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Alocação de energia celular de *Chironomus riparius* sob stress tóxico

Cellular energy allocation of *Chironomus riparius* under toxic stress

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ALOCAÇÃO DE ENERGIA CELULAR DE *Chironomus riparius* SOB STRESS TÓXICO

CELLULAR ENERGY ALLOCATION OF *Chironomus riparius* UNDER TOXIC STRESS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor João Luís Teixeira Pestana, Investigador do Departamento de Biologia/CESAM - Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro e co-orientação do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro e do Professor Doutor Marco Filipe Loureiro Lemos, Professor Adjunto da Escola Superior de Turismo e Tecnologia do Mar do Instituto Politécnico de Leiria

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Aos meus pais.

o júri

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palavras-chave balanço energético, metabolismo, reservas, biomarcadores, cádmio, Movento®, ecotoxicologia

resumo

A alocação de energia celular (CEA) é um biomarcador energético que integra a análise das reservas energéticas disponíveis (conteúdo total de proteínas, lípidos e carbohidratos) e da energia consumida pelo organismo (sistema de transporte de eletrões), fornecendo uma análise global do estado metabólico dos organismos.

Neste trabalho, este biomarcador foi usado como uma potencial ferramenta para a avaliação de efeitos do Cádmio e do inseticida Movento® em larvas da espécie *Chironomus riparius*. Alterações no CEA foram comparadas com efeitos no crescimento e emergência dos organismos, de modo a avaliar a sensibilidade e relevância do CEA como uma ferramenta na avaliação de risco ecológico. Cádmio e Movento® tiveram efeitos negativos no crescimento e na emergência dos organismos e o biomarcador energético revelou uma menor sensibilidade que os outros parâmetros para ambos os contaminantes. Contrariamente à exposição ao inseticida, onde não foram observadas diferenças na alocação de energia, o cádmio causou um aumento na energia consumida – provavelmente devido a um maior gasto de energia para a destoxificação.

Uma menor quantidade de energia disponível irá, provavelmente, provocar uma redução nas taxas de desenvolvimento com consequências na emergência, afetando assim a reprodução e a dinâmica da população.

Alterações na alocação de energia, dependendo da sua magnitude, podem estar intimamente relacionadas com alterações nos parâmetros individuais do ciclo de vida dos organismos (crescimento e reprodução), podendo ter consequências negativas em níveis de organização biológica superiores. Isto pode reforçar a importância de utilizar estes biomarcadores energéticos como uma potencial ferramenta na avaliação de risco ambiental. Contudo, e apesar do CEA ser utilizado como um biomarcador sensível em diferentes espécies de invertebrados, os resultados deste trabalho mostram que a sua utilização e interpretação em organismos modelo com ciclos de vida rápidos e complexos, como *C. riparius*, deve ser feita de forma cuidadosa.

keywords energy balance, metabolism, reserves, biomarkers, cadmium, Movento®, ecotoxicology

abstract

The Cellular Energy Allocation (CEA) is an energetic biomarker that integrates the assessment of the available energy reserves (total content of protein, lipids and carbohydrates) and the energy consumed by the organism (electron transport system), providing an overall assessment of the metabolic status of the organisms.

In this work, this biomarker was used as a prospective tool to assess the effects of Cadmium and the insecticide Movento® to exposed midge larvae, *Chironomus riparius*. Alterations in CEA were compared to effects on growth and emergence in order to evaluate CEA's relevance and sensitiveness as a tool in environmental risk assessment. Cadmium and Movento® impaired the growth and emergence of the organisms and the energetic biomarker revealed less sensitivity than the other endpoints for both contaminants. Contrary to the insecticide exposure, where no differences in the energy allocation were observed, cadmium caused an energy allocation shift – towards the increase of the consumed energy - probably due to a higher energy demand for toxic defense purposes.

Less energy available will most probably reduce development rates with consequences on the emergence thus affecting reproduction and population dynamics.

Changes in the energy allocation, depending on their magnitude, are closely related to changes in the individual life cycle traits (growth and reproduction) and may therefore have major impacts in higher levels of biological organization. This may strengthen these energy based biomarkers as prospective tools in environmental risk assessment. Nevertheless and despite CEA's potential use as a sensitive biomarker in different invertebrates species the results of this work call for a careful use and interpretation of CEA assessment in model test species, such as *C. riparius*, which have a rapid and complex life-cycle.

CONTENTS

CHAPTER I

1. Main Introduction

1.1. Ecotoxicology testing

The increasing pressure over ecosystems due to widespread contamination has led to a global concern about its immediate and longer-term effects on the environment and even on human health. In the past decades, this concern has led to growing efforts to develop tools to suit the environmental risk assessment needs.

Ecotoxicology tests do not reproduce faithfully all natural environmental conditions of the organisms and its interactions, but, at the laboratory, they allow us to know the effect of toxic compounds in specific components of the ecosystems in a reproducible and controlled way (Di Giulio 2008). Short-time laboratory assays with the use of single species are advantageous for a fast acquisition of information about compounds' toxicity and selectivity. They are also good for the selection of the most suitable parameters to be assessed in longer exposure tests. Long-time (or chronic) assays allow the evaluation of the effects of compounds over extended periods of time. They can also include the testing of substances in different stages of development e.g. throughout the whole life-cycle of the organisms, assessing the effects on the organisms' reproduction and in sequential generations (Di Giulio 2008; Mortensen et al. 2010).

In these assays, parameters such as reproduction, respiration and feeding rates, growth, development rate and biomarkers are analyzed (Vogt et al. 2007; Jungmann et al. 2009; Paumen et al. 2008; Lee and Choi 2009). The assessment of these life-cycle traits become relevant to the attempt to link the effects on lower levels of biological organization to changes in population dynamics (Forbes et al. 2010).

1.2. Biomarkers in ecotoxicology

It is known that stress triggers the activation of cellular, physiological and/or behavioral mechanisms in order to minimize possible adverse effects to the organism (Servia et al. 2006; Sancho et al. 2009; Ribeiro et al. 2001; Erk et al. 2011). These mechanisms require the use of some part of the energy accumulated by the organism, which together with the energy needed to maintain regular homeostasis affects the energetic balance of the organism, leading to less energy available for growth and reproduction (Smolders et al. 2004; Sokolova et al. 2012).

The growing knowledge of invertebrate's biochemistry allows, nowadays, a good interpretation of the information obtained by biochemical responses to a better evaluation of ecological risks (Choi 2004). Several types of biochemical markers have been increasingly used in investigations to obtain early signs of exposure to toxic substances. These markers enable the identification of contaminants' effects at a sub-cellular level, even before they are visible at higher levels of biological organization (Lemos et al. 2010). However, in contrast to what it is often said in ecotoxicology, biomarkers do not provide direct information about the contaminants' impacts on higher organization levels (Di Giulio 2008). In fact, they still require integration with a series of other biological, physiological and biochemical parameters in way to establish a connection between the different responses in different levels of biological organization (Domingues 2007; Crane 2002). When it comes to organisms with complex life-cycles, there is a need to know the potential differential effects on the distinct stages of development (Forbes et al. 2006). This is particularly relevant once the impacts observed in other life-cycle traits (e.g. survival, fecundity, time to first reproduction) may have impact on population growth, although the effects on these life-cycle traits might not be linear or consistently causative of alterations on population dynamics (Forbes et al. 2010). Still, biomarkers are worthy evaluating since they can provide early information about the pollutant's effect in such a way that become complementary tools to methods utilized in the ecological risk monitoring (Walker 2004).

1.2.1. Energetic markers: Scope For Growth

One of these biomarker tools is the Scope for Growth (SfG). This method rationale is based on the energetic balance of the organism and it quantifies the energy available for growth and reproduction, through the measurement of the energy gained after feeding and the energy expended by metabolic processes. SfG is given by the following formula as described by Winberg (1960):

$$SfG = A - (R + U)$$

where *A* is the energy assimilated through food consumption *R* is the energy lost through respiration and *U* is the energy lost through excretion.

Scope for growth has been used to evaluate the effects of metals such as zinc, copper, lead and cadmium (Mubiana and Blust 2007; Naylor et al. 1989; Smolders et al. 2005; Munari and Mistri 2007) and also natural stressors as temperature, salinity, food limitation and oxygen levels (Wang et al. 2011; Smolders et al. 2005; Guzmán-Agüero et al. 2012). SfG has been successfully applied in laboratory and in situ ecotoxicity studies using, clams, mussels, bivalves and freshwater crustaceans (Juhel et al. 2006; Wang et al. 2011; Munari and Mistri 2007; Verslycke et al. 2004b; Smolders et al. 2005; Naylor et al. 1989; Maltby et al. 1990a, b). Results in terms of SfG values can be either a positive or negative, meaning the organisms are able to grow under the implied conditions or not.

1.2.2. Energetic markers: Feeding and respiration

Over time SfG showed to be disadvantageous for being very laborious, because energy absorbed, assimilation efficiency, energy excreted and lost by respiration had to be measured in each organism (Bossuyt et al. 2005). This way, the assessment of energy changes evolved to separate measures of feeding rates and respiration.

Feeding, the major component in SfG, is a parameter that is easily assessed and at the same time is sensitive and reliable to be used in laboratory and field situations. Feeding rate has been used in laboratory and in field studies as an indicator of environmental alterations with several test organisms, such as crustaceans, bivalves and insects (Sancho et al. 2009; De Jonge et al. 2012; Felten et al. 2008; Buffet et al. 2011; Macedo-Sousa et al. 2007; Nilin et al. 2012; Leppänen et al. 1998; Soares et al. 2005; Pestana et al. 2007).

In their study, Leppänen et al. (1998) have investigated the effect of metal-polluted sediments on the feeding behavior of *Chironomus riparius*. The organisms were exposed to sediments collected from different polluted sites for 96h. The egestion rates of the organisms exposed to sediments with higher metal contamination were lower, suggesting a decreased feeding activity. Soares et al. (2005) addressed the postexposure feeding rates on *C. riparius* under light and dark conditions, concluding that their feeding rate was higher with light rather than in darkness. They also investigated the effect of temperature on feeding rate, observing a decrease in food consumption under exposure to 5ºC (Soares et al. 2005).

Along with feeding behavior, respiration is also a parameter tested in ecotoxicological studies. Respiration rates can be used as a proxy for metabolism and although laborious and implying the use of specific equipment are also sensitive measures used to address effects of chemical exposure in laboratory toxicity investigations (De Coen and Janssen 2003). This parameter has been assessed in organisms such as mussels, cockles, insects and crustaceans, exposed to e.g. metals, pesticides, acid mine drainage, hydrocarbons, biotic stressors and under different seasonal and spatial conditions (Widdows et al. 2002; Widdows et al. 1997; Smaal et al. 1997; Pestana et al. 2009; Pestana et al. 2007; Nilin et al. 2012; Macedo-Sousa et al. 2007).

Respiration rates of *C. riparius* have already been successfully used as a sensitive indicator of stress caused by simultaneous exposure to chemical (pesticide) and biotic (predation risk) stress (Pestana et al. 2009). Here, exposure to sub-lethal concentrations of the insecticide imidacloprid and of chemical cues

from predators caused significant increases of respiration rates of *C. riparius* larvae showing the metabolic costs of such exposures (Pestana et al. 2009).

1.2.3. Cellular Energy Allocation

The measurement of the amount of the different components of the energy budget (i.e. proteins, carbohydrates and lipids) is one possible tool to assess the effects of stressors on the energetic balance of the organisms (Verslycke et al. 2003; Wang et al. 2012).

Cellular Energy Allocation method (CEA) (De Coen et al. 1995) assesses the energy budget of the organisms by measuring the energy reserves available (E_a - total sugar, lipid and protein content) and the energy consumption (E_c - by electron transport system – ETS - activity) (Olsen et al. 2007). The ETS measurement is based on the activity of enzymes associated with oxidative phosphorylation and can be considered as a good indicator of energy consumption (Packard 1971; Packard 1985). Consumption of oxygen depends on biotic and/or abiotic factors that have specific effects and that may interact and influence the respiratory metabolism. Within biotic variables, age, weight and sex may have a major influence in respiration rates (Verslycke 2003). Glycogen is considered the main source of energy (Hamburger 1996), therefore, a decrease in glycogen content after stress conditions is related to an increase in its consumption derived from the need of higher amounts of energy in response to stress (Choi et al. 2001). Also, changes in lipid and protein content reflect a response to perturbations of the organism homeostasis that lead to a higher consumption of energy reserves (Verslycke et al. 2004b). So, the energy consumption must be measured simultaneously with lipid, sugar and protein content (Verslycke et al. 2004).

Cellular Energy Allocation can be calculated by different forms. Samples for CEA analysis may be taken at different moments of the test, calculating the final CEA value by integration of the changes over the total time of exposure as suggested by De Coen and Janssen (2003). It can also be given by the difference

between E_a and E_c (CEA=E_a-E_c) (Rueda-Jasso et al. 2004) or by the ratio between E_a and E_c (CEA=E_a/E_c) (Verslycke et al. 2004b).

The method of CEA has been applied in many ecotoxicology studies to assess the effect of chemical stressors such as metals, insecticides, biocides, (Muyssen et al. 2002; Verslycke et al. 2004b; Erk et al. 2008) as well as natural stressors as salinity, temperature and oxygen levels (Erk et al. 2011; Verslycke and Janssen 2002; Choi et al. 2001).

The freshwater crustacean *Daphnia magna* has been object of several studies for the assessment of alterations in the energy budget caused by exposure to different stressors (De Coen and Janssen 2003; De Coen et al. 1995; Filho et al. 2011; Vandenbrouck et al. 2009; Bossuyt et al. 2005; De Coen et al. 2006). Other studies were performed with the mysid shrimp *Neomysis integer* (Erk et al. 2008; Verslycke et al. 2003; Verslycke and Janssen 2002; Verslycke et al. 2004b; Verslycke et al. 2004a), gastropods (Moolman et al. 2007), mussels (Erk et al. 2011; Gagne et al. 2007; Smolders et al. 2004) and insects (Choi et al. 2001; Bagheri et al. 2010).

CEA has become a commom parameter in ecotoxicity studies since it measures energy consumption and energy allocation together, giving an energetic integrative analysis (Verslycke and Janssen 2002), i.e. instead of enzymatic measures that give a more instantaneous image of the organism's status, CEA provides the visualization of changes in organism's energy budget caused by different stressors . Other advantage is the transformation of the energy reserves available and energy consumption into energetic equivalents, allowing its integration in an overall energy budget value that can be compared between different organisms and stressors (De Coen and Janssen 2003). The simple measurement of ETS activity as a respiration parameter is also advantageous because it is much less laborious than measuring the whole-organism respiration rates, as it is done in SFG, and equally reliable since the electron transport system is directly related to the process of oxygen consumption (De Coen and Janssen 2003). In fact, CEA has shown similar sensitivity when compared to the SfG technique (Verslycke et al. 2004b).

1.3. Test-organism: *Chironomus riparius*

The family Chironomidae includes a group of determinant species in aquatic systems, including the most ubiquitous species of insects (Faria et al. 2007). The species *Chironomus riparius* has been largely used in toxicity assays due to its wide geographic distribution and sensitivity to environmental stress, together with its easy laboratory culture and relative short life-cycle (Péry et al. 2003; Ha and Choi 2008). These organisms have an important role in the food chain, serving as food to fish and other vertebrates (Ha and Choi 2008). Communities of benthic organisms as *C. riparius* are exposed to different kind of organic and inorganic contamination suspended in the water or deposited in the sediment they inhabit (Mäenpää 2003). Their close association with sediment, where pollutant compounds can be accumulated, makes essential the study of the impact of toxic substances in these communities, because they are responsible for the transfer of energy, nutrients, and even contaminants to higher trophic levels (Langer-Jaesrich 2010; Mäenpää 2003).

Several studies have been performed on the biological and molecular responses of *Chironomus riparius* exposed to pesticides, metals, nanoparticles, antibiotics (Park 2009, 2010; Nair and Choi 2011; Nowak 2008; Dias et al. 2008; Azevedo-Pereira et al. 2011) and abiotic stressors as temperature, salinity and oxygen levels (Bervoets et al. 1995; Choi et al. 2000; Pestana et al. 2009). Some of the analyzed parameters include mortality (or survival), growth (larvae body size and body weight), feeding rate, respiration, adult sex ratio, adult emergence (time to emergence, emergence rate), reproduction (oviposition, number of egg per egg-mass, number of egg-mass per female) and biomarkers (enzymatic/energetic responses) (De Haas et al. 2004; Leppänen et al. 1998; Marinković et al. 2011; Paumen et al. 2008; Péry and Garric 2006; Vogt et al. 2007; Jungmann et al. 2009).

1.4. Contaminants

1.4.1. Cadmium

Cadmium is a toxic and widespread metal which has harmful effects on organisms (Henson and Chedrese 2004; Waisberg et al. 2003) and it is originated from different sources, such as industrial and anthropogenic (Korte 1982). Cadmium accumulates in sediments (Jung et al. 2005) and affects the benthic biota causing negative effects such as oxidative stress, genotoxicity and impairment of growth, behaviour, emergence, reproduction and population growth rate of chironomids (Barata et al. 2005; Vogt et al. 2010; Marinković et al. 2011; Nair and Choi 2011; Nair et al. 2011; Lee et al. 2006).

1.4.2. Insecticide Movento®

Pesticides differ from other environmental pollutants by entering the environment due to their deliberate use by Man for specific purposes. When a pesticide is applied onto a crop, its majority is absorbed by plants and animals (Slikker 2010; Pretty 2005). Still, some of it is spread through the environment by being vaporized and rainfall leads to its accumulation in the soil. It also may reach surface and ground waters by runoff and leaching (Slikker 2010). This makes monitoring and minimization of the harmful effects of these compounds to the environment and to humans crucial (Costa 2008).

Spirotetramat (Movento®) is a new insecticide of the tetramic acids' class which acts by inhibiting lipid biosynthesis (Maus 2008). As a consequence of lower lipid content, juvenile organisms suffer an inhibition of their growth and see their reproduction compromised (Chen 2010). Spirotetramat is thus highly effective against juvenile insects and decreases the fecundity and fertility of adult females, promoting a decrease in insect populations (Brück 2009; Nauen 2008). Such properties make it an adequate compound to the control of persistent species and it is applied worldwide to eliminate pests as aphids (*Aphis* spp., *Myzus* spp.,

Dysaphis spp., *Toxoptera* spp., *Phorodon humuli*), psyllids (*Psylla* spp., *Paratrioza cockerelli*) and whiteflies (Bemisia spp., *Trialeurodes vaporariorum*) (Nauen 2008). This insecticide remains active through a large range of temperatures and has a high residual activity, i.e., after its implementation on leaves it moves through the vascular system of the plants, acting in several sites during long periods of time. In this way, allows the control of roots attacking pests, also protecting leaves and new shoots after its implementation (Brück 2009).

There is scarce information on the toxicity and ecological risk of Movento®. The insecticide has shown a relatively fast degradation on sediment and water and a low toxicity for *Daphnia magna* and *Chironomus riparius*. In table I are described some of the properties of Movento®.

Table I. Properties of the insecticide Movento® (adapted from Bayer CropScience (2010), Brück et al. (2009) and Austrian Agency for Health and Food Safety (2012)).

Commercial name		**Movento®**
Comum name		Spirotetramat
Chemical name (IUPAC)		ethyl-cis-3-(2,5-dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl carbonate
Chemical formula		$C_{21}H_{27}NO_5$
Molecular weight		373.45g/mol
Melting point		142ºC
Vapor pressure		$1.5x10^{-8}$ Pa (25ºC)
Water solubility		29.9 mg/L (pH=7)
Log K_{ow}		2.5 (pH=4, 7 and 9)
Half-life		32.5d (pH=4, 25ºC) 8.6d (pH=7, 25ºC) 0.32d (pH=9, 25ºC)
Toxicity	*Daphnia magna*	NOEC = 20.3 mg a.i./L; EC_{50} > 42.7 mg a.i./L
	Chironomus riparius	NOEC = 0.1 mg a.i./L; LC_{50} = 1.6 mg a.i./L

1.5. Aim of the study

The work presented in this thesis focused on the adaptation of cellular energy allocation methodology to *C. riparius*, a model species in ecotoxicological studies. After that the effects of Cadmium and Movento® (spirotetramat) on CEA components were assessed on *C. riparius* larvae. To evaluate the sensitivity and relevance of this energetic biomarker, effects were simultaneously assessed with classic parameters such as growth or emergence. The objective was to investigate the suitability of CEA methodologies in ecotoxicological studies using *C. riparius* as test organisms.

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CHAPTER II

2. Effects of Cadmium and the pesticide Movento® on *Chironomus riparius'* cellular energy allocation

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2.1. Abstract

Cellular Energy Allocation is an energetic biomarker which is addressed through the ratio between the available energy and the energy consumed. The sum of total protein, carbohydrate and lipid content represents the energy of the available reserves and the energy consumed is given by the activity of the electron transport system. In this work this biomarker was used as a tool to assess the effects of sublethal concentrations of Cadmium Chloride and the insecticide Movento® in exposed *Chironomus riparius*. The changes in the Cellular Energy Allocation were compared with classical endpoints (growth and emergence) in order to assess the sensitivity and relevance of this biomarker. The energetic budget biomarker revealed less sensitivity than the classic endpoints for both contaminants. Contrary to the insecticide exposure, where no differences in energy allocation were observed, exposure to cadmium resulted in an energetic allocation shift, caused by increased metabolic energy demand.

Although CEA is used as a sensitive biomarker in different invertebrate species, care should be taken when using these methodologies in model test species, such as *C. riparius*, which have a rapid and complex life-cycle.

2.2. Introduction

Cellular Energy Allocation (CEA) is a biomarker approach that allows the determination of the energy reserves available and the energy consumed by the organism, when exposed to any type of condition. Initially described by De Coen et al. (1995), CEA is an integrative measure of the total content of proteins, lipids and carbohydrates as the energy reserves available and the electron transport system (ETS) activity as the energy consumed, giving an overall assessment of the metabolic status of the organisms.

When under stress, either by the presence of contaminants or other type of stressors, organisms tend to expend more energy in order to resist their action (Wang et al. 2012). As a result, the energy reserves decrease and essential functions such as growth or reproduction may be negatively affected (Moolman et al. 2007). CEA analysis provides us information about the energy costs, thus the metabolic status of the organism, through quantification of both fractions: energy available after stress exposure and energy consumption (Olsen et al. 2008; Choi et al. 2001).

This methodology has been applied to different species and it has shown to be a sensitive endpoint in *Daphnia magna* exposed to contaminants such as copper, mercury, lindane, TBT, and binary metal mixtures (Bossuyt et al. 2005; Vandenbrouck et al. 2009; De Coen and Janssen 2003), for the estuarine mysid shrimp *Neomysis integer* exposed to the pesticide chlorpyrifos (Verslycke et al. 2004), and for insects (Tassou and Schulz 2009) and silkworm larvae (Etebari et al. 2007) exposed to the insecticide pyriproxyfen. CEA has also been successfully tested on fish, mussels and gastropods exposed to different contaminants (Ayuningtias 2011; Erk et al. 2011; Wang et al. 2012; Rueda-Jasso et al. 2004).

In this study, CEA methodology for *Chironomus riparius* was developed and the impacts of Cadmium and Movento®'s short exposure on this integrative energetic biomarker are assessed.

The midge larvae of the species *Chironomus riparius* have been shown to be a good sentinel of environmental pollution and are commonly used in laboratory assays to test the effect of contaminants due to their easy maintenance in the laboratory, short-life cycle, wide geographic distribution and sensitivity to

environmental stress (Faria et al. 2007; Dias et al. 2008; Vogt et al. 2007). Surprisingly, CEA methodology was never adapted for this model species.

Cadmium is a non-essential metal which free ionic form (Cd^{2+}) makes it more bioavailable to aquatic organisms, and thus more toxic (Vellinger et al. 2012). Cadmium has been proved to cause several effects on organisms, such as decreasing feeding rate and locomotor activity in the amphipod *Gammarus pulex* (Vellinger et al. 2012) and reduced growth, survival and reproduction in *Daphnia magna* (Smolders et al. 2005). Also *C. riparius* are known to suffer effects such as delayed emergence, decreased number of egg-masses per female and decreased population growth rate when exposed to cadmium (Vogt et al. 2010).

There is scarce information about the effects of Movento® (Spirotetramat) on aquatic organisms. It acts by lipid synthesis inhibition and it has been labeled has a low-risk insecticide (Klempner 2008). It is effective against aphids and whiteflies, especially juveniles, decreasing the females' fecundity and fertility (Varenhorst 2011; Klempner 2008). It is known to have no acute toxic effects on mammals and birds, however, it is toxic to fish (Maus 2008) and to fresh water invertebrates (National Registration Authority for Agricultural and Veterinary Chemicals 2009). Its toxicity for invertebrates as *Daphnia magna* and *Chironomus riparius* is considered to be low (Austrian Agency for Health and Food Safety 2012).

By using these two dissimilar acting contaminants, our main objective is to evaluate CEA sensitivity and relevance compared to classical endpoints (growth and midge emergence) in order to assess its suitability in ecotoxicity studies with *C. riparius*.

2.3. Materials & Methods

2.3.1. Laboratory culture and maintenance of *Chironomus riparius*

Chironomus riparius Meigen (Diptera, Chironomidae) larvae were obtained from a laboratory culture established at the Biology Department, University of Aveiro, for more than 10 years. The culture is maintained in ASTM hard water medium in plastic containers with a 2cm sediment layer. The culture medium is changed every week and the larvae are fed every two days with a suspension of grounded TetraMin®. The culture is maintained at constant temperature (20 ± 1ºC) and light conditions (16h light/ 8h dark).

2.3.2. Organisms' exposure to Cadmium and Spirotetramat (Movento®)

Organisms from the 3rd instar (8 days) were exposed to a gradient of concentrations of Cadmium chloride (technical grade, CASNo. 10108-64-2, Sigma-Aldrich, USA) (50, 100 and 200 µg/L) and Spirotetramat (Movento® 150 OD Insecticide, Bayer CropScience) (0.5, 3 and 18 µg/L). For each concentration and controls, 16 replicates were used, each with 15 organisms, as it is showed below:

8 replicates x 15 org	+	4 replicates x 15 org	+	4 replicates x 15 org
CEA		Growth		Emergence
48h		6 days		28 days

Eight replicates were used to assess CEA after 48 hours of exposure, four replicates to assess growth after 6 days and the remaining four replicates were used to check for emergence during 28 days of exposure. Mortality was registered after 2 and 6 days of exposure and at the end of the test. All the tests were performed in the same conditions as described for culturing.

The organisms were placed in 1L flasks with a 2cm sediment layer each containing 600ml of each test solution. Water spiking was made from a stock solution of 15mg Cadmium/L and of 0.5mg Spirotetramat/L.

2.3.3. Growth and emergence

After 6 days of exposure, organisms were removed from each treatment and total body length and head capsule width were measured under a stereo dissecting microscope. The emergence of adults was recorded daily throughout the experiment, in order to calculate the cumulative percentage of emergence and the mean time to emergence of the organisms from all treatments.

2.3.4. Cellular energy allocation analysis

Organisms for CEA measurements were collected after 48h of exposure. Animals were quickly passed through filter paper to remove any superficial water, promptly weighed (fresh weight), and frozen in liquid nitrogen and kept at -80ºC until analysis.

Energy available (sugars, lipids, and proteins) and energy consumption (ETS) were quantified following the method of De Coen et al. (1995) with minor modifications. Samples with pools of 15 larvae each were homogenized by sonication in 1000µl of ultra-pure water from which 300µl

portions each were taken for the analysis of sugar content, lipid content and ETS activity, and the remaining 100µl for protein quantification.

All reagents used were of analytical or molecular grade and purchased from various suppliers (such as Scharlau, Panreac, Fisher Scientific and Sigma-Aldrich). All spectrofotometric measurements were performed in the microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA)

2.3.4.1. Energy available (E_a)

The available energy (Ea) includes the following components: total carbohydrates, lipids and proteins.

Total protein content was quantified by Bradford's method after a 4:1 dilution of the sample in ultra-pure water. After 30min incubation at 20ºC the absorbance was measured in the microplate at 592nm with bovine serum albumin as a standard.

Carbohydrate content was quantified by adding 100µl of 15% TCA to the 300µl of homogenate and incubating at -20ºC for 10 min. After centrifugation (3500rpm, 10min, 4ºC) the supernatant was used for carbohydrate quantification. The measurement of carbohydrate content was made by adding 50µl of 5% phenol and 200µl of H_2SO_4 to 50µl of the treated sample in the microplate. The samples were incubated at 20ºC for 30min and the absorbance was measured at 492nm in the microplate with glucose used as a standard.

Total lipid content was quantified by adding 500µl of chloroform (119.38M; ACS spectrophotometric grade, ≥99.8%, contains 0.5-1.0% ethanol as stabilizer) and methanol (32.04M; ACS reagent, ≥99.8%) to 300µl of the homogenized sample. After centrifugation (3500rpm, 5min, 4ºC) the top phase and the thin layer were discarded and 100µl of the remaining phase were transferred to glass tubes for lipid quantification. Then, 500µl of H_2SO_4 were added to each sample and incubated at 200ºC for 15min. After cooling

down to room temperature, 1500µl of ultra-pure water were added to each tube and the absorbance measured in the microplate at 375nm and tripalmitin used as a lipid standard.

2.3.4.2. Energy consumed (E_c)

Electron transport system activity was measured by adding 150µl of homogenization buffer (0.3M Tris; 150% (w/v) Poly Vinyl Pyrrolidone; 8mM $MgSO_4$; 0.6% (v/v) Triton X-100) to 300µl of the initial homogenate. After centrifugation (3500rpm, 10min, 4ºC) the supernatant was removed for ETS activity measurement. Then, 50µl of each sample were transferred to the microplate and 150µl of a buffered solution B [2% (v/v) solution A (6.67M Tris; 0.27% (v/v) Triton X-100); 1.8mM NADH; 280µM NADPH], and 100µl of INT solution (p-IodoNitroTetrazolium; 8mM) added. The absorbance was immediately measured kinetically at 490nm over a 3 min period.

2.3.4.3. Cellular energy allocation calculation

The fractions of the energy available were transformed into energetic values using the corresponding energy of combustion: 39500 mJ/g lipid, 17500 mJ/g glycogen, 24000 mJ/g protein (De Coen & Janssen, 1997). The cellular oxygen consumption rate was determined by following the stoichiometrical relationship in which for 2µmol of formazan formed, 1 µmol of oxygen is consumed. The calculation of the quantity of oxygen consumed was determined by using the formula of Lambert-Beer: $A = \varepsilon \times l \times c$ (A = absorbance; ε for INT-formazan = 15900/M.cm; l = 0.9; c = oxygen consumed). The values obtained were then transformed into energetic values using the specific oxyenthalpic equivalent for an average lipid, protein and

carbohydrate mixture of 480 kJ/mol O_2, giving us the final E_c value. E_a value is given by the sum of protein, carbohydrate and lipid fractions, and the final CEA value is obtained by the formula: CEA= E_a/E_c

The energetic values obtained for lipid, protein and sugar content and ETS activity were adjusted to the weight of the organisms using the following allometric equation as suggested by (Penttinen and Holopainen 1995):

$$Z = Y\ (M^{-0.71})$$

where Y is the energetic value of lipid, protein or sugar content or ETS activity after transformation with the respective energy combustion values, M is the fresh weight of the sample and Z is the final value corrected to the weight of the organisms.

2.3.5. Statistical analysis

All data were checked for normality and homoscedascity. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison of means was employed to determine differences relatively to control treatment. For all statistical tests the significance level was set at $p \leq 0.05$ and all calculations were performed with SigmaStat software package (Systat Software Inc., 2006).

2.4. Results

2.4.1. Effects on *C. riparius* growth and emergence

After 6 days exposed to cadmium the mean body length of larvae was 11.4 ±0.49mm in the control with a significant decrease throughout the concentrations and the lowest size of 5.85 ±0.13mm in the highest concentration (table II). The same effect was observed for head capsule width, which decreased from a mean value of 0.52 ±0.006mm in the control treatment to 0.32 ±0.007mm at 200µg/L of cadmium. Larvae from the control, 50 and 100µg/L treatments changed to the 4th instar while the larvae from the highest concentration of cadmium remained in the 3rd instar of development.

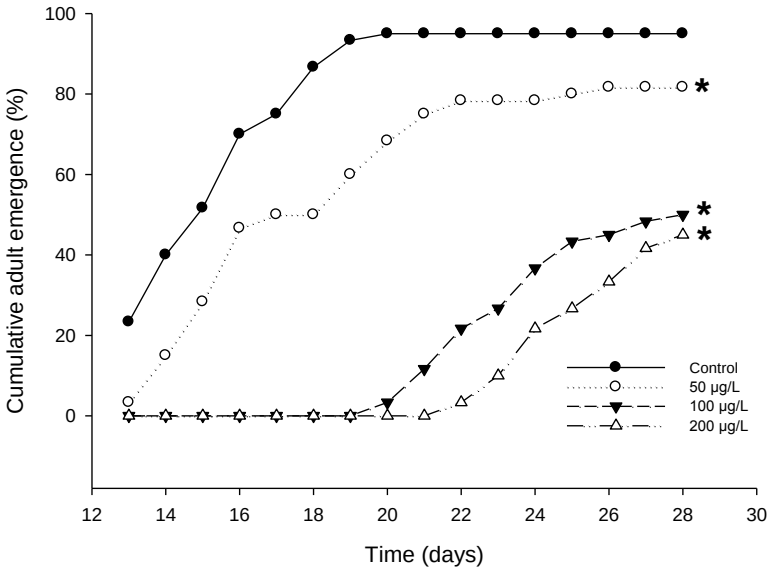

Fig.1. Cumulative percentage of emergence of *Chironomus riparius* exposed to cadmium. An asterisk indicates a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's test).

Organisms exposed to cadmium also suffered a significant increase in the time to emergence which reached a mean value of 25.4 ±1.6 days in the highest concentration tested compared to 15.4 ±2.0 days in the control treatment (table II). The cumulative percentage of emergence suffered a significant decrease with cadmium concentrations from 95% in the control treatment to a minimum value of 45% at 200µg/L (fig. 1).

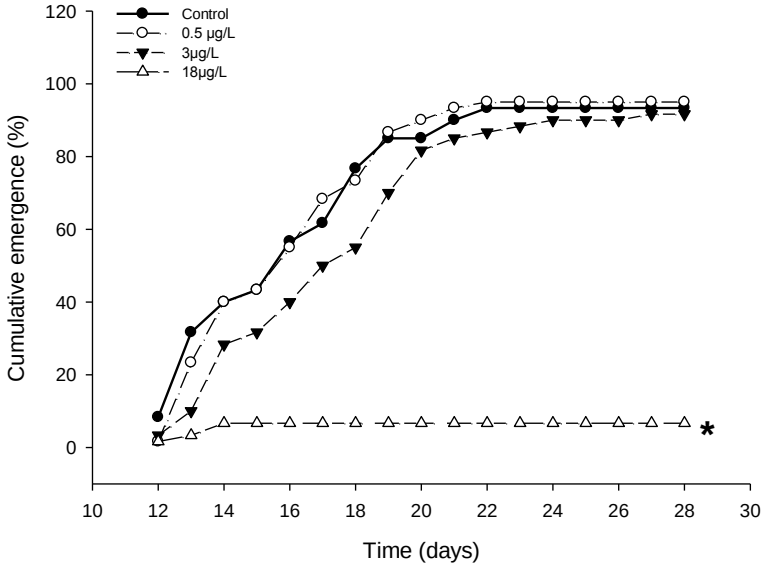

Fig. 2. Cumulative percentage of emergence of *Chironomus riparius* exposed to Movento®. An asterisk indicates a significant difference from the control at p ≤ 0.05 (ANOVA, Dunnett's test)

Organisms exposed to Movento® only showed significant effects in the body length of larvae exposed to 18µg/L which decreased from a mean value of 11.54 ±0.26mm in the control treatment to 9.16 ±0.19mm (table II). Despite the absence of statistically significant differences, Movento® promoted a decrease in the number of surviving organisms at 18µg/L, both after 6 days of exposure and throughout the experiment, which consequently led to a low percentage of emerged adults in the highest concentration. Because only four larvae

successfully emerged in this treatment time to emergence cannot be presented. (table II, fig. 2). No differences were observed for the other parameters and concentrations tested (table II).

Table II. Mean values (± SD) for survival and growth after 6 days of exposure and time to emergence of *Chironomus riparius* exposed to cadmium and Movento®. An asterisk indicates a significant difference from the control at p≤0.05 (ANOVA, Dunnett's test).

	Concentration (µg/L)	% Survival	Body length (mm)	Head capsule width (mm)	Time to emergence (days)
Cadmium	0	94.44 ± 0.96	11.4 ± 0.49	0.52 ± 0.006	15.37 ± 2.04
	50	91.11 ± 9.80	10.21 ± 0.19*	0.49 ± 0.016*	17.17 ± 3.06*
	100	81.67 ± 27.4	7.01 ± 0.29*	0.35 ± 0.029*	23.27 ± 2.12*
	200	84.17 ± 20.9	5.85 ± 0.13*	0.32 ± 0.007*	25.35 ± 1.64*
Movento®	0	95.28 ± 3.37	11.54 ± 0.26	0.50 ± 0.006	15.71 ± 2.82
	0.5	94.44 ± 4.19	11.37 ± 0.78	0.50 ± 0.018	15.95 ± 2.62
	3	95.00 ± 5.00	11.62 ± 0.31	0.50 ± 0.008	17.26 ± 3.22
	18	60.46 ± 46.5	9.16 ± 0.19*	0.50 ± 0.004	--- a)

a) only four adults

2.4.2. Effects on *C. riparius* cellular energy allocation

The levels of the different energy fractions of *C. riparius* exposed to cadmium and Movento® are presented in table III. In the cadmium experiment, the carbohydrate content of the organisms significantly rose to a mean value of 715.3 ±66.3 mJ/org at 50µg/L of cadmium, when comparing to control. Carbohydrate content began to decreased in the next concentration reaching a mean value of 367.7 ±66.2 mJ/org at 200µg/L. Protein values remained similar among the concentrations, with no statistical differences. Lipid content significantly increased in the highest concentration of cadmium with a mean value of 1316.2 ±194.6 mJ/org compared to control (1031.1 ±157.0). E_a values for cadmium (fig. 3) showed no differences between treatments and E_c values were significantly higher than control at 100 and 200µg/L (fig. 4). CEA suffered a significant reduction at 100 and 200µg/L of cadmium as a result of the higher energy consumed (ETS activity) (fig. 5).

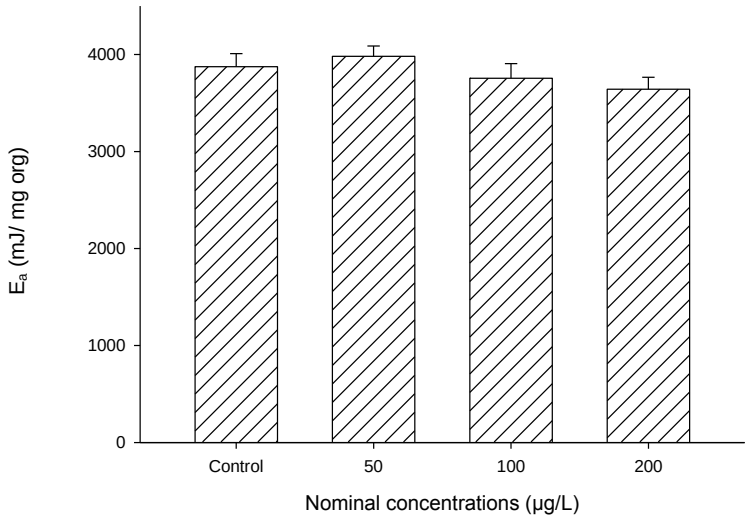

Fig. 3. Effect of 48h exposure to sub-lethal concentrations of cadmium on the energy available of *Chironomus riparius.* Error bars represent standard error of the mean.

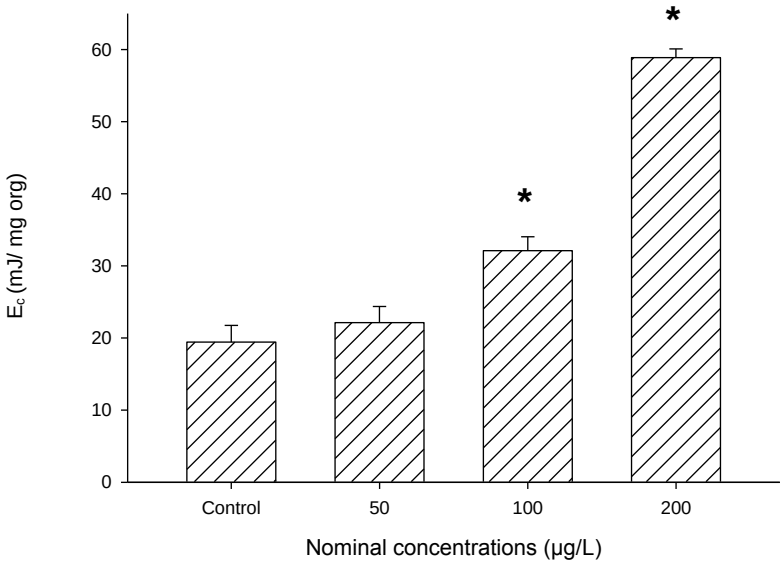

Fig. 4. Effect of 48h exposure to sub-lethal concentrations of cadmium on the energy consumption of *Chironomus riparius.* Error bars represent standard error of the mean. An asterisk indicates a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's test).

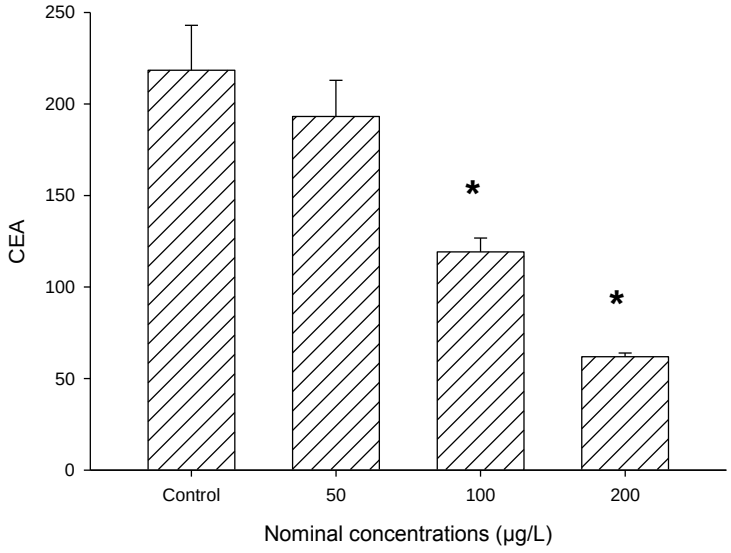

Fig. 5. Effect of 48h exposure to sub-lethal concentrations of cadmium on the cellular energy allocation of *Chironomus riparius.* Error bars represent standard error of the mean. An asterisk indicates a significant difference from the control at $p \leq 0.05$ (ANOVA, Dunnett's test).

Table III.

Available energy fractions (mean ± SD) in the total body of *C. riparius* exposed to sub-lethal concentrations of cadmium and Movento®. An asterisk indicates a significant difference from the control at p ≤ 0.05 (ANOVA, Dunnett's test).

	Concentration (µg/L)	$E_{(carbohydrates)}$ (mJ/ mg org)	$E_{(proteins)}$ (mJ/ mg org)	$E_{(lipids)}$ (mJ/ mg org)
Cadmium	0	514.8 ± 189.2	2329.0 ± 319.9	1031.1 ± 157.0
	50	715.3 ± 66.3*	2408.7 ± 256.8	857.5 ± 94.2
	100	607.2 ± 83.3	2194.0 ± 382.1	954.1 ± 115.7
	200	367.7 ± 66.2*	1958.0 ± 306.2	1316.2 ± 194.6*
Movento®	0	1171.0 ± 437.8	3406.0 ± 201.8	995.8 ± 135.1
	0.5	869.0 ± 446.6	3455.0 ± 346.9	1137.6 ± 261.2
	3	1226.5 ± 258.9	3400.6 ± 205.5	948.4 ± 78.8
	18	1011.0 ± 418.9	3529.0 ± 395.2	1003.4 ± 145.1

No differences were observed in any energy fraction of the organisms exposed to Movento®, in any of the concentrations tested (table III). E_c and E_a values for the organisms exposed to Movento® presented thus no significant differences comparing to control (fig. 6 and fig. 7) which consequently resulted in the absence of effects also in CEA (fig. 8).

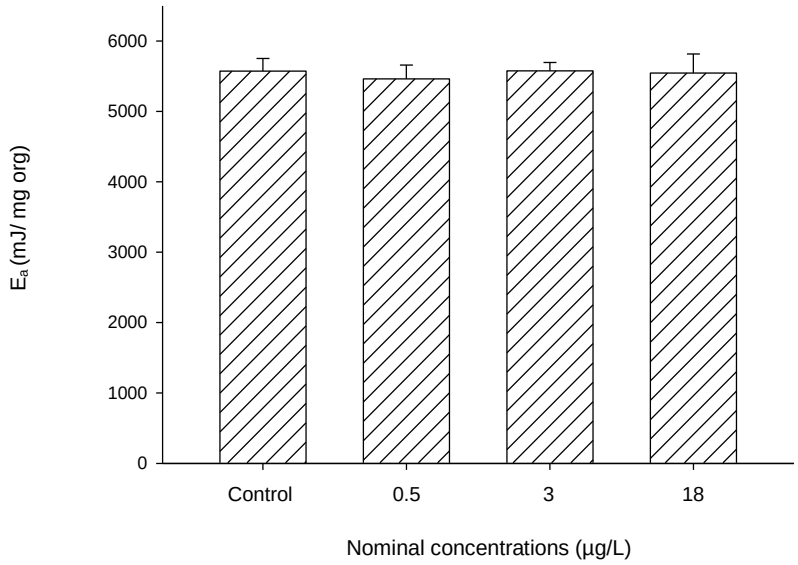

Fig. 6. Effect of 48h exposure to sub-lethal concentrations of Movento® on the energy available of *Chironomus riparius.* Error bars represent standard error of the mean.

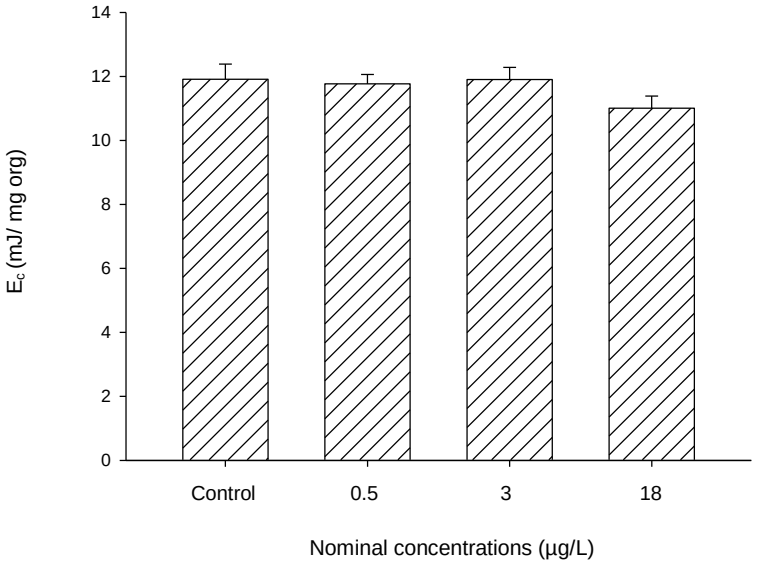

Fig. 7. Effect of 48h exposure to sub-lethal concentrations of Movento® on the energy consumption of *Chironomus riparius.* Error bars represent standard error of the mean.

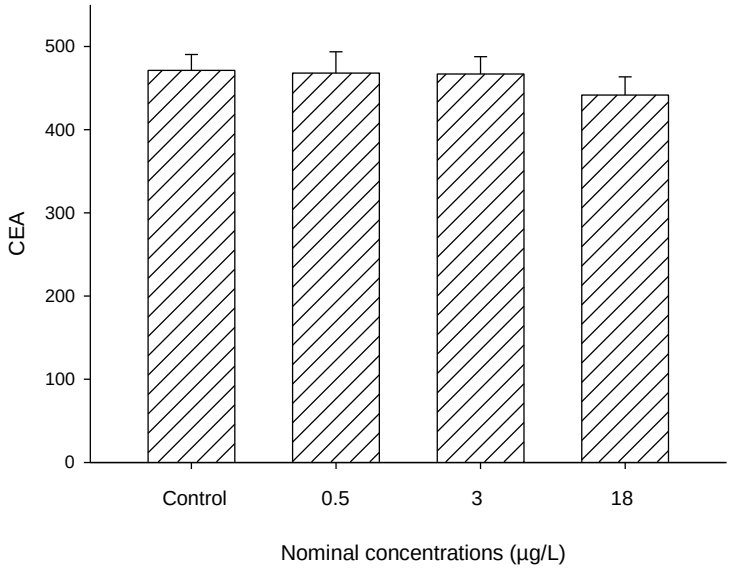

Fig. 8. Effect of 48h exposure to sub-lethal concentrations of Movento® on the cellular energy allocation of *Chironomus riparius.* Error bars represent standard error of the mean.

2.5. Discussion

Cellular Energy Allocation has been used to assess the effect of contaminants on the energy balance of several organisms (Erk et al. 2008; Smolders et al. 2004; Stomperudhaugen et al. 2009; Verslycke et al. 2004; De Coen et al. 1995; De Coen et al. 2001; De Coen and Janssen 2003). Its relevance before other biomarkers resides in the fact of combining the energy reserves of the organisms with the energy consumption, reflecting the energy status and giving a perspective of their overall condition (De Coen and Janssen 2003).

Cadmium has been proved to impair the development of *Chironomus riparius* (Vogt et al. 2010; Nair and Choi 2011). The present work supports this fact by showing a clear effect of cadmium exposure on the development and metabolism of *C. riparius*. A decrease of CEA may be due either to a reduction in the energy reserves (E_a) and/or increase of the energy consumed (E_c) (Olsen et al. 2007). As expected, under cadmium contamination, *C. riparius* presented higher energy consumption than in control. This is coincident with a higher metabolic activity used to fight the contamination effects (Gagne et al. 2007; De Coen and Janssen 2003). So, despite no changes in the energy reserves were detected, the increasing ETS activity suggests a higher energy demand that caused a reduction in the energy available for growth (decrease in CEA values) (De Coen et al. 2000; De Coen and Janssen 2003; Smolders et al. 2004). The responses of *C. riparius* in terms of energy allocation are in line with what is observed in terms of reduced growth and development meaning that CEA results for cadmium were correlated with effects at higher levels of biological organization. It was also clear that metabolic expenditure (E_c) is the component responsible for this effect. The absence of similar effects on energy reserves (E_a) might be explained by compensatory mechanisms in terms of feeding and assimilation efficiency or by the reduced exposure period (48 hours).

The primary source of energy are the carbohydrates followed by lipids and then proteins (Ayuningtias 2011). Thus, the absence of effects on the protein content in the presence of cadmium may be explained by the fact that proteins are the last fraction of energy that is consumed combined with the short exposure

period tested (Ayuningtias 2011; Erk et al. 2011; Smolders et al. 2004). However, there was an unexpected increase in carbohydrate and lipid content. Other studies have also reported unexpected shifts in the energy budget of test organisms (Verslycke and Janssen 2002; Verslycke et al. 2004; Bagheri et al. 2010; Filho et al. 2011). Increase in carbohydrate content was also observed in Bagheri et al. (2010) along with the absence of effect on lipid content. Here the authors assumed the argument from Cymborowski (1992) who has associated increasing lipid content to lipid accumulation resultant from changes in metabolic pathways.

In the experiment with Movento® the organisms did not suffer alterations in the energy budget and effects were only significant for body size and time to emergence for the highest concentration (18µg/L). The lack of a value for the time to emergence for the highest concentration of Movento® is due to few adult organisms used for the calculation of the mean. In this concentration, only four larvae emerged, which was a result of the lower number of pupae found. In this concentration, 22 of the 60 larvae initially used were dead after 28 days of exposure (data not shown), suggesting that few larvae resisted the effect of the insecticide. However, 8 larvae were alive at day 28, which means that, if they were able to pupate and emerge, the mean time to emergence of the organisms from this concentration of Movento® (18µg/L) would be higher than for the other concentrations. Spirotetramat thus clearly reduced growth and development rates of *C. riparius*.

In the same reasoning as for the cadmium exposure, it was expected that Movento® would induce an increase of energy consumption and/or decrease of the energy available. The absence of alterations in E_a and *Ec* components of organisms under Spirotetramat exposure may be associated with organisms' defense strategy as described by De Coen et al. (1998). They have linked the conservation of energy reserves to an energy-saving strategy where the organisms under stress diminish their metabolic and feeding activity in order to save their available energy reserves. Another reason for the difference in ETS activity between cadmium and spirotetramat exposure may be the different mode of action of the contaminants. Cadmium it is known to cause oxidative stress, promoting several reactions with oxygen and reactive oxygen species, production

of enzymes that prevent oxidative damage (superoxide dismutase, peroxidase, catalase, glutathione peroxidase) and disruption of membrane-bound electron transport (Sun and Zhou 2008). This way it was expected that cadmium would readily affect the electron transport system due to a higher metabolic activity contrarily to spirotetramat which acts primarily by lipid biosynthesis inhibition. Again, altered assimilation efficiency could have contributed for the absence of effects on the energy allocation under spirotetramat exposure (Stoks et al. 2005). Moreover, a constant ETS activity may be explained by a short time of exposure to the contaminant (Filho et al. 2011; Moolman et al. 2007). In fact, Moolman et al. (2007) have applied longer exposure periods to their organisms so changes in energy reserves could be detected and associated with a chronic exposure. After a 2-week exposure of *Melanoides tuberculata* to different levels of cadmium and zinc it was observed a decrease of the energy reserves, except for lipids. The different exposure periods tested for CEA and growth measurements can thus explain why reduced growth was not correlated with effects on energy allocation despite the specific mode of action of Spirotetramat.

Toxicity values of Movento® (Spirotetramat) for *C. riparius* show a LC_{50} value of 1.6mg/L (1600µg/L) according to the Austrian Agency for Health and Food Safety (2012). Following chronic endpoints, in here the organisms showed more sensitivity to this insecticide, being the growth and emergence significantly affected at 18µg/L.

In general, and for both cadmium and Spirotetramat exposures, growth and emergence were more sensitive endpoints than CEA analysis.

It should be noted that the absence of results in terms of E_a or E_c might be related to different factors related to the experimental methods used and with physiological characteristics of the test species used.

First it should be noted that effects on CEA were assessed after 48 hours of exposure, whereas the other parameters were analyzed after longer exposure periods. Thus, prolonging the time of exposure for CEA analysis could allow the observation of effects on the energy allocation in lower concentrations.

In terms of organisms' physiology and as stated above, it should be kept in mind that physiological and or behavioral adaptations such as changes in feeding

or altered assimilation efficiency could affect energy acquisition and assimilation and thus complicate the interpretation of the effects of short exposure to contaminants. Food was provided during the exposure period mainly because it allowed us to exclude the hypothesis of changes in energy available as a result of starvation and to assess CEA, growth and emergence in the same conditions, in order to compare the results between the different parameters (Moolman et al. 2007). Moreover providing food avoids cannibalism of larvae.

It is also important to consider that in organisms with rapid and complex life-cycles as *C. riparius*, which go through molting and metamorphosis, energy allocation is dependent on the molting period. Molting is an energy consuming process and it is assumed that organisms store energy reserves prior to molt, since during molt larvae stop feeding and spend the previous stored energy in the process of new cuticle synthesis (Lorenz and Gade 2009). In our experiments we tried to use a short exposure period (48 hours) but it might not have been sufficient to eliminate these differences even if the organisms were in the same instar.

2.6. Conclusion

The results presented show that classic endpoints such emergence or larvae growth are more sensitive than cellular energy allocation. Moreover only with exposure to cadmium it was possible to relate effects at the different levels of biological organization.

Analysis of CEA suggests that Spirotetramat (Movento®) did not cause effects on the metabolism of the organisms, since no dose-response relationship was observed. *C.riparius*' growth and emergence were significantly reduced at 18 µg/L.

Although cellular energy allocation has been suggested as a sensitive and non specific energetic biomarker we advocated that for model species with rapid life cycles, classic parameters are more suitable to be used in environmental risk assessment. Growth and emergence of *C. riparius* are easily assessed in the

laboratory and showed higher sensitivity compared to effects of short exposures of cadmium and Spirotetramat on CEA.

CEA is nevertheless a useful ecotoxicological tool and it can be used to investigate how organisms respond to chemical stressors with specific modes of action, as a proxy for growth in organisms with long life-cycles and to study resistance mechanisms comparing different energy allocation strategies in organisms showing different tolerance to contaminants.

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CHAPTER III

3. General Discussion & Conclusion

The permanent contamination of the environment has increased the need of ecotoxicological testing in order to find new tools to predict effects at higher levels of biological organization and serve the environmental risk assessment purpose.

Energy based biomarkers like cellular energy allocation (CEA) have been proposed as non-specific biomarkers that are sensitive to a wide range of chemical stressors (Smolders et al. 2004) and that are directly linked with higher levels of biological organization (Smolders et al. 2004; De Coen and Janssen 2003; Olsen et al. 2007). However, CEA methodology was until now never applied to *C. riparius*, a model test species in ecotoxicological investigations. This is surprising since one advantage of CEA is the fact that it can be directly linked with higher levels of biological organization (Smolders et al. 2004; Stomperudhaugen et al. 2009; De Coen and Janssen 2003). This energy based biomarker can thus be used to assess effects of any type of stressors, which gives the possibility to be compared between different stress situations. Still, the great advantage is based on the transformation of the results into energetic values that can be used to compare effects between different species.

Thus the work presented consisted in adapting the CEA methodology to *C. riparius* evaluating the effects of two dissimilarity acting contaminants, cadmium and the insecticide spirotetramat.

The focus was the simultaneous assessment of three parameters: *C. riparius* larval growth (body size), emergence (cumulative percentage of emergence and time to emergence) and the energy based biomarker analysis. This allowed us to assess CEA's sensitivity and relevance before classical endpoints commonly used in standard guidelines with this species. The results showed that cadmium and Movento® caused negative effects on *C. riparius* development rates.

The Cellular Energy Allocation method has proved to be relevant for the identification of changes in the metabolic status of these organisms caused by exposure to cadmium, but irresponsive for spirotetramat exposure.

With this work we also were able to gather new information on the toxicity of spirotetramat for aquatic biota. Despite the absence of effects on the cellular energy allocation, *C. riparius* showed to be sensitive to low concentrations of this insecticide.

Concerning the suitability of using CEA in *C. riparius*, the results showed that classic endpoints such emergence or larvae growth are more sensitive than energy components of cellular energy allocation. Thus we conclude that CEA, at least for environmental risk purposes is not adequate. It is laborious compared to classic endpoints and no dose-response results were observed in energy reserves at least for the contaminants and exposure duration tested.

The results presented and gathered during this thesis revealed that further research is needed in terms of using energy based biomarkers in species with rapid and complex life cycles. Particularly important would be to evaluate how the different components of CEA respond to stress across different larval stages and even in different stages within intermolt periods. The process of molting is an energetically costly physiological process and energy allocation during molting can complicate the evaluation of CEA results.

On the other hand changes in feeding behavior and assimilation rates can also add confusion to the interpretation of results and thus a more refined investigation is critical. In order to correctly validate and understand the mechanism of action of contaminants on an organism energy budget, we suggest that at least effects on feeding rates should be evaluated simultaneously with CEA. Only then can we correctly link energy based responses to effects at higher levels of biological organization and improve environmental risk assessment strategies.

Nevertheless and when properly validated, CEA can be a sensitive and relevant ecotoxicological tool to investigate how organisms respond to chemical stressors with specific modes of action, as a proxy for growth in organisms with long life cycles and to study resistance mechanisms comparing different energy allocation strategies in organisms showing different tolerance to contaminants.

3.1. References

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