J. H. (1954). The determination of dextran in blood and urine with anthrone reagent. *Journal of Biological chemistry*, *208*(2), 889-896. https://doi.org/10.1016/S0021-9258(18)65614-5

Roe, J. H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. *Journal of Biological chemistry*, *212*, 335-343. https://doi.org/10.1016/S0021-9258(18)71120-4

Rogalski, M. A. (2017). Maladaptation to acute metal exposure in resurrected Daphnia ambigua clones after decades of increasing contamination. *American Naturalist*, *189*(4), 443–452. https://doi.org/10.1086/691077

Rosenfeldt, R. R., Seitz, F., Schulz, R., and Bundschuh, M. (2014). Heavy metal uptake and toxicity in the presence of titanium dioxide nanoparticles: A factorial approach using Daphnia magna. *Environmental Science and Technology*, *48*(12), 6965–6972. https://doi.org/10.1021/es405396a

Saebelfeld, M., Minguez, L., Griebel, J., Gessner, M. O., and Wolinska, J. (2017). Humic dissolved organic carbon drives oxidative stress and severe fitness impairments in Daphnia. *Aquatic Toxicology*, *182*, 31–38. https://doi.org/10.1016/j.aquatox.2016.11.006

Samanta, P., Im, H., Shim, T., Na, J., and Jung, J. (2020). Linking multiple biomarker responses

in Daphnia magna under thermal stress. *Environmental Pollution*, *263*, 114432. https://doi.org/10.1016/j.envpol.2020.114432

Sancho, E., Villarroel, M. J., Andreu, E., and Ferrando, M. D. (2009). Disturbances in energy metabolism of Daphnia magna after exposure to tebuconazole. *Chemosphere*, *74*(9), 1171–1178. https://doi.org/10.1016/j.chemosphere.2008.11.076

Sanders, T., Widdicombe, S., Calder-Potts, R., & Spicer, J. I. (2014). Environmental hypoxia but not minor shell damage affects scope for growth and body condition in the blue mussel *Mytilus edulis* (L.). *Marine Environmental Research, 95*, 74-80. https://doi.org/10.1016/j.marenvres.2013.12.013

Santiago, A., & Moreira, R. (2020). Drying of edible seaweeds. In *Sustainable seaweed technologies* (pp. 131-154). https://doi.org/10.1016/B978-0-12-817943-7.00004-4

Santos-Medrano, G. E., & Rico-Martínez, R. (2013). Lethal effects of five metals on the freshwater rotifers *Asplanchna brigthwellii* and *Brachionus calyciflorus*. *Hidrobiológica*, *23*(1), 82-86.

Sapan, C. V., Lundblad, R. L., & Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnology and applied Biochemistry*, *29*(2), 99-108. https://doi.org/10.1111/j.1470-8744.1999.tb00538.x

Sarma, S. S. S., Azuara-García, R., & Nandini, S. (2007). Combined effects of zinc and algal food on the competition between planktonic rotifers, *Anuraeopsis fissa* and *Brachionus rubens* (Rotifera). *Aquatic ecology*, *41*(4), 631-638. https://doi-org.sdl.idm.oclc.org/10.1007/s10452-007-9120-4

Sarma, S. S. S., Brena-Bustamante, P., & Nandini, S. (2008). Body size and population growth of Brachionus patulus (Rotifera) in relation to heavy metal (copper and mercury) concentrations. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *43*(5), 547–553. https://doi.org/10.1080/10934520701796606

Sarma, S. S. S., Corral-Jácquez, F. I., Nandini, S., and Brena-Bustamante, P. (2010). Population level indicators of stress: Effects of two heavy metals (copper and mercury) on the growth of lecane quadridentata (Ehrenberg, 1830) (Rotifera: Lecanidae). *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *45*(1), 32–36. https://doi.org/10.1080/10934520903388632

Sarma, S. S. S., Martinez-Jeronimo, F., Ramírez-Pérez, T., & Nandini, S. (2006). Effect of cadmium and chromium toxicity on the demography and population growth of *Brachionus calyciflorus* and *Brachionus patulus* (Rotifera). *Journal of Environmental Science and Health, Part A*, *41*(4), 543-558. https://doi.org/10.1080/10934520600564311

Sarma, S. S. S., Ramírez Pérez, T., and Nandini, S. (2000). Comparison of the sensitivity of Brachionus calyciflorus and Brachionus patulus (Rotifera) to selected heavy metals

under low and high food (Chlorella vulgaris) levels. *Bulletin of Environmental Contamination and Toxicology*, *64*(5), 735–739. https://doi.org/10.1007/s001280000065

Schanz, F. R., Sommer, S., Lami, A., Fontaneto, D., and Ozgul, A. (2021). Life-history responses of a freshwater rotifer to copper pollution. *Ecology and Evolution*, *11*(16), 10947–10955. https://doi.org/10.1002/ece3.7877

Schettino, T., Caricato, R., Calisi, A., Giordano, M. E., & Lionetto, M. G. (2012). Biomarker approach in marine monitoring and assessment: new insights and perspectives. *Open Environmental Sciences, 6*(1), 20–27. http://dx.doi.org/10.2174/1876325101206010020

Schuijt, L. M., Peng, F. J., van den Berg, S. J., Dingemans, M. M., & Van den Brink, P. J. (2021). (Eco) toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: facts, challenges, and future. *Science of the total environment*, *795*, 148776. https://doi.org/10.1016/j.scitotenv.2021.148776

Schwarzenbach, R. P., Escher, B. I., Fenner, K., Hofstetter, T. B., Johnson, C. A., Von Gunten, U., & Wehrli, B. (2006). The challenge of micropollutants in aquatic systems. *Science, 313*(5790), 1072-1077. https://doi.org/10.1126/science.1127291

Scott Jr, T. A., & Melvin, E. H. (1953). Determination of dextran with anthrone. *Analytical Chemistry*, *25*(11), 1656-1661. http:// doi.org/ 10.1021/ac60083a023

Scott, A. P., & Baynes, S. M. (1978). Effect of algal diet and temperature on the biochemical composition of the rotifer, *Brachionus plicatilis*. *Aquaculture*, *14*(3), 247-260. https://doi.org/10.1016/0044-8486(78)90098-4

Shang, Y., Wang, X., Chang, X., Sokolova, I. M., Wei, S., Liu, W., ... & Wang, Y. (2021). The effect of microplastics on the bioenergetics of the mussel Mytilus coruscus assessed by cellular energy allocation approach. *Frontiers in Marine Science*, *8*, 754789. https://doi.org/10.3389/fmars.2021.754789

Shang, Y., Wang, X., Shi, Y., Huang, W., Sokolova, I., Chang, X., ... & Wang, Y. (2023). Ocean acidificationf affects the bioenergetics of marine mussels as revealed by high-coverage quantitative metabolomics. *Science of The Total Environment*, *858*, 160090. https://doi.org/10.1016/j.scitotenv.2022.160090

Sheng, J., Vannela, R., & Rittmann, B. E. (2011). Evaluation of methods to extract and quantify lipids from Synechocystis PCC 6803. *Bioresource technology*, *102*(2), 1697-1703. https://doi.org/10.1016/j.biortech.2010.08.007

Shertzer, K. W., and Ellner, S. P. (2002). State-dependent energy allocation in variable environments: Life history evolution of a rotifer. *Ecology*, *83*(8), 2181–2193. https://doi.org/10.1890/0012-9658(2002)083[2181:SDEAIV]2.0.CO;2

Silva, A. R. R., Santos, C. S. A., Ferreira, N. G. C., Morgado, R., Cardoso, D. N., Cruz, A., Mendo, S., Soares, A. M. V. M., and Loureiro, S. (2019). Multigenerational effects of carbendazim in Daphnia magna: From a subcellular to a population level. *Environmental*

Toxicology and Chemistry, *38*(2), 412–422. https://doi.org/10.1002/etc.4307

Silva, M., Feijão, E., da Cruz de Carvalho, R., Duarte, I. A., Matos, A. R., Cabrita, M. T., Barreiro, A., Lemos, M. F. L., Novais, S. C., Marques, J. C., Caçador, I., Reis-Santos, P., Fonseca, V. F., & Duarte, B. (2020). Comfortably numb: Ecotoxicity of the non-steroidal anti-inflammatory drug ibuprofen on *Phaeodactylum tricornutum*. *Marine Environmental Research*, *161*, 105109. https://doi.org/10.1016/j.marenvres.2020.105109

Sitepu, I. R., Ignatia, L., Franz, A. K., Wong, D. M., Faulina, S. A., Tsui, M., ... & Boundy-Mills, K. (2012). An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of microbiological methods*, *91*(2), 321-328. https://doi.org/10.1016/j.mimet.2012.09.001

Smedes, F., & k Askland, T. (1999). Revisiting the development of the Bligh and Dyer total lipid determination method. *Marine Pollution Bulletin*, *38*(3), 193-201. https://doi.org/10.1016/S0025-326X(98)00170-2

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical biochemistry*, *150*(1), 76–85. https://doi.org/10.1016/0003-2697(85)90442-7

Smolders, R., Baillieul, M., and Blust, R. (2005). Relationship between the energy status of Daphnia magna and its sensitivity to environmental stress. *Aquatic Toxicology*, *73*(2), 155–170. https://doi.org/10.1016/j.aquatox.2005.03.006

Smolders, R., Bervoets, L., De Coen, W., & Blust, R. (2004). Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. *Environmental Pollution, 129*(1), 99-112. http://dx.doi.org/10.1016/j.envpol.2003.09.027

Smolders, R., De Boeck, G., & Blust, R. (2003). Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). *Environmental Toxicology and Chemistry: An International Journal, 22*(4), 890-899. https://doi.org/10.1002/etc.5620220429

Snell, T. W. (2014). Rotifers as models for the biology of aging. *International review of hydrobiology*, *99*(1-2), 84-95. https://doi.org/10.1002/iroh.201301707

Snell, T. W., & Carmona, M. J. (1995). Comparative toxicant sensitivity of sexual and asexual reproduction in the rotifer Brachionus calyciflorus. *Environmental Toxicology and Chemistry: An International Journal*, *14*(3), 415-420. https://doi.org/10.1002/etc.5620140310

Snell, T. W., & Janssen, C. R. (1995). Rotifers in ecotoxicology: a review. *Hydrobiologia, 313*(1), 231-247. https://doi.org/10.1007/BF00025956

Snell, T. W., & Joaquim-Justo, C. (2007). Workshop on rotifers in ecotoxicology. *Hydrobiologia, 593*(1), 227-232. https://doi.org/10.1007/s10750-007-9045-x

Snell, T. W., & Marcial, H. S. (2017). Using rotifers to diagnosis the ecological impacts of toxicants. *Rotifers: aquaculture, ecology, gerontology, and ecotoxicology*, 129-147. https://doi.org/10.1007/978-981-10-5635-2_9

Snell, T. W., & Moffat, B. D. (1992). A 2-d life cycle test with the rotifer *Brachionus calyciflorus*. *Environmental Toxicology and Chemistry: An International Journal*, *11*(9), 1249-1257. https://doi.org/10.1002/etc.5620110905

Snell, T. W., & Persoone, G. (1989a). Acute toxicity bioassays using rotifers. II. A freshwater test with *Brachionus rubens*. *Aquatic toxicology*, *14*(1), 81-91. https://doi.org/10.1016/0166-445X(89)90056-8

Snell, T. W., & Persoone, G. (1989b). Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatilis*. *Aquatic toxicology*, *14*(1),65-80. https://doi.org/10.1016/0166-445X(89)90055-6

Snell, T. W., Johnston, R. K., and Matthews, A. B. (2017). Freshwater toxicity testing using rehydrated Philodina sp. (Rotifera) as test animals. *Environmental Toxicology*, *32*(10), 2267–2276. https://doi.org/10.1002/tox.22442

Snell, T. W., Moffat, B. D., Janssen, C., & Persoone, G. (1991a). Acute toxicity tests using rotifers: IV. Effects of cyst age, temperature, and salinity on the sensitivity of *Brachionus calyciflorus*. *Ecotoxicology and environmental safety*, *21*(3), 308-317. https://doi.org/10.1016/0147-6513(91)90070-6

Snell, T. W., Moffat, B. D., Janssen, C., & Persoone, G. (1991b). Acute toxicity tests using rotifers. III. Effects of temperature, strain, and exposure time on the sensitivity of *Brachionus plicatilis*. *Environmental Toxicology and Water Quality*, *6*(1), 63-75. https://doi.org/10.1002/tox.2530060106

Snell, T.W. & Hoff, F.A. (1986). Rotifer resting cysts: their prospects for use in aquaculture. Presented at meeting of World Aquaculture Society, Reno, NV.

Sobral, P., & Widdows, J. (1997). Effects of copper exposure on the scope for growth of the clam *Ruditapes decussatus* from southern Portugal. *Marine Pollution Bulletin, 34*(12), 992-1000. https://doi.org/10.1016/S0025-326X(97)00116-1

Soetaert, A., Vandenbrouck, T., van der Ven, K., Maras, M., van Remortel, P., Blust, R., & De Coen, W. M. (2007). Molecular responses during cadmium-induced stress in *Daphnia magna*: integration of differential gene expression with higher-level effects. *Aquatic Toxicology, 83*(3), 212-222. http://dx.doi.org/10.1016/j.aquatox.2007.04.010

Sokolova, I. (2021). Bioenergetics in environmental adaptation and stress tolerance of aquatic ectotherms : linking physiology and ecology in a multi-stressor landscape. *Experimental Biology*, *224*(Suppl_1), jeb236802. https://doi.org/10.1242/jeb.236802

Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology, 53*(4), 597-608. https://doi.org/10.1093/icb/ict028

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G., & Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine environmental research*, *79*, 1-15. https://doi.org/10.1016/j.marenvres.2012.04.003

Solomon, F. (2009). Impacts of copper on aquatic ecosystems and human health. *Environment and Communities, 25*(8), 25-28.

Sorokin, N., Johnson, I., Rockett, L, & Aldous, E. (2012). *Proposed EQS for Water Framework Directive Annex VIII substances: 3,4- dichloroaniline (For consultation)*. Edinburgh, UK: Water Framework Directive United Kingdom Technical Advisory Group (WFD-UKTAG). Retrieved from https://www.wfduk.org/sites/default/files/Media/34-DCA%20-%20UKTAG.pdf

Sorvari, J., and Sillanpää, M. (1996). Influence of metal complex formation on heavy metal and free EDTA and DTPA acute toxicity determined by Daphnia magna. *Chemosphere*, *33*(6), 1119–1127. https://doi.org/10.1016/0045-6535(96)00251-2

Soto, E., Oyarce, G., Inzunza, B., & Bay-Schmith, E. (2003). Acute toxicity of organic and inorganic compounds on the freshwater cyclopoid copepod *Eucyclops neumani neumani* (Pesta, 1927). *Bulletin of environmental contamination and toxicology, 70*(5), 1017-1021. https://doi.org/10.1007/s00128-003-0084-3

Sousa, T., Domingos, T., Poggiale, J. C., & Kooijman, S. A. L. M. (2010). Dynamic energy budget theory restores coherence in biology. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*(1557), 3413-3428. https://doi.org/10.1098/rstb.2010.0166

Srivastava, A., Hamre, K., Stoss, J., Chakrabarti, R., & Tonheim, S. K. (2006). Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): With emphasis on the water soluble fraction. *Aquaculture*, *254*(1-4), 534-543. https://doi.org/10.1016/j.aquaculture.2005.11.014

Steinberg, C. E. W., Ouerghemmi, N., Herrmann, S., Bouchnak, R., Timofeyev, M. A., and Menzel, R. (2010). Stress by poor food quality and exposure to humic substances: Daphnia magna responds with oxidative stress, lifespan extension, but reduced offspring numbers. *Hydrobiologia*, *652*(1), 223–236. https://doi.org/10.1007/s10750-010-0334-4

Stelzer, C. P. (2001). Resource limitation and reproductive effort in a planktonic rotifer. *Ecology*, *82*(9), 2521–2533. https://doi.org/10.1890/0012-9658(2001)082[2521:RLAREI]2.0.CO;2

Stevenson, L. M., Krattenmaker, K. E., Johnson, E., Bowers, A. J., Adeleye, A. S., McCauley, E., and Nisbet, R. M. (2017). Standardized toxicity testing may underestimate

ecotoxicity: Environmentally relevant food rations increase the toxicity of silver nanoparticles to Daphnia. *Environmental Toxicology and Chemistry*, *36*(11), 3008–3018. https://doi.org/10.1002/etc.3869

Stoeppler, M. (1991). Cadmium. In: E. Merian, & T. W. Clarkson (Eds). *Metals and their compounds in the environment* (pp. 803 – 851). Weinheim, Germany: VCH.

Stomperudhaugen, E. S., Hanssen Øverås, N. H., Langford, K., De Coen, W., Smolders, R., and Hylland, K. (2009). Cellular energy allocation in Hediste diversicolor exposed to sediment contaminants. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, *72*(3–4), 244–253. https://doi.org/10.1080/15287390802539178

Storey, K. B. (2015). Regulation of hypometabolism: insights into epigenetic controls. *Journal of Experimental Biology*, *218*(1), 150-159. https://doi.org/10.1242/jeb.106369

Stoscheck, C. M. (1990). Increased uniformity in the response of the Coomassie blue G protein assay to different proteins. *Analytical Biochemistry*, *184*(1), 111-116. https://doi.org/10.1016/0003-2697(90)90021-Z

Strickland, J.D.H. & Parsons, T.R. (1968). *A Practical Handbook of Seawater Analysis*. Ottawa, Canada: Fisheries Research Board of Canada.

Sun, X., Cui, Y., Wang, Q., Tang, S., Cao, X., Luo, H., ... & Wang, T. (2018). Proteogenomic analyses revealed favorable metabolism pattern alterations in rotifer Brachionus plicatilis fed with selenium-rich chlorella. *Journal of agricultural and food chemistry*, *66*(26), 6699-6707. https://doi.org/10.1021/acs.jafc.8b00139

Suter, G.W., 1992. *Ecological Risk Assessment*. Lewis Publishers, London (p. 509).

Sylvestre, C., & Le Gal, Y. (1987). In situ measurements of adenylate energy charge and assessment of pollution. *Marine pollution bulletin*, *18*(1), 36-39. https://doi.org/10.1016/0025-326X(87)90656-4

Tait, T. N., Cooper, C. A., McGeer, J. C., Wood, C. M., & Smith, D. S. (2016). Influence of dissolved organic matter (DOM) source on copper speciation and toxicity to Brachionus plicatilis. *Environmental Chemistry*, *13*(3), 496-506. https://doi.org/10.1071/EN15123

Tang, B., Zhu, L., and Zhou, Q. (2011). Joint effects of Penta-BDE and heavy metals on Daphnia magna survival, its antioxidant enzyme activities and lipid peroxidation. *Frontiers of Environmental Science and Engineering in China*, *5*(1), 99–110. https://doi.org/10.1007/s11783-010-0260-8

Thébault, M. T., Raffin, J. P., Picado, A. M., Mendonça, E., Skorkowski, E. F., & Le Gal, Y. (2000). Coordinated changes of adenylate energy charge and ATP/ADP: use in ecotoxicological studies. *Ecotoxicology and Environmental Safety, 46*(1), 23-28. https://doi.org/10.1006/eesa.1999.1870

Theegala, C. S., Suleiman, A. A., and Carriere, P. A. (2007). Toxicity and biouptake of lead and arsenic by Daphnia pulex. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, *42*(1), 27–31. https://doi.org/10.1080/10934520601015404

Thermo Fisher Scientific Incorporated. (2017). *A rapid, sensitive protein assay for the accurate analysis of protein concentrations.* Retrieved DEC 19, 2021, from https://assets.thermofisher.com/TFS-Assets/BID/Application-Notes/rapid-sensitive-protein-assay-white-paper.pdf.

Thermo Fisher Scientific Incorporated. (n.d.). *Overview of protein assays methods*. Retrieved DEC 19, 2021, from https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-protein-assays.html

Thoré, E. S., Van Hooreweghe, F., Philippe, C., Brendonck, L., & Pinceel, T. (2021). Generation-specific and interactive effects of pesticide and antidepressant exposure in a fish model call for multi-stressor and multigenerational testing. *Aquatic Toxicology*, *232*, 105743. https://doi.org/10.1016/j.aquatox.2021.105743

Thorp, J. H., & Covich, A. P. (Eds.). (2009). *Ecology and classification of North American freshwater invertebrates*. Academic press.

Todgham, A. E., & Stillman, J. H. (2013). Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. *Integrative and comparative biology*, *53*(4), 539-544. https://doi.org/10.1093/icb/ict086

Tornero, V., & Hanke, G. (2016). Chemical contaminants entering the marine environment from sea-based sources: A review with a focus on European seas. *Marine Pollution Bulletin, 112*(1-2), 17-38. http://dx.doi.org/10.1016/j.marpolbul.2016.06.091

Tourinho, P. S., Silva, A. R. R., Santos, C. S., Prodana, M., Ferreira, V., Habibullah, G., ... & Loureiro, S. (2022). Microplastic fibers increase sublethal effects of AgNP and AgNO3 in Daphnia magna by changing cellular energy allocation. *Environmental toxicology and chemistry*, *41*(4), 896-904. https://doi.org/10.1002/etc.5136

Treuner, A.B. (2002). *Ecotoxicology of neotropical freshwater zooplankton species exposed to toxic mixtures.* PhD thesis, University of Stirling.

Udochi, S. (2020). *Predicting the ecological effects of metals in marine sediments* (Doctoral dissertation, University of East Anglia)

USEPA [United States Environmental Environmental Protection Agency]. (2002). *Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms* (EPA-821-R- 02-013). US Environmental Protection Agency,

Washington, DC. Available at https://www.epa.gov/sites/production/files/2015-08/documents/short-term-chronic-freshwater-wet-manual_2002.pdf

USEPA [United States Environmental Protection Agency]. (1994). Eco Update, Using Toxicity Tests in Ecological Risk Assessment. Publication 9345.0-05I. *Intermittent Bulletin 2*(1)

Van Dievel, M., Janssens, L., & Stoks, R. (2019). Additive bioenergetic responses to a pesticide and predation risk in an aquatic insect. *Aquatic Toxicology*, *212*, 205-213. https://doi.org/10.1016/j.aquatox.2019.05.010

Vandenbrouck, T., Soetaert, A., van der Ven, K., Blust, R., & De Coen, W. (2009). Nickel and binary metal mixture responses in *Daphnia magna*: Molecular fingerprints and (sub)organismal effects. *Aquatic Toxicology*, *92*(1), 18-29. https://doi.org/10.1016/j.aquatox.2008.12.012

Verheyen, J., & Stoks, R. (2020). Negative bioenergetic responses to pesticides in damselfly larvae are more likely when it is hotter and when temperatures fluctuate. *Chemosphere*, *243*. https://doi.org/10.1016/j.chemosphere.2019.125369

Verschraegen, K., Herman, P. M. J., Van Gansbeke, D., & Braeckman, A. (1985). Measurement of the adenylate energy charge in *Nereis diversicolor* and *Nephtys* sp. (Polychaeta: Annelida): Evaluation of the usefulness of AEC in pollution monitoring. *Marine Biology, 86*, 233−240. https://doi.org/10.1007/BF00397509

Verslycke, T., & Janssen, C. R. (2002). Effects of a changing abiotic environment on the energy metabolism in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). *Journal of Experimental Marine Biology and Ecology, 279*(1-2), 61-72. http://dx.doi.org/10.1016/S0022-0981(02)00339-8

Verslycke, T., Ghekiere, A., & Janssen, C. R. (2004b). Seasonal and spatial patterns in cellular energy allocation in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) of the Scheldt estuary (The Netherlands). *Journal of Experimental Marine Biology and Ecology, 306*(2): 245−267. https://doi.org/10.1016/j.jembe.2003.12.022

Verslycke, T., Roast, S. D., Widdows, J., Jones, M. B., & Janssen, C. R. (2004a). Cellular energy allocation and scope for growth in the estuarine mysid Neomysis integer (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. *Journal of experimental marine biology and ecology*, *306*(1), 1-16. https://doi.org/10.1016/j.jembe.2003.12.022

Verslycke, T., Vercauteren, J., Devos, C., Moens, L., Sandra, P., & Janssen, C. R. (2003). Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure. *Journal of Experimental Marine Biology and Ecology*, *288*(2), 167-179. https://doi.org/10.1016/S0022-0981(03)00006-6

Vijayavel, K., & Balasubramanian, M. P. (2006). Changes in oxygen consumption and respiratory enzymes as stress indicators in an estuarine edible crab *Scylla serrata* exposed to naphthalene. *Chemosphere, 63*(9), 1523-1531. https://doi.org/10.1016/j.chemosphere.2005.09.010

Villarroel, M. J., Sancho, E., Andreu-Moliner, E., & Ferrando, M. D. (2013). Caloric content of *Daphnia magna* as reflect of propanil stress during a short-term exposure and its relationship to long-term responses. *Environmental Toxicology and Pharmacology*, *35*(3), 465-472. https://doi.org/10.1016/j.etap.2013.02.012

Villarroel, M. J., Sancho, E., Andreu-Moliner, E., & Ferrando, M. D. (2009). Biochemical stress response in tetradifon exposed *Daphnia magna* and its relationship to individual growth and reproduction. *Science of the Total Environment, 407*(21), 5537-5542. https://doi.org/10.1016/j.scitotenv.2009.06.032

Vutukuru, S. S. (2005). Acute effects of hexavalent chromium on survival, oxygen consumption, hematological parameters and some biochemical profiles of the Indian major carp, *Labeo rohita*. *International Journal of environmental Research and Public health, 2*(3), 456-462. https://doi.org/10.3390/ijerph2005030010

Walker, J. M. (2009). The Bicinchoninic Acid (BCA) Assay for Protein Quantitation. In: Walker, J.M. (Ed), *The protein protocols handbook* (pp. 11–15). Totowa, NJ: Humana Press. https://doi:10.1007/978-1-59745-198-7_3

Wallace, R. L., Snell, T. W., & Smith, H. A. (2015). Phylum rotifera. In: J.H. Thorp & D.C. Rogers (Eds). *Thorp and Covich's freshwater invertebrates* (pp. 225-271). Academic Press. https://doi.org/10.1016/B978-0-12-385026-3.00013-9

Wallace, R. L., Snell, T. W., Ricci, C., & Nogrady, T. (2006). Rotifera: Vol. 1: biology, ecology and systematics (2nd edition). In: S. H., & H. J. Dumont (Eds). *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World, 23*. Ghent, Belgium: Kenobi Productions & The Hague, The Netherlands: Backhuys Academic Publishing BV. https://doi.org/10.1002/iroh.19950800111

Wallace, R.L. & Snell, T.W. (2001). Phylum Rotifera. In: J.H. Thorp & A.P. Covich (Eds.). *Ecology and Systematics of North American Freshwater Invertebrates*, 2nd ed (pp. 195 – 254). New York, USA: Academic Press. https://doi.org/10.1016/B978-0-12-690647-9.X5000-5

Wallace, R.L. (2002). Rotifers: Exquisite Metazoans, *Integrative and Comparative Biology*. *42*(3), 660–667. https://doi.org/10.1093/icb/42.3.660

Wang, H., Tang, X., Sha, J., Chen, H., Sun, T., and Wang, Y. (2015). The reproductive toxicity on the rotifer Brachionus plicatilis induced by BDE-47 and studies on the effective mechanism based on antioxidant defense system changes. *Chemosphere*, *135*, 129–137. https://doi.org/10.1016/j.chemosphere.2015.03.090

Wang, Y., Hu, M., Wong, W. H., Shin, P. K., & Cheung, S. G. (2011). The combined effects of oxygen availability and salinity on physiological responses and scope for growth in the green-lipped mussel *Perna viridis. Marine pollution bulletin, 63*(5-12), 255-261. https://doi.org/10.1016/j.marpolbul.2011.02.004

Wang, Y., Niu, J., Zhang, L., & Shi, J. (2014). Toxicity assessment of perfluorinated carboxylic acids (PFCAs) towards the rotifer *Brachionus calyciflorus*. *Science of the Total Environment*, *491*, 266-270. https://doi.org/10.1016/j.scitotenv.2014.02.028

Ware, G. W. (1982). Pesticides: Theory and application. *Ed. Freeman, WH*, *308*.

Warren, C. E., & Davis, G. E. (1967). Laboratory studies on feeding, bioenergetics and growth. In *The biological basis of freshwater fish production.* Blackwell, Oxford (pp. 175-214).

Watanabe, T. (1978). Nutritional quality of rotifer *Brachionus plicatilis* as a living feed from the viewpoint of essential fatty acids for fish. *Bull. Jpn. Soc. Sci. Fish.*, *44*, 1109-1114.

Watanabe, T., Arakawa, T., Kitajima, C., & Fujita, S. (1978). Nutritional evaluation of proteins of living feeds used in seed production of fish. *Bulletin of the Japanese Society of Scientific Fisheries, 44*(9), 985 – 988.

Watanabe, T., Kitajima, C., & Fujita, S. (1983). Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, *34*(1-2), 115-143. https://doi.org/10.1016/0044-8486(83)90296-X

Waterborg, J. H. (2009). The Lowry method for protein quantitation. In J.M. Walker (Ed) *The Protein Protocols Handbook* (pp. 7-10). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-59745-198-7_2

Widdows, J. (1983). Field measurements of the biological impact of pollutants. In: J. C. Su & T.C. Hung. (Eds.) *Proceedings of a Pacific Regional workshop on the assimilative capacity of the oceans for man's wastes*, April 26-30, 1982. Taipei, Republic of China: Scope/ICSU Academia Sinica, 111–129.

Widdows, J., & Johnson, D. (1988). Physiological energetics of *Mytilus edulis*: scope for growth. *Marine Ecology Progress Series*, *46*(1-3) 113-121. https://www.jstor.org/stable/24827572

Widdows, J., & Salkeld, P. (1993). Role of Scope for Growth in environmental toxicology and pollution monitoring. Map Technical reports series No. 71 – Selected techniques for monitoring bio- logical effects of pollutants inmarine organisms, UNEP, Athens (pp. 115–146).

Widdows, J., Donkin, P., 1992. Mussels and environmental con- taminants: bioaccumulation and physiological aspects. In: Gosling, E. (Ed.), The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Elsevier, Amserdam, The Netherlands

Widdows, J., Donkin, P., Brinsley, M. D., Evans, S. V., Salkeld, P. N., Franklin, A., ... & Waldock, M. J. (1995). Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis. Marine Ecology Progress Series*, *127*, 131-148. https://doi.org/10.3354/meps127131

Widdows, J., Donkin, P., Salkeld, P. N., & Evans, S. V. (1987). Measurement of scope for growth and tissue hydrocarbon concentrations of mussels (*Mytilus edulis*) at sites in the vicinity of the Sullom Voe oil terminal: A case study. In: J. Kuiper & W. J. van den Brink (Eds.). *Fate and effects of oil in marine ecosystems* (pp. 269–277). Dordrecht, Netherlands: Martinus Nijhoff Publishers.

Wiechelman, K. J., Braun, R. D., & Fitzpatrick, J. D. (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Analytical biochemistry*, *175*(1), 231-237. https://doi.org/10.1016/0003-2697(88)90383-1

Wilder, S. M., & Jeyasingh, P. D. (2016). Merging elemental and macronutrient approaches for a comprehensive study of energy and nutrient flows. *Journal of Animal Ecology*, *85*(6), 1427-1430. https://doi.org/10.1111/1365-2656.12573

Williams, M. B., Powell, M. L., & Watts, S. A. (2016). Combined effects of Corexit EC 9500A with secondary abiotic and biotic factors in the rotifer *Brachionus plicatilis*. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, *188*, 39–44. https://doi.org/10.1016/j.cbpc.2016.06.005

Willis, K. J. (1999). Acute and chronic bioassays with New Zealand freshwater copepods using pentachlorophenol. *Environmental Toxicology and Chemistry, 18*(11), 2580-2586. https://doi.org/10.1002/etc.5620181127

Won, E., Han, J., Kim, D., Dahms, H., & Lee, J. (2017). Rotifers in Ecotoxicology. In A. Hagiwara & T. Yoshinaga (Eds). *Rotifers: Aquaculture, Ecology, Gerontology, and Ecotoxicology* (pp. 149 – 176). Fisheries Science Series. Singapore: Springer. https://doi.org/10.1007/978-981-10-5635-2_10

Wong, C. K. (1992). Effects of chromium, copper, nickel, and zinc on survival and feeding of the cladoceran Moina macrocopa. *Bulletin of environmental contamination and toxicology*, *49*(4), 593-599. https://doi.org/10.1007/BF00196304

Xi, Y. L., Chu, Z. X., & Xu, X. P. (2007). Effect of four organochlorine pesticides on the reproduction of freshwater rotifer *Brachionus calyciflorus* pallas. *Environmental Toxicology and Chemistry: An International Journal*, *26*(8), 1695-1699. https://doi.org/10.1897/06-415R.1

Xu, X. P., Xi, Y. L., Huang, L., & Xiang, X. L. (2014). The life-table demographic response of freshwater rotifer *Brachionus calyciflorus* to multi-metal (Cu, Zn, Cd, Cr, and Mn) mixture interaction. *Bulletin of environmental contamination and toxicology*, *93*(2), 165-170. https://doi.org/10.1007/s00128-014-1281-y

Xu, X. P., Xi, Y. L., Huang, L., & Xiang, X. L. (2015). Effects of multi-metal (Cu, Zn, Cd, Cr, and Mn) mixtures on the reproduction of freshwater rotifer *Brachionus calyciflorus*. *Bulletin of environmental contamination and toxicology*, *95*(6), 714-720. https://doi.org/10.1007/s00128-015-1675-5

Xu, X. P., Yang, X. F., Wei, X. Y., Xi, Y. L., & Wang, X. M. (2021b). Studies on toxicity and bioaccumulation of Cu2+ in alga Scenedesmus obliquus and its effect on life table demography of the rotifer Brachionus calyciflorus. *Polish Journal of Environmental Studies*, *30*(1), 893-901. https://doi.org/10.15244/pjoes/123605

Xu, X., Chen, T., Xie, A., Yang, X., & Wei, X. (2021a). Chronic effects of bromate on sexual reproduction of freshwater rotifer Brachionus calyciflorus. *Bulletin of Environmental Contamination and Toxicology*, *106*, 270-277. https://doi.org/10.1007/s00128-021-03103-z

Xue, Y. H., Sun, Z. X., Feng, L. S., Jin, T., Xing, J. C., and Wen, X. L. (2021). Algal density affects the influences of polyethylene microplastics on the freshwater rotifer Brachionus calyciflorus. *Chemosphere*, *270*. https://doi.org/10.1016/j.chemosphere.2020.128613

Yang, H., Han, X., Ma, Z., Wang, R., Liu, J., & Ji, X. (2010). Palladium-guanidine complex immobilized on SBA-16: a highly active and recyclable catalyst for Suzuki coupling and alcohol oxidation. *Green Chemistry*, *12*(3), 441-451. https://doi.org/10.1039/B919175E

Yang, Y., Jian, X., Tang, X., Ma, W., Sun, Z., Zhang, X., Fang, K., and Zhang, X. (2021). Feeding behavior toxicity in the marine rotifer Brachionus plicatilis caused by 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47): Characteristics and mechanisms. *Chemosphere*, *271*, 129512. https://doi.org/10.1016/j.chemosphere.2020.129512

Yousuf, A. (2020). Fundamentals of microalgae cultivation. In *Microalgae cultivation for biofuels production* (pp. 1-9). Academic Press. https://doi.org/10.1016/B978-0-12-817536-1.00001-1

Yuan, W., Zhou, Y., Chen, Y., Liu, X., & Wang, J. (2020). Toxicological effects of microplastics and heavy metals on the *Daphnia magna*. *Science of the Total Environment*, *746*, 141254. https://doi.org/10.1016/j.scitotenv.2020.141254

Zanders, I. P., & Rodríguez, J. (1992). Effects of temperature and salinity stress on osmoionic regulation in adults and on oxygen consumption in larvae and adults of *Macrobrachium amazonicum* (Decapoda, Palaemonidae). *Comparative Biochemistry and Physiology Part A: Physiology, 101*(3), 505-509. https://doi.org/10.1016/0300-9629(92)90502-H

Zeman, F. A., Gilbin, R., Alonzo, F., Lecomte-Pradines, C., Garnier-Laplace, J., and Aliaume, C. (2008). Effects of waterborne uranium on survival, growth, reproduction and physiological processes of the freshwater cladoceran Daphnia magna. *Aquatic Toxicology*, *86*(3), 370–378. https://doi.org/10.1016/j.aquatox.2007.11.018

Zhang, H., Shin, P. K., & Cheung, S. G. (2015). Physiological responses and scope for growth upon medium-term exposure to the combined effects of ocean acidification and temperature in a subtidal scavenger *Nassarius conoidalis. Marine Environmental Research, 106*, 51-60. https://doi.org/10.1016/j.marenvres.2015.03.001

Zhang, H., Shin, P. K., & Cheung, S. G. (2016a). Physiological responses and scope for growth in a marine scavenging gastropod, *Nassarius festivus* (Powys, 1835), are affected by salinity and temperature but not by ocean acidification. *ICES Journal of Marine Science, 73*(3), 814-824. https://doi.org/10.1093/icesjms/fsv208

Zhang, L., Niu, J., & Wang, Y. (2016b). Full life-cycle toxicity assessment on triclosan using rotifer *Brachionus calyciflorus*. *Ecotoxicology and Environmental Safety*, *127*, 30-35. https://doi.org/10.1016/j.ecoenv.2015.12.043

Zhang, L., Niu, J., Li, Y., Wang, Y., & Sun, D. (2013). Evaluating the sub-lethal toxicity of PFOS and PFOA using rotifer *Brachionus calyciflorus*. *Environmental pollution*, *180*, 34-40. https://doi.org/1

Zhang, X., Tang, X., Yang, Y., Sun, Z., Ma, W., Tong, X., ... & Zhang, X. (2021). Responses of the reproduction, population growth and metabolome of the marine rotifer Brachionus plicatilis to tributyl phosphate (TnBP). *Environmental Pollution*, *273*, 116462. https://doi.org/10.1016/j.envpol.2021.116462

Zhou, Q., Lu, N., Gu, L., Sun, Y., Zhang, L., Huang, Y., Chen, Y., and Yang, Z. (2020). Daphnia enhances relative reproductive allocation in response to toxic microcystis: Changes in the performance of parthenogenetic and sexual reproduction. *Environmental Pollution*, *259*. https://doi.org/10.1016/j.envpol.2019.113890

Zhou, T., Zhang, L., Wang, Y., Mu, Q., and Yin, J. (2019). Effects of LaCoO3 perovskite nanoparticle on: Daphnia magna: Accumulation, distribution and biomarker responses. *RSC Advances*, *9*(42), 24617–24626. https://doi.org/10.1039/c9ra03513c

Zipf, R. E., & Waldo, A. L. (1952). Spectrophotometric analysis of carbohydrates and study of anthrone reagent. *Journal of Laboratory and Clinical Medicine*, *39*(3), 497-502.