Universidade de Aveiro
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André Vinhas Mendes Fernandes

CONSCIÊNCIA DE PERIGOS QUÍMICOS EM INSETOS COMESTÍVEIS: TOXICOCINÉTICA DO MERCÚRIO EM *TENEBRIO MOLITOR*

AWARENESS OF CHEMICAL HAZARDS IN EDIBLE INSECTS: TOXICOKINETICS OF MERCURY IN *TENEBRIO MOLITOR*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Prof. Doutora Susana Patrícia Mendes Loureiro, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e do Doutor Diogo Filipe Nunes Cardoso, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

Presidente	Prof. Doutora Maria Helena Abreu Silva, Professora Auxiliar, Departamento de Biologia, Universidade de Aveiro
Vogal - Arguente	Doutora Paula da Silva Tourinho, Investigadora de Pós-Doutoramento, RECETOX, Masaryk University
Vogal - Orientador	Doutor Diogo Filipe Nunes Cardoso, Investigador Júnior, CESAM & Departamento de Biologia, Universidade de Aveiro

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palavras-chave

bioacumulação, exposição a comida, crescimento, absorção de contaminantes, eleminação de contaminantes, segurança alimentar

resumo

A população humana está a aumentar significativamente e, como resultado, há uma demanda crescente por uma fonte proteíca alternativa para aliviar a pressão do setor pecuário. Os insetos comestíveis, desde a publicação da Organização para a Alimentação e Agricultura das Nações Unidas em 2013, sobre o uso de insetos como alimento e alimento para animais, tem sido um assunto com muito debate e crescimento na comunidade científica. As larvas-de-farinha (*Tenebrio molitor*) são usadas como alimento para animais de estimação, no entanto, estudos concluíram que estas são adequadas para consumo humano e para a alimentação no setor pecuário e avícola. Esta espécie pode ser criada em resíduos agrícolas reduzindo, assim, o desperdício alimentar e contribuindo para um planeta mais sustentável e para a circularidade ambiental. Uma das principais vias de exposição a poluentes químicos é o substrato utilizado na criação de animais para alimentação humana e animal. O mercúrio (Hg) é um metal com elevado potencial de toxicidade, bioacumulação e biomagnificação em organismos e pode estar presente no substrato de insetos comestíveis devido a atividades antropogénicas. Posto isto, o objetivo deste estudo foi investigar a toxicocinética do Hg em larvas-de-farinha expostas a comida contaminada como substrato. Para avaliar o meio ideal de exposição e estágio larvar para os estudos de bioacumulação, foram realizados testes de aquisição de biomassa. Durante 21 dias, dois estádios larvares (20-40 mg e 60-80 mg) foram expostos a quatro tratamentos com: solo e aveia; apenas solo (sem aveia); apenas aveia (sem solo); sem solo e aveia. Após verificar que o tratamento “apenas aveia” era o melhor substrato a utilizar, os testes de bioacumulação foram executados em duas fases, recorrendo a este tratamento de aveia: uma fase de absorção e uma de eliminação, cada uma com 21 dias. Na primeira fase, as larvas de *T. molitor* foram expostas a aveia contaminada com Hg, passando após os 21 dias para meio com aveia não contaminada.

resumo (cont)

Nos testes de biomassa para ambos os estados larvares apenas a aveia, não demonstrou induzir mortalidade e observou-se ainda um maior ganho de biomassa. No entanto, as larvas no intervalo de peso 60-80 mg tiveram o maior ganho de biomassa e do ponto de vista do setor de insetos comestíveis será a mais adequada. As larvas expostas a solo apresentaram maiores taxas de mortalidade e, além disso, a presença de fungos. O modelo toxicocinético elegido para explicar a acumulação de Hg em *Tenebrio molitor* foi o modelo de compartimento único de primeira ordem. O modelo toxicocinético escolhido permitiu observar o comportamento do Hg. Observou-se assim, uma constante de absorção (k1) de 0.056 e uma constante de eliminação (k2) de 0.316. Concluindo, desta forma, que o mercúrio mantém-se no organismo com a retenção de aproximadamente 70% num compartimento, mesmo depois de um período de eliminação em substrato não contaminado durante 21 dias. Este estudo revela que os testes de bioacumulação com *T. molitor* devem ser realizados apenas com alimento como substrato e ainda, que a utilização de modelos toxicocinéticos são um método viável para a compreensão das taxas de absorção e eliminação de Hg na larva-da-farinha.

keywords bioaccumulation, food exposure, growth, contaminants uptake, safety, food safety, contaminants elimination

abstract

The human population is increasing significantly, and, as a result, there is a growing demand for an alternative protein source to relieve the pressure from the livestock sector. Discussion regarding edible insects has grown in the scientific community since the report concerning its use as food and feed, from the Food and Agriculture Organization of the United Nations in 2013. The Yellow mealworm larvae (*Tenebrio molitor*) is considered one of the best solutions to feed pets, livestock and poultry sectors, and human consumption. When reared in agricultural leftovers, mealworms convert waste into valuable nutritional material, reducing waste and contributing to a more sustainable planet. One of the major concerns on rearing insects as a food source is related to food safety aspects. Contaminants can be uptaken by insects through the substrate they feed on, entering into the farming cycle and, consequently, into the food chain. Understanding these compounds' pathways and their effects on insects and generations' and different development stages is crucial for the continuous farming production process. After looking at previous studies where mercury was observed to accumulate in edible insect species, this thesis aimed to apply an innovative and more complex approach to understand how mercury is uptaken and eliminated by insects. Considering that, this thesis aimed to investigate the toxicokinetics of Hg in *T. molitor* larvae exposed through food. This thesis combined a series of ecotoxicological and bioaccumulation assays with previous biomass gain experiments, followed by the Hg bioaccumulation assays. To assess the optimal medium of exposure and larval stage for the bioaccumulation studies, biomass gain experiments were carried out: two larval stages (20-40 mg and 60-80 mg) were exposed to four treatments for 21 days: with soil and oat; with soil only (no oat); with oat only (no soil); no soil and oat. Results revealed a high mortality rate in the treatments where insect larvae growth in soil (with presence of fungi), contrasting with a very low mortality rate and highest biomass gain when both larval stages were exposed to oat and no soil as a substrate.

abstract (cont)

Considering this, the bioaccumulation studies were conducted only with oat (no soil present), using larvae with 60-80 mg, which presented the highest biomass gain, being also the most suitable size from the perspective of the producers. Bioaccumulation experiments consisted of an uptake and elimination phase of 21 days each, providing Hg contaminated and “clean” oat in each phase, respectively. A first-order one-compartment model had the best fit, to explain the uptake and elimination of Hg, in *Tenebrio molitor*. Through this, it was observed that Hg stays in the organism (Fi=0.682) even after an elimination period in clean oat. In addition, this model had an uptake (k1) and elimination (k2) constant of 0.056 and 0.316, respectively. Lastly, to the best of our knowledge, this was the first time that the absorption and elimination rate were measured through time. To conclude, these experiments should be conducted without soil and toxicokinetic models are a viable method to comprehend the behavior of Hg in mealworms.

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Chapter 1

1. General Introduction

1.1. Population growth and its impacts on food and feed

The human population is growing at an alarming pace, and The Food and Agriculture Organization (FAO) of the United Nations predicts that it will grow to 9 billion by 2050 (FAO, 2017). The demand for food and feed production of animal-derived protein is continuously increasing due to the human dietary transformation. The consumption of animal products grows due to higher incomes and increasing urbanization (Boland et al., 2013; van Huis, 2015). In developing countries, this demand is even more prominent since traditional diets were substituted by more western ones, characterized by a high intake of saturated and omega-6 fatty acids, reduced omega-3 fat intake, overuse of salt, and refined sugar (Myles, 2014). This tendency can lead to a nutritional disequilibrium due to a reduction in the consumption of diverse, nutritionally rich, and functionally healthy foods (Belluco et al., 2013; Johns & Eyzaguirre, 2006).

Moreover, the increase in meat consumption leads to a livestock sector's growth, negatively impacting the ecosystems. For example, the decline in land availability through deforestation, soil erosion, and desertification are among other consequences of this livestock sector's growth (Steinfield 2006; Tabassum-Abbasi et al. 2016). Simultaneously, the livestock sector is one of the largest contributors to water resources' decline due to intense water usage and consequent pollution. Apart from the intensive need for water to directly use as a water source for animals and plants, water contamination can occur from animal wastes, fertilizers and pesticides used for feed crops, antibiotics and hormones (Steinfield, 2006). Furthermore, livestock activities are responsible for 18 percent of total greenhouse gases (GHG) emissions by emitting three of the main GHG: carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) (Steinfield, 2006). This factor is of top-most importance since GHG are one of the major contributors to global warming. Considering all this, the need for more clean and sustainable food sources has been discussed and unveiled by the scientific community and regulatory agencies, pointing out all the concerns related to environmental, food safety, and animal welfare aspects (van Huis, 2015).

Food safety has many definitions, and throughout the years, it has been the subject of much debate. According to the 1996 World Food Summit definition (FAO, 1996), food safety is met when “all people, at all times, have physical and economic access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences for an active and healthy life.” The growing demand for more resource-intensive protein products requires an estimated 60% increase in agricultural production by weight from 2005 to match the human population's needs (Alexandratos & Bruinsma, 2012). Besides, climate change will affect agriculture production due to impacts on land suitability and crop yields, and consequently, livestock

productivity. As a result, food safety is adversely affected in its four dimensions: availability, stability, access, and utilization (Schmidhuber & Tubiello, 2007).

1.2. Alternative protein sources

Food production must be improved to meet the growing human population's requirements while at the same time, the environmental impact of both agriculture and livestock must decrease (FAO, 2013; Foley et al., 2011). Soybean, fish, and fish oils are the conventional food and feed sources of protein; however, they are expensive, have high water and carbon footprints, and are no longer considered sustainable due to overexploitation and fluctuation of feedstuff prices (FAO, 2013; Sánchez-Muros et al., 2016; van Huis, 2015). As a result, alternative, less expensive, and more sustainable food and feed sources of protein are needed to answer the demand for protein-rich food and livestock feed (FAO 2013; Grau et al. 2017).

Insects as a potential sustainable source of protein have been proposed and gained much interest over the recent years due to numerous advantages that could help respond to global hunger, malnutrition, and no food safety. Nutritionally, insects present a higher proportion of protein content, already part of several animal species (Makkar, 2017; Sogari et al., 2019). Several studies have already compared the use of insects as novel feed additives to soymeal or fishmeal, and positive results have been observed in terms of animal health, growth, product quality, and good feed conversion ratio (Bovera et al., 2015; Iaconisi et al., 2017; Ng et al., 2001; Ramos-Elorduy et al., 2002).

In the present day, promoting a circular economy is essential for a sustainable planet. Eliminating waste and reducing the impact of food production in ecosystems are the bases for circular economy and, in this specific case, circular agriculture. This is well represented in Figure 1, a scheme of how the use of insect larvae for food and feed can promote circular agriculture by using all the products and by-products produced during the processes, promoting a zero-waste policy (Cappellozza et al., 2019). Insect larvae can grow on biodigesters of fruit and vegetable leftovers that were not used by wholesale markets. Within this, the leftovers of agriculture are used as a food source for insect larvae's growth (Figure 1A). After a certain period of growth, larvae can be used to produce insects flour or other components that will be used to feed animals, substituting the conventional feed sources (Figure 1B). Currently, the use of larvae for human consumption is not a priority due to the developed countries' current difficulties in using this food source. After their growth on biodigesters with agricultural leftovers, insects generate a compost consisting of molting skins (exuviae), degraded organic matter and insect feces (“frass”) (Dicke, 2018). This bio-compost could be used in agriculture as a partial or a complete substitute of mineral NPK fertilizer due to its rapid mineralization and high content in readily available nutrients. This will increase soil biodiversity and microbial metabolic activity, resulting in better soil functioning (Houben et al., 2020) (Figure 1C). Within

this process, food waste is converted into higher-value products in a zero-waste policy with a lower environmental impact (Oonincx et al., 2010).

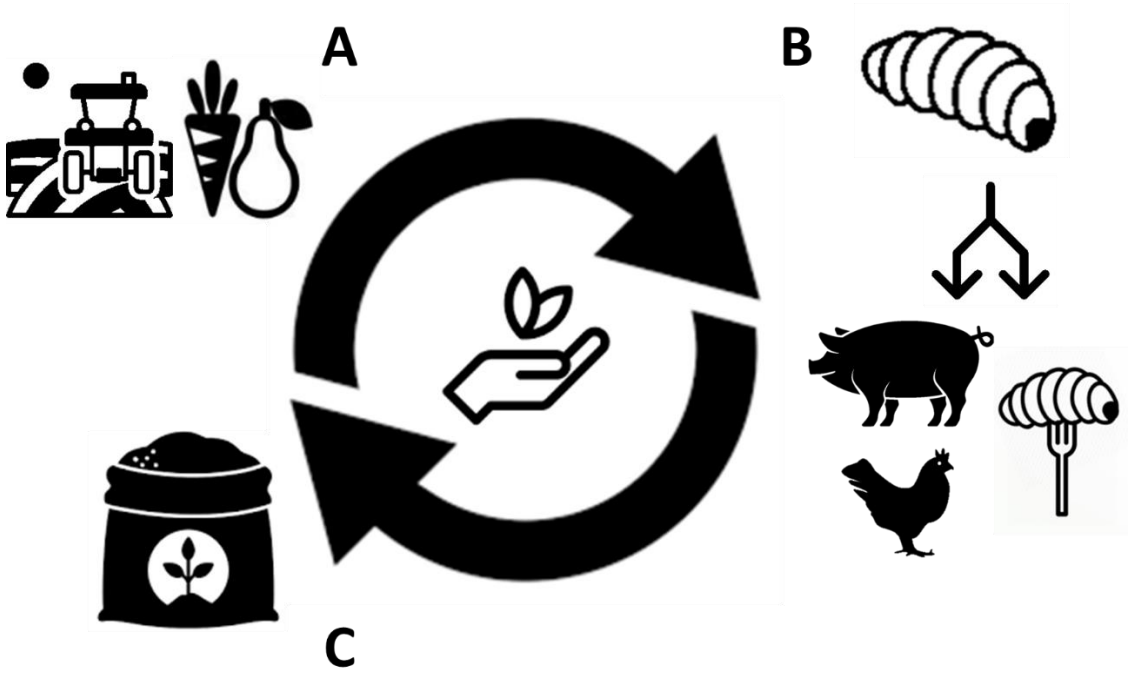

Figure 1. Scheme of circular agriculture. (A) Organic residues produced in the agrifood sector used as feed for insects larvae. (B) Insects larvae products or components used as feed for livestock animals. (C) Insects larvae by-products (exuviae, degraded organic matter and insect feces) used as a mineral NPK fertilizer.

Entomophagy (the consumption of insects) is common worldwide, and close to 2100 species of edible insects were identified (Jongema, 2017). This culture predominates in developing tropical countries where insects can be collected from nature since they are larger, facilitating its harvest. These countries suffer from a shortage of nutritious food, so edible insects offer a cheap and efficient opportunity to minimize food insecurity. They could also help improve livelihood for some of the poorest members because they could easily become involved in their gathering, rearing, processing, and sale (FAO, 2013; Kelemu et al., 2015). According to FAO, (2013) edible insects belong to the following orders: orders Coleoptera (31%), Lepidoptera (18%) and Hymenoptera (14%), with others in Orthoptera (13%), Hemiptera (10%), Isoptera (3%), Odonata (3%), Diptera (2%) and other orders (5%).

While in tropical countries, insects' consumption is practiced, in western countries, entomophagy still has a lot to grow due to people's reluctance towards the inclusion of insects in their diets (van Huis, 2015, 2016). This reluctance originates from insects' perception as pests, dirty, disgusting, harmful, and adverse taste expectations (Looy et al. 2014). Also, in a recent study, a high percentage of people were classified as

having food neophobia, which results in the rejection of eating insects due to the unfamiliarity and the novelty of unconventional foods (La Barbera et al., 2018). The acceptance of insect-based products in western diets is a significant barrier to its introduction as a more sustainable protein source, bringing nutritional, environmental, and economic benefits. However, the interest in edible insects has gained momentum in western societies, especially in Europe, due to the growing awareness about the nutritional and environmental benefits associated with their consumption (Schouteten et al., 2016). In Europe, insects for human consumption or animal feed are already being produced by some companies. In Portugal (Santarém), for example, Ingedient Odysseys, Lda. is a company based on Research & Development, especially in the development of EntoGreen® (registered trademark), often presented as the name of the company itself. It takes advantage of the ability the Black soldier fly (*Hermetia illucens*) has to convert low-value waste streams into novel nutrient alternatives for animal feed, also producing soil fertilizer that will improve agriculture sustainability.

A review of 236 edible insects' nutritional composition reported a large variation between species and developmental stage and their diet and habitat (Rumpold & Schlüter, 2013). In general, edible insects are rich in proteins providing essential amino acids for humans due to their amino acid score (the essential amino acids requirement expressed as a percentage in an ideal protein) ranging from 46% to 96%. They are also highly digestible, making them suitable for all age groups (Belluco et al., 2013; Ramos-Elorduy et al., 1997). Furthermore, insects present high levels of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), having a ratio between the 3 fat categories (saturated fatty acid (SFA): MUFA: and PUFA = 3: 4: 3) within the suggested range for health purposes (1: 1.3: 1) (Belluco et al., 2013). Insects also contain a high content of minerals such as copper, magnesium, manganese, phosphorous, iron, zinc, riboflavin, pantothenic acid, and biotin (Rumpold & Schlüter, 2013).

1.3. Mealworms as European novel food

A variety of insect species have been tested for animal feed and human consumption, revealing that the most promising species are larvae from the Black soldier fly (*Hermetia illucens*), the Common housefly (*Musca domestica*), and the Yellow mealworm (*Tenebrio molitor*) on which this study will focus. Mealworms are well known and widely cultured for use as a nutritious meal for pets such as reptiles, amphibians, fish, and birds. Furthermore, they are a holometabolous insect known as a pest that infests stored grain, flour, and food products, by contaminating them with exuviates, excrements, and dead organisms. This species' life cycle consists of four life stages: egg, larva, pupae, and adult. Females start to lay eggs 4-17 days after copulation, and a female may lay up to 500 eggs. In general, larvae hatch after 10-12 days (at 18-20ºC), with a whitish color turning yellowish after a few days, which may last up to 18

months. The larval stage becomes mature after undergoing a variable number of moults (8 to 20). Larvae produce a hard, chitinous exoskeleton turning into pupae with a duration between 7 to 9 days at 25ºC and up to 20 days at lower temperatures. Finally, it turns into a darkling beetle (adult stage) living between 2 to 3 months (Costa et al., 2019; Park et al., 2014; Siemianowska et al., 2013).

The yellow mealworm is a promising alternative as a mini-livestock due to its short life cycle, minimal physical space needed, high feed conversion efficiency, and access to previous knowledge from the pet food industry. Besides, mealworms have good nutritional composition, and it could replace fishmeal or soymeal (FAO, 2013; Ramos-Elorduy et al., 2002; Sánchez-Muros et al., 2016). Additionally, mealworms can be used as human food or food additives (e.g. cereal bars, pasta, meat imitates and bakery products) containing a high protein content, polyunsaturated fatty acids, calcium, zinc, iron, and high in magnesium (EFSA, 2021; Finke, 2002; Nowak et al., 2016). *T. molitor* larvae are commonly reared on a substrate of wheat bran. However, they can be produced on diets containing organic side streams with increased growth performance and enhancement of their nutritional value (Ramos-Elorduy et al., 2002; van Broekhoven et al., 2015). Although the energy needed to produce 1 kg of *T. molitor* is similar to that of livestock, the amount of water per edible ton, 4341 m^3/t, is comparable to poultry and 3.5 times lower than that of livestock. Furthermore, from a life cycle assessment, it was shown that mealworms produce much less GHG's and require much less land area than poultry and livestock (Miglietta et al., 2015; Oonincx & de Boer, 2012).

1.4. The legal framework in Europe

Adopting edible insects to answer the growing food demand and environmental sustainability is a growing topic of concern and discussion (FAO, 2013). Nevertheless, the legislative framework concerning edible insects in Europe is still a challenge and under development. In this way, the yellow mealworm (*Tenebrio molitor*) is the only insect species, included in the Union list of authorised novel foods for marketing and consumption in accordance with Regulation (EU) 2015/2283. These safety policies and regulations should be a priority to governments of both developing and developed countries for a safer and sustainable consumption of this novel food source. Within the European Union, insect-based foods meant for human consumption are regarded as novel foods. According to the old Regulation (EC) No 258/97 "one of the criteria for food to be considered a novel food should continue to be the absence of use for human consumption to a significant degree within the Union before the date of entry into force of that Regulation, namely 15 May 1997" (Imathiu, 2020). This novel food regulation has been updated to the Regulation (EC) No 2015/2283, which expands categories of novel foods to include whole insects, insect parts, and other foods not produced or used before 1997. Thus, EU consumers have access to a wide range of safe, unique,

and innovative food choices, including those from countries where entomophagy is practiced. At present, only seven insect species are allowed as feed only to aquaculture by the Regulation (EU) No 2017/893 (Annex II): the yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), common house fly (*Musca domestica*) black soldier fly (*Hermetia illucens*), field cricket (*Gryllus assimilis*), banded cricket (*Gryllodes sigillatus*) and the house cricket (*Acheta domesticus*).

Food safety is one of the important topics of concern regarding edible insects' consumption due to a lack of information concerning potential hazards on the rearing and use of insects for food and feeds production (Camenzuli et al., 2018; Schlüter et al., 2017). According to van Huis (2016) the risks of using insects as food and feed can result from: 1) the insect itself could be toxic; 2) the insect could have acquired toxic substances or human pathogens from its environment during its life cycle; 3) the insect could become spoiled after harvest; 4) consumers could experience an allergic reaction to the insect. Using this novel food source, a risk profile was conducted by the European Food Safety Authority (EFSA), assessing the microbiological, chemical, and environmental hazards and allergenicity, processing, and storage impacts and production chain. This risk profile concluded that the production and processing methods, the substrate used, stage of harvest, insect species, and the developmental stage would all influence the possible presence of biological and chemical contaminants in insect food and feed products (EFSA, 2015).

Based on the circular agriculture that is presented before, the process of rearing insects for food and feed is based on their growth in a substrate composed of leftovers from agriculture. The use of agricultural leftovers (organic waste) as a substrate for the rearing of edible insects is one of the leading chemical exposure routes due to biological and chemical contaminants' possible presence, that could be hazardous to public health (van der Spiegel et al. 2013; EFSA 2015). That organic waste often contains persistent chemical residues such is the case of metals (Schrögel & Wätjen, 2019), since pollutants are continuously added into soils via atmospheric emissions or inappropriate disposal of metal-containing waste, accumulating and transferring to plants and, therefore, entering into the food chain (Carbonell et al., 2011). Although metals are present naturally in the soil, their concentration can increase through anthropogenic activities, as is the case of cadmium (Cd), lead (Pb), arsenic (As), chromium (Cr), cobalt (Cb), nickel (Ni), copper (Cu) and mercury (Hg). Metals can bioaccumulate in the organisms' bodies, entering then into the food chain. Even knowing the different toxicity mechanisms from the different metals, the majority interfere with vital cellular components (blocking the essential functional groups of biomolecules such as enzymes; displace essential metal ions from biomolecules; modify the active conformation of biomolecules or some other biologically active agents; disrupt the integrity of biomembranes; binding with bioanions) (Ochiai, 1995).

Mercury (Hg) is a critical pollutant because it is highly toxic and can (bio)accumulate in organisms (Morel et al. 1998; Truzzi et al. 2019). This metal in nature can occur in its elemental state mercury (Hg^0),

inorganic mercury (Hg^{+} and Hg^{2+}) as well as in organic form, most frequently as methylmercury (Schrögel & Wätjen, 2019). The organic form is the most frequently encountered compound due to inorganic Hg forms' methylation through microbial activity (Valko et al. 2005). Mercury ions have a high affinity for thiol groups due to sulfur (S) atoms, which will be reduced. In this way, Hg can form stable complexes with sulfhydryl-containing molecules, such as the cysteine residues of cellular proteins and nonprotein molecules (Hultberg et al. 2001; Carvalho et al. 2008). Furthermore, mercury can biomagnify along food chains posing a serious threat, particularly with the consumption of contaminated fish and wildlife at the top of the food chains. Accordingly, maximum levels of certain contaminants such as mycotoxins and metals in specific foodstuffs are set by Regulation (EC) No 1881/2006 on which the maximum level for mercury is 1,0 mg/kg. The EU maximum limits in animal feed for mercury (Hg) is stated in the Regulation (EC) No 2002/32/EC as 0,1 mg/kg. In the new Regulation (EU) 2021/882, the specification concerning metals reports that Cd and Pb levels are equal or below 0.1 and 0-075 mg/kg respectively.

1.5. Bioaccumulation studies

Bioaccumulation occurs when the chemical uptake of a pollutant present in the environment accumulates in tissues, by an organism, from an external phase (water, food, or substrate). Depending on the chemical and the organism, the bioaccumulation of a substance can have toxic effects. These substances are metabolized in different ways related to the type of substance, particularly its hydrophobicity/hydrophilicity (Chojnacka & Mikulewicz, 2014; Hoffman et al., 2002). Bioaccumulation according to Petersen et al., (2019) is the process and phenomenon of accumulation of metals in or on an organism, regardless of exposure regime (*i.e.* whether ingesting or otherwise taking up metals *via* water, food, sediment, soil, or air.

The use of these bioaccumulation studies is essential to understand the hazards posed by chemicals to the environment and assess the risks associated. These tests are designed to identify all the potential uptake pathways, including food and aquatic sources of exposure, and determine the bioavailability of toxic elements to the biota in a mid-to-long-term (10-28 days) laboratory study (Marigómez, 2014). Metals undergo different processes in soil organisms such as uptake, internal distribution, storage and excretion (Ardestani et al. 2014). Furthermore, bioaccumulation models were designed to understand and/or predict the uptake, and elimination rate constants of metals (or other substances) in the organism studied, which could help in the extrapolation to field scenarios (Veltman et al., 2007).

A considerable amount of research on the accumulation of pollutants such as metals in insects from rearing substrates are available (Maryanski et al. 2002; Zhang et al. 2012; Charlton et al. 2015; Diener et al. 2015; Tschirner and Simon 2015; Purschke et al. 2017). Vijver et al. (2003) studied the uptake kinetics

of Cd, Cu, Pb and Zn from soils by the larvae of *T. molitor*. It was observed that essential metals were normally regulated regardless of the concentration in the soils they were exposed to. In contrast, uptake of non-essential metals was determined by the total metal concentration in the soils. A study from Truzzi et al. (2019) on the accumulation of heavy metals (Cd, Pb, Hg, Ni and As), on the yellow mealworm present in different mixtures of organic wheatmeal and organic olive-pomace, concluded that the former showed the lowest Hg content, whereas the latter showed the highest. It was observed only bioaccumulation of Hg in larvae of *T. molitor* however, the level of heavy metal content complied with European Union regulations. Another research from van der Fels-Klerx et al. (2016) on Cd's uptake, Pb and As by *Tenebrio molitor* and *Hermetia illucens* from contaminated substrates observed, comparatively to the yellow mealworm, that Cd did not bioaccumulate and after being provided with the original feed it was excreted. Additionally, the Pb concentration was far lower than in their feed whereas, this species accumulated As with increasing feed concentrations.

1.6. Objectives and Contributions to the field

An important element to food and feed safety is the regular monitoring of pollutants, especially in the rearing substrate as it is the main route of exposure. Moreover, European regulations on maximum limits for heavy metals in food and feed materials should be updated to match insects' metabolism that may differ from conventional livestock.

This master dissertation investigates the toxicokinetics of Hg in mealworms exposed through food as a rearing substrate. The yellow mealworm *Tenebrio molitor* (order: *Coleoptera*, family: *Tenebrionidae*) was chosen as a test organism in this study due to it being the first insect species authorised in the European market and a source of high-quality protein, vitamins, and minerals (EFSA, 2021; Finke, 2002; Nowak et al., 2016). This species also goes through various life stages, having a life strategy that favors this type of study, where different metabolic processes are expected to occur at different larval stages. Therefore, as a prior approach, a biomass gain test at different larval stages was carried out to choose the best/optimal larval stage and medium of exposure for the toxicokinetics study.

After choosing the best larval stage to use, a bioaccumulation test was carried out. Several studies have already been conducted measuring the amount of the different metals in this species after a short exposure period (Truzzi et al., 2019; van der Fels-Klerx et al., 2018; M. Vijver et al., 2003). To the best of our knowledge, this is the first time that the uptake of a metal element (using Hg as model chemical substance) is measured in time, during a 21day period. In addition, an elimination phase was also carried out by exposing insect larvae to non-contaminated food for the same period (21d), evaluating in time the insects' elimination capacity.

1.7. Thesis structure

To achieve the proposed objectives, the overall structure of this thesis was divided three chapters.

- Chapter 1 – General introduction to the thematic of insects as food and feed, its risks, potential as an alternative protein source and objectives of this thesis
- Chapter 2 – Awareness of chemical hazards in edible insects: Toxicokinetics of Hg in *Tenebrio molitor*
- Chapter 3 – Provides a short discussion and conclusions of the thesis

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Chapter 2

2. Awareness of chemical hazard in edible insects: Toxicokinetics of Hg in *Tenebrio molitor*

2.1. Abstract

Yellow mealworm larvae (*Tenebrio molitor*) has a potential to be an alternative protein source for humans and animals. In the rearing substrate of edible insects, metals can be present and constitute a concern since they are prone to bioaccumulate in insect species. One of those metals is mercury, which was previously found to accumulate in insect species. This study aimed to understand the uptake and elimination of mercury in mealworm larvae exposed to contaminated food. Furthermore, the optimal larval stage for the bioaccumulation studies was chosen through biomass gain experiments. Two larval stages were chosen to assess larvae biomass gain for 21 days: 20-40 mg and 60-80 mg. They were exposed to 4 treatments: with soil and oat; 2) with soil only (no oat); 3) with oat only (no soil); 4) no soil and oat and each treatment had seven organisms. The treatment with oat showed no mortality and larvae with 60-80 mg had the highest biomass gain. Based on that, larvae with 60-80 mg were chosen for the bioaccumulation experiments and exposed to Hg contaminated oat for 21 days, being thereafter moved to clean oat for another 21 days. A first-order one-compartment toxicokinetic model was applied to the data (concentration of Hg in mealworms, in time) and it was observed that during the elimination phase, Hg remained in the organism. Future research regarding the results of this study proposes a subcellular partitioning of Hg in *T. molitor* to further understand how and where this metal is stored. Furthermore, it is crucial to assess its potential for biomagnification in higher trophic levels to promote food and feed safety.

2.2. Introduction

The world population is continuously increasing, resulting in a growing demand for food worldwide, mainly for animal-derived products (FAO, 2013; Ochoa-Sanabria, 2019). In developed countries, the rapid growth of animal proteins is restricted to a few selected sources such as poultry, pigs and cattle. However, this trend leads to a negative impact on both on the environment and human health (Belluco et al., 2013; Johns & Eyzaguirre, 2006). Additionally, the livestock sector has a considerable environmental impact due to the intense demand for land and water usages, such as deforestation, soil erosion, and the use of water as a source for the animals and feed crops (Steinfield 2006; Tabassum-Abbasi et al. 2016). This brings serious implications concerning the process sustainability, food safety issues, and animal welfare. Furthermore, livestock production is one of the significant contributors to global warming due to high total greenhouse gas (GHG) emissions (18% of global human-induced emissions) (Steinfield, 2006; van Huis, 2015). Besides the effects caused by the described above to human health, intensive livestock production leads to

the use of several pharmaceuticals (e.g. pesticides, hormones, etc) that can enter into the human biological system with foreseen health issues (Steinfield, 2006).

A dietary change in the current populations should involve replacing the major animal protein sources for human consumption for novel ones such as insects, cultured meat, seaweed, and fungi (FAO, 2013; van Huis, 2015). The consumption of insects, or entomophagy, has always been a part of the human diet in developing countries and, recently, it is gaining relevance in Western countries (FAO, 2013; van Huis, 2015, 2016). Recently, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) stated that frozen and dried formulations from whole yellow mealworm (*T. molitor*) is safe under the proposed uses and use levels which is a step towards entomophagy approval. Simultaneously, the use of insects as feed for animals has been studied and is considered a substitute in broiler diets for example (Bovera et al., 2015). Insects as food and feed production can be considered more sustainable than livestock production with several environmental benefits. The use of reared insects for food and feed is suggested to lead to lower greenhouse gas and ammonia emissions (Oonincx et al., 2010), less land area needed (Oonincx & de Boer, 2012), more efficient feed conversion (FAO, 2013; Oonincx et al., 2015) and can be used as powerful bio-converters of waste substrates into high-quality protein sources (Bovera et al., 2015; FAO, 2013; Makkar, 2017; Sogari et al., 2019; van Huis, 2013). Many insects can reproduce quickly, providing large biomass with healthy and high nutritional value (FAO, 2013; Rumpold & Schlüter, 2013). Insect-derived proteins can be then used to entirely or partly substitute the increased use of fishmeal, and soymeal for aquaculture reducing the importation into the European Union (EU) (Bovera et al., 2015; EFSA, 2015).

Although an insect species is included in the Union list of novel foods authorised for food, it is a tiny niche market with considerable potential to grow. Concerning the market of edible insects in Eastern countries, Europe still has a developing legislative framework delaying farming insects' industrial development to supply the food and feed sectors. There is a need to establish a safety profile regarding the insects themselves and the feed or substrate fed to the insects, which may pose a risk to human and animal health (Camenzuli et al., 2018; EFSA, 2015; FAO, 2013; Schlüter et al., 2017). When looking at insects as a potential new protein source, it is essential to consider the different food safety hazards related to this new protein source. Microbial (e.g. mycotoxins) and chemical hazards (e.g. metals, pesticides, veterinary pharmaceuticals, etc.) are some of the topics of significant concern, as well as allergen concerns related to the ingestion of insects. Also, processing methods of rearing insects and their environmental impacts should be well studied before large-scale production occurs. According to the European Food Safety Authority (EFSA) risk profile, the presence of biological and chemical pollutants is related to the substrate used, stage of harvest, insect species, and their developmental stage.

The larvae of the yellow mealworm *Tenebrio molitor* (order: *Coleoptera*, family: *Tenebrionidae*) is one of the insect species already tested as a food and feed source (FAO, 2013; Ramos-Elorduy et al., 2002;

Sánchez-Muros et al., 2016). This species is commonly used as pet food since they have the potential for massive production due to its short life cycle, diverse rearing substrates, high conversion efficiency, and minimal physical space required for production (Klasingph et al., 2000; Ramos-Elorduy et al., 2002). Additionally, the larvae are rich in fat, fiber, and a good source of trace minerals and amino acids, providing protein quality similar to soybean (FAO, 2013; Finke, 2002; Ramos-Elorduy et al., 2002; Sánchez-Muros et al., 2016). At present, mealworms are the only insect species allowed to be in the European market and consumed due to the factors mentioned before (EFSA, 2021). However, regarding their contaminants (bio)accumulation capacity there is still a gap to be filled. Contaminants can be present in insects rearing substrates (agricultural leftovers, for example), which can accumulate by different routes of exposure (e.g. uptake via pore water, via the skin, soil ingestion and/or ingestion of food) (EFSA, 2015; Schrögel & Wätjen, 2019; M. Vijver et al., 2003).

Some authors already conducted research on how metals can affect or accumulate in insect species with potential to be used as food and feed via contaminated substrate (Charlton et al., 2015; Diener et al., 2015; Maryanski et al., 2002; Truzzi et al., 2019; Tschirner & Simon, 2015; van der Fels-Klerx et al., 2016; M. Vijver et al., 2003; Z. Zhang et al., 2012). However, only a few studied those effects on *T. molitor* (Truzzi et al., 2019; van der Fels-Klerx et al., 2016; M. Vijver et al., 2003). In a recent study from Truzzi et al. (2019), the accumulation of metals such as Cd, Pb, Hg, Ni, and As by mealworms was tested, observing that only mercury (Hg) accumulated in larvae of this insect. Despite the observed accumulation, the Hg measured values were in compliance with the EU regulations' levels permitted.

Considering the previous reports that Hg can accumulate in insects larvae (Truzzi et al., 2019), this study aimed to go forward, investigating the toxicokinetics of Hg in mealworms exposed to Hg contaminated food. This study is a step forward in the literature since we propose a complete bioaccumulation design, where *T. molitor* is exposed for 21 days to contaminated Hg food, followed by the same period with non-contaminated food. With this, it is possible to follow the uptake and elimination rates of Hg in *T. molitor*, which is extremely important for the knowledge on how insects larvae of *T. molitor* deal with Hg contaminated substrate/food and consequent uptake and elimination. Previous to the complete bioaccumulation assay, and knowing that different metabolic processes are expected to occur at different larval stages, a biomass assay without Hg contamination was conducted for better knowledge on the use of *T. molitor* in bioaccumulation studies.

2.3. Materials and methods

2.3.1. Test organisms and soil

The individuals from the species yellow mealworm (*Tenebrio molitor*) were obtained from laboratory cultures maintained at the applEE- applied Ecology and Ecotoxicology laboratory, CESAM, University of Aveiro (Portugal), and previously purchased from the Pet-Blink, Lda.. The organisms were cultured in plastic boxes on a dry substrate of wheat bran, egg trays, and oat. Organisms were maintained at a fixed temperature (20 ± 2 ºC) with a 16:8 (light:dark) photoperiod. Larvae organisms between 20-40 and 60-80 mg were used in the biomass gain experiments. For the bioaccumulation experiments, only 60-80 mg *T. molitor* larvae were used.

In the present work, two assays were conducted: 1) a biomass gain experiment, assessing differences in biomass gain using two different larvae weight ranges (20-40 mg and 60-80 mg), testing also the influence of soil and oat presence; 2) a complete bioaccumulation study, where larvae were exposed for 21 days to contaminated Hg oat, followed by a 21 days non-contaminated food source. For the biomass gain experiments, the standard soil LUFA 2.2 (LUFA-Speyer, Germany, with the following properties: organic carbon (1.71 ± 0.30 % C), pH (5.6 ± 0.4 0.01 M $CaCl_2$), and maximum water holding capacity (44.8 ± 2.9 g/100g)) was used.

2.3.2. Experimental setup

2.3.2.1. Biomass gain experiments

Biomass gain experiments were performed in cylindrical plastic boxes (ø 65 mm) with one *T. molitor* larvae per box. To assess possible differences in biomass gain, two different larvae weight ranges were tested (20-40 mg and 60-80 mg). Four different conditions were assessed for each weight range with seven replicates each, making a total of 56 test boxes and organisms: 1) with Soil and Oat (SO); 2) with Soil only, No Oat (SNO); 3) No Soil, Oat only (NSO); 4) No Soil and No Oat (NSNO). This test lasted for 21 days, where each organism was weighed, at day 0, 1, 3, 7, 14, and 21. In those treatments where food was provided, oat grains were added *ad libitum* (changing every two days in the replicates with soil to avoid fungi contamination). In the treatments where larvae were on the soil, 20 g of natural LUFA 2.2 soil was added to the test pots. The test soil had 40% of maximum water holding capacity, and to assure it remained the same, five extra cylindrical plastic boxes with soil were prepared. These containers were weighed at the beginning of the test and weighted every week to confirm water. As necessary, ultrapure water was added to every box test with soil.

Mealworm biomass gain (%) was calculated as:

$B=(W_f - W_i) / W_i \times 100$

Where W_f is the individual weight at each sampling time and W_i is the weight at the start of the experiment.

2.3.2.2. Bioaccumulation experiments

The bioaccumulation tests were performed in individual cylindrical plastic boxes (ø 65 mm) with one test organism per box ranging between 60-80 mg. *T. molitor* larvae were exposed for 21 days to contaminated Hg oat (0.7 mg/kg Hg), provided *ad libitum.*

T. molitor larvae were exposed to oat contaminated with 0.7 mg/kg Hg in the bioaccumulation experiments using Mercury (II) chloride ($HgCl_2$-CAS no: 7487-94-7) purchased from Merck Millipore (99.5% purity). 30 g of oat was spiked in plastic boxes by adding 35 mL of the work solution (0.017 mg/L Hg). This process was done to guarantee the spiking homogenization. Simultaneously, non-contaminated oat was submitted to the same process, adding only 35 mL of ultra-pure water (without Hg). After mixing accordingly, the boxes containing oat were left to dry for a minimum of 4 days at air dry.

This test consisted of an uptake phase followed by an elimination phase, each with 21 days. In the uptake phase, the organisms were exposed to Hg-contaminated oat and five organisms (replicates) were sampled at day 1, 3, 7, 14, and 21. At the beginning of the elimination phase (day 21) the contaminated oat was replaced by non-contaminated oat, and the organisms were sampled after 22, 24, 28, 35 and 42 days. The same procedure used in the uptake phase was conducted for the elimination phase. As a control, *T. molitor* larvae were exposed to non-contaminated oat and 3 sampling times were performed (0, 21, and 42 days of the test) in the same conditions previously described. At each sampling time, 5 replicates were collected, weighed, and immediately frozen at -80 ºC for Hg measurements.

2.3.3. Mercury analysis

To determine the total mercury content in the mealworms, and oat the Advanced Mercury Analyzer (AMA254) LECO was used. This system is an atomic absorption spectrometer that detects and quantifies the volatilized mercury derived from the combustion of the sample (Costley et al., 2000). The total flow of the operation is 5 min with a drying time of 60 s, decomposition time of 150 s and waiting time of 45 s.

The analytical quality and accuracy of the procedure were certified using the reference material TORT-3 (Lobster Hepatopancreas Reference Material for Trace Metals, National Research Council of

Canada) and DOLT-5 (Dogfish Liver Reference Material for Trace Metals and other Constituents, National Research Council of Canada). The calibration blanks were run in the beginning, and between samples from different sampling times to check for and avoid possible sample contaminations.

2.3.4. Data analysis

The statistical analysis of the biomass test data was carried out using the SigmaPlot 14.0 software. To assess differences in biomass gain between the 4 different treatments (Soil and Oat; No Soil, Oat only; with Soil only, No Oat; No Soil, No Oat.) in time, a two-way ANOVA was conducted with multiple comparisons examined by Tukey method ($\alpha = 0.05$). The 4 different types of exposures and the different sampling times (0, 1, 3, 7, 14, and 21 days) were defined as fixed factors. Some of the data comparisons that failed both the Normality Test (Shapiro-Wilk) and the Equal Variance Test (Brown-Forsythe) were subjected to simple transformations such as log(10) (for SO vs SNO in both larval stages 20-40 and 60-80 mg) and exponential (for NSO vs SNO – 20-40 mg). Differences between internal body concentrations of Hg on organisms exposed to contaminated (0.7 ppm Hg) and non-contaminated oat at day 42 (last day of elimination) were confirmed using a T-test.

Three toxicokinetics models were tested to describe the uptake and elimination rates of Hg in mealworms, named here as models 1 and 2. Model 1 is a first-order one compartment model which considers the animal as a homogenous compartment with single uptake and elimination rates. Model 2 is a variant of the first-order one compartment model, adapted from Vijver et al. (2006), in which an inert fraction (Fi) was added to account for metal translocation inside the mealworm forming storage pools from which no elimination occurs.

Models 1 and 2 used the following equation for the uptake phase (Eq. 1):

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times (1 - e^{-k2 \times t}) \quad (1)$$

where Q(t) is the concentration of Hg in the animal measured in time (t) (µg Hg/g_{animal}); C_0 the basal internal Hg concentration (µg Hg/g_{animal}) calculated from the mean measured Hg body concentration at t = 0; k1 the uptake rate constant ($g_{food}/g_{animal}/day$); k2 the elimination rate constant (day^{-1}); C_{exp} the measured Hg concentration in the exposure medium (mg Hg/kg_{food}); and t = time (days).

For the elimination phase, Model 1 used Eq. 2 and Model 2 used Eq. 3 adapted from Vijver et al. (2006)):

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times (e^{-k2 \times (t - t_c)} - e^{-k2 \times t}) \quad (2)$$

$$Q_{(t)} = C_0 + \frac{k1}{k2} \times C_{exp} \times (Fi + (1 - Fi) \times (e^{-k2 \times (t - t_c)}) \quad (3)$$

where t_c is the last day of the uptake phase when the animals are transferred to clean food and Fi is the inert fraction (ranging from 0 to 1). The selection of the best toxicokinetic model was conducted using an information-based approach with the Akaike Information Criteria (AIC), with correction for small sampling sizes – AICc. Toxicokinetics models were ranked and the model with ΔAICc < 2 (i.e. difference between AICc and the lowest AICc for all models) was considered the best model. This method determines the fit of how a data set supports each model, taking into consideration the number of parameters in the model and the goodness-of-fit (sum-of-squares) (Motulsky, 2007).

$$\Delta\text{AIC} = N \times \ln\left(\frac{SS2}{SS1}\right) + 2\Delta DF$$

where N is the sample size; SS1 and SS2 are the sum-of-squares of the two models; ΔDF is the difference between the number of degrees of freedom of the two models.

2.4. Results

2.4.1. Biomass gain experiments

Table 1 presents the mean weight (mg ± SD) and biomass gain/loss (% ± SD) of mealworms in the larvae stage 20-40 mg. It is possible to observe that higher biomass gains are related to the treatments where mealworms were in soil (see supplemental data), particularly when oat was present (219.5 % biomass gain related to T0). However, in the treatments where soil was present, high mortality rates (81.57 and 57.15% with oat and no oat, respectively) were also observed. By opposition, in the treatments without soil, survival was higher, but mealworms' growth was lower. Mealworms only gained 36.3% of their biomass in oat only (and no soil), compared with the 219.5 % when soil was available with oat. Mealworms experienced a biomass loss when maintained without substrate for 21 days (-16%), comparing with the gain of 93.6% when in soil and no oat provided. It is also possible to observe a complete absence of mortality in the treatment with no substrate provided (however, with biomass loss).

Figure 2 shows the different biomass changes related to exposure with and without soil and oat. It is possible to observe that mealworms when exposed to soil with or without oat had higher biomass changes. This demonstrates the influence that soil has in the gain of mealworms biomass, even at early and short exposures (1 day of exposure). The same did not happen when mealworms were without soil, with a slight increase in their biomass through time. In addition, there was a decrease in biomass through time when no substrate was provided.

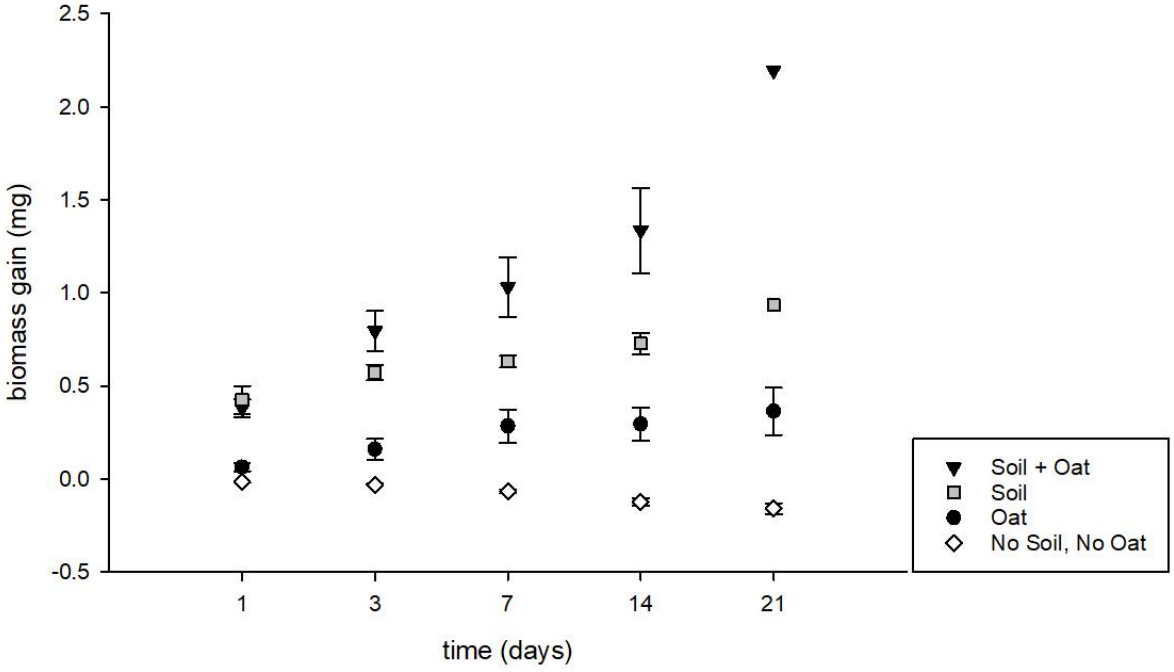

Figure 2. Biomass gain (mean (± Standard error) in mg) of *Tenebrio molitor* larvae (20-40 mg initial weight) exposed to different conditions for 21 days. SO: Soil + Oat; NSO: Oat only; SNO: Soil only, No Oat; NSNO: No Soil, No Oat.

Table 1. Weight (mg ± SD) and biomass gain (% ± SD) of mealworms in the larvae stage 20-40 mg, exposed to the different treatments during a period of 21 days. The total mortality rate calculated was calculated at the end of the test (%) for each treatment.

Treatment	0 d		1 d		3 d		7 d		14 d		21 d		Mortality rate (%)
	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	
SO	29.9 (±5.40)	-	40.8 (±6.17)	37.9 (±11.69)	53.8 (±13.68)	79.5 (±26.53)	61.1 (±17.96)	103.1 (±39.54)	77.0 (±17.48)	133.6 (±45.75)	100.0 (±0.00)	219.5 (±0.00)[$]	85.71
NSO	27.5 (±6.55)	-	29.2 (±6.67)	6.3 (±5.42)	31.6 (±7.00)	15.9 (±13.67)	35.2 (±9.55)	28.4 (±22.10)	35.8 (±10.84)	29.4 (±22.15)	37.5 (±11.32)	36.3 (±31.39)	0.00
SNO	31.6 (±6.15)	-	43.9 (±4.52)	42.4 (±18.58)	49.1 (±7.56)	57.2 (±10.44)	51.3 (±9.58)	63.0 (±7.79)	57.2 (±9.43)	72.7 (±13.35)	69.7 (±0.00)	93.6 (±0.00)[$]	57.14
NSNO	34.6 (±5.15)	-	34.0 (±5.26)	-1.7 (±0.95)	33.5 (±5.19)	-3.1 (±1.08)	32.3 (±5.27)	-6.7 (±2.64)	30.4 (±5.34)	-12.4 (±4.49)	29.3 (±5.95)	-16.0 (±5.99)	28.57

SO – soil and oat; NSO – no soil, oat; SNO – soil, no oat; NSNO – no soil, no oat

[$]- only one organism

For larvae weighing between 60-80 mg (Table 2), it was observed that the mortality was lower compared with the smaller organisms (20-40 mg), even when the soil was present. Again, the presence of soil was related to an increase in insect's biomass (see supplemental data), but also with an increase in mortality compared with treatments without soil – 28.57 and 42.86% of mortality in the case of the experiments with soil and oat and no oat, respectively, while 0 and 14.29% mortality were observed without soil with oat and no substrate, respectively. The complete absence of mortality when organisms were exposed to oat without soil was consistent with the 20-40 mg larvae weight range (Table 2). In the presence of oat, larvae mealworms almost double their biomass gain when in the presence of soil (67.1 to 113.6% biomass gain without soil and with soil, respectively). Without the presence of oat, mealworms increased their biomass only when the soil was present, with a biomass gain of 60.7%, compared with a loss of 8.5% when without soil.

Figure 3 plotted the biomass changes when mealworms were exposed to/without the presence of soil and oat. In this larval stage, biomass gains were similar however, it was observed a decline in biomass when no substrate was present. Looking to figure 3, it is also possible to observe that when without soil, day 7 of exposure was the first of the sampling times where we observed the effect of oats presence in the biomass gain of mealworms.

Table 2. Weight (mg ± SD) and biomass gain (% ± SD) of mealworms in the larvae stage 60-80 mg, exposed to the different treatments during a period of 21 days. The total mortality rate calculated was calculated at the end of the test (%) for each treatment.

	0 d		1 d		3 d		7 d		14 d		21 d		
Treatment	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mean weight (mg)	biomass gain (%)	Mortality rate (%)
SO	63.2 (±2.52)	-	80.7 (±3.06)	27.8 (±6.27)	110.2 (±9.39)	74.3 (±10.62)	131.7 (±10.10)	108.4 (±12.28)	132.5 (±16.60)	106.1 (±22.01)	133.9 (± 11.84)	113.6 (±22.79)	28.57
NSO	65.6 (± 3.13)	-	69.0 (±2.81)	5.3 (±2.86)	74.1 (± 4.26)	12.9 (±5.07)	94.3 (±4.47)	44.0 (±9.95)	113.5 (±9.34)	73.2 (±16.16)	109.4 (±8.84)	67.1 (±16.67)	0.00
SNO	70.1 (± 4.19)	-	85.2 (±3.69)	21.6 (±3.25)	107.0 (± 8.12)	52.6 (±6.67)	110.8 (± 8.00)	58.0 (±6.96)	123.8 (± 13.30)	74.8 (±14.49)	120.4 (± 12.05)	60.7 (±9.68)	42.86
NSNO	70.1 (± 6.15)	-	69.6 (±6.04)	-0.8 (±0.25)	68.7 (± 6.29)	-2.0 (±0.88)	67.5 (± 6.64)	-3.9 (±1.89)	66.6 (± 5.86)	-5.7 (±1.74)	65.5 (±5.99)	-8.5 (±2.53)	14.29

SO – soil and oat; NSO – no soil, oat; SNO – soil, no oat; NSNO – no soil, no oat

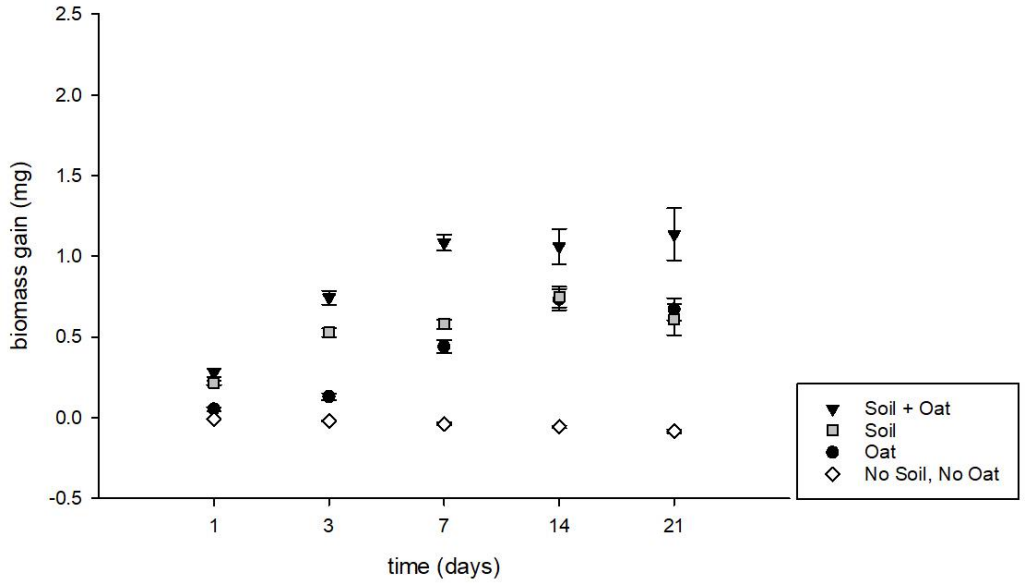

Figure 3. Biomass gain (mean (± Standard error) in mg) of *Tenebrio molitor* larvae (40-60 mg initial weight) exposed to different conditions for 21 days. SO: Soil + Oat; NSO: Oat only; SNO: Soil only; NSNO: No Soil, No Oat.

2.4.2. Hg concentration in mealworms and substrate

The background Hg concentration in oat flakes was 0.002 (±0,0006; n=8) ppm. The measured Hg concentration in the spiked oat with 0.7 Hg ppm solution was 0,637 (±0,166; n = 8) ppm. The toxicokinetic models used the measured concentrations to calculate Hg uptake and elimination rate constants in the mealworms.

At the beginning of the bioaccumulation test, the background Hg concentration in the mealworms was 0.0078 (±0.005) fresh body weight (mean ± SD, n =5). According to table 3, it is possible to observe that the Hg concentration in larvae increased through the time when exposed to Hg contaminated substrate, reaching the maximum Hg content at day 22 of exposure (0.1936 Hg ppm). After that, the Hg concentration in larvae decreases but maintains the Hg levels until the 42d of the test. Table 3 also shows that larvae increased their weight through time, reaching their maximum size at day 22 (118.58 mg), stabilizing their growth until the end of the test. On day 42, the Hg content in mealworms exposed to Hg in the first 21 days was also statistically higher than the Hg content of mealworms exposed to clean food during the whole 42 days (0.0194 mg/kg Hg) (T-test, 0.030).

Table 3. Initial and final weight values (mg ± SD), Hg content (ng ± SD) and Hg concentration (ppm ± SD) of *Tenebrio molitor* larvae during different times of exposure to oat contaminated with 0.7 ppm of Hg. At each sampling time (days of exposure), larvae were weighted, and according to their Hg content, the concentration (ppm) of Hg in the body of larvae was assessed.

Days of exposure	Initial weigh (mg)	Final weigh (mg)	Hg concentration (ng)	Hg (ppm)
0	76.56 (±2.01)	76.56 (±2.01)	0.553 (±0.365)	0.0078 (±0.005)
1	71.94 (±4.41)	76.60 (±6.18)	4.43 (±4.132)	0.0603 (±0.050)
3	72.12 (±2.52)	78.02 (±9.49)	6.966 (±9.19)	0.0842 (±0.108)
7	70.74 (±3.34)	77.96 (±15.02)	7.769 (±7.821)	0.0929 (±0.085)
14	71.98 (±4.13)	85.40 (±20.53)	10.27 (±8.881)	0.1121 (±0.074)
21	70.28 (±6.07)	85.02 (±29.46)	8.40 (±8.867)	0.0851 (±0.059)
22	70.08 (±6.94)	118.58 (±18.70)	21.83 (±3.638)	0.1936 (±0.053)
24	70.84 (±3.95)	73.04 (±3.08)	5.333 (±3.134)	0.0765 (±0.042)
28	70.94 (±5.25)	93.06 (±33.71)	9.736 (±9.529)	0.0835 (±0.071)
35	73.90 (±4.34)	90.20 (±18.29)	6.467 (±5.359)	0.0634 (±0.049)
42	69.80 (±6.98)	113.07 (±33.04)	12.629 (±8.222)	0.1037 (±0.041)

2.4.3. Uptake and elimination kinetics

The kinetic parameters for the model 1 was k1 = 0.018 g_{food}/g_{animal}/day and k2 = 0.067 day^{-1}. For the model 2 the uptake constant differed slightly from model 1 (k1 = 0.056 g_{food}/g_{animal}/day) and had a distinct elimination constant, k2 = 0.316 day^{-1} (Table 4). In addition, the parameter inert fraction (Fi) had a value of 0.682. Using this toxicokinetic approach, the best that fits our data is the classic one-compartment model, with an AICc of 2.84, the lowest obtained value (Figure 3 – top graph, Table 4). Considering this, and assuming this one compartment model approach, we derived a bioaccumulation factor of 0.134 (uptake) and 0.163 (elimination) with our results. Even though it was not the best model fitting our data, model 2 (Eq. 2) revealed an inert fraction of almost 70% inside the organism (Table 4, Figure 3 - bottom graph). Regarding the Hg uptake, we tried to confirm if our organisms achieved a steady state. Comparing our 21d to day 14, we found statistical differences in the Hg concentrations (ppm) in the organisms, confirming that organisms did not reach the steady-state during the exposure to contaminated Hg food (T-test, 0.029).

Table 4. Uptake and elimination kinetic parameters for Hg in mealworms (*Tenebrio molitor*) exposed to contaminated 0.7 ppm Hg oat. k_1 is the uptake rate constant, k_2 the elimination rate constant and Fi the inert fraction. AICc was used to select the best toxicokinetic model using an information-based approach with the Akaike Information Criteria (the lowest AICc was considered the best model). 95% confidence intervals are given in brackets.

	k1 (kg_{food}/kg_{org}/day)	k2 (day^{-1})	Fi	AICc
Model 1	0.018 (±0.005)	0.067 (±0.029)	-	2.84
Model 2	0.056 (±0.030)	0.316 (±0.189)	0.682	4.99

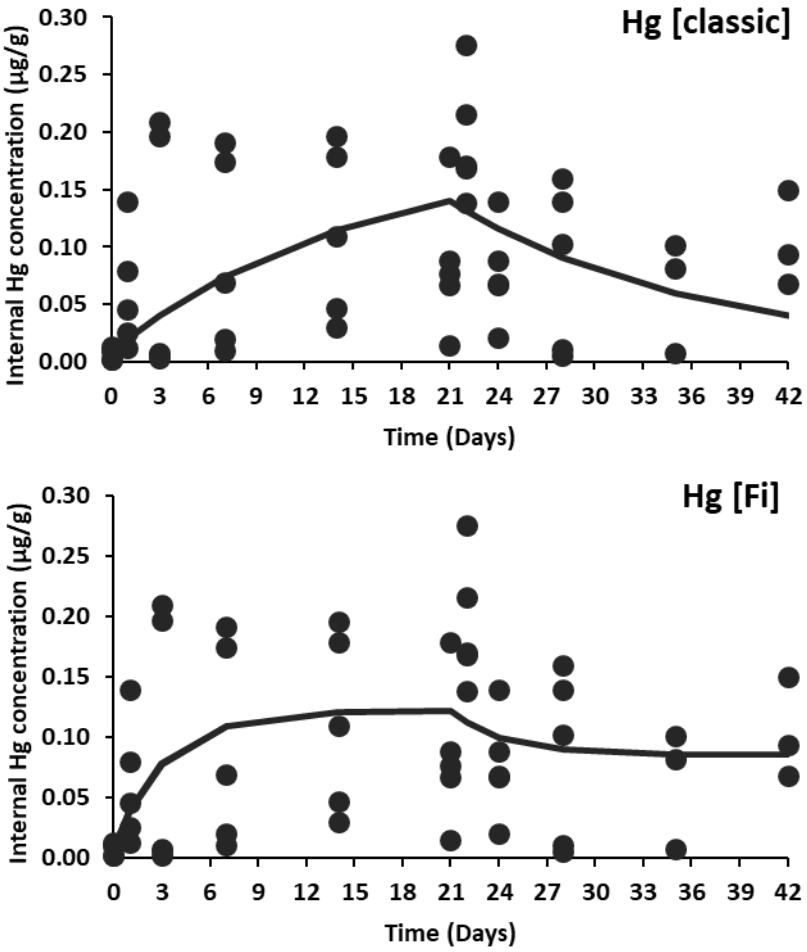

Figure 4. Uptake and elimination kinetics of Hg in mealworms (*Tenebrio molitor*) exposed to oat contaminated with 0.7 ppm of Hg. Points show the obtained Hg values in mealworms (n= 3). On the top graph, lines show the fit of a classic one-compartment model to the Hg concentrations measured in the mealworms (Eq. 1 and 2), and the lines on the bottom graph show the fit to one-compartment first-order model with inert fraction (Fi) (Eq. 1 and 3).

2.5. Discussion

As an initial step, this work investigated the differences in the biomass gain of the Yellow mealworm (*Tenebrio molitor*), at different larval stages and in the presence of soil and oat, combining different conditions. In the treatment where oat and soil were present, both larval stages presented the highest average weight on the last day of exposure. However, that treatment had the highest mortality rate, especially for the 20-40 mg weight range larvae. A recent study from Khodaparast et al. (2021) investigated the Ag toxicokinetics in *T. molitor* exposed via soil or food to different silver nanoparticles, evaluating different routes of exposure (pore water exposure and soil particle

ingestion). The authors observed that mealworms exposed to dyed soil showed guts completely tinted and that collected faeces from mealworm contained soil. With this, and considering our results, we can discuss that mealworms ingest soil, contributing significantly to their biomass growth. However, despite the high mass gains when in soil, those treatments revealed to be responsible for the high mortality rates. Vijver et al. (2003) assessed the metal uptake of Cd, Cu, Pb, and Zn from different soils by the larvae of *T. molitor*, reporting negligible mortality rates, compared the obtained ones in this study. Also, Khodaparast et al. (2021) reported maximum mortalities of 34% and 27% in mealworms exposed to spiked soil and food, respectively. Similar mortality rates were found in control situations, concluding that mortality was not related to the presence of silver nanoparticles.

Daily larvae observations enabled to detect the presence of fungi in larvae body and soil near the larvae. This observation seems to agree with a review on some of the primary insect diseases caused by microorganisms and viral pathogens among insects already used for food and feed (Eilenberg et al., 2015). For example, Eilenberg et al. (2015) highlighted the high susceptibility of the yellow mealworm in the presence of fungi of the genera Beauveria, particularly *Beauveria bassiana* causing the white muscardine disease. Fungi belonging to this genera are pathogenic to insects and are commonly found in the soil, so, for this reason, this insect has been extensively used as "bait" to isolate them from soil (Kim et al., 2018; Sun & Liu, 2008). Through the isolation and culture of samples of fungi present in the soil (data not shown), it was found two entomopathogenic species *Mortierella sp. and Metarhizium anisopliae.* In addition, species of *Mucor moelleri and Mucor circinelloides* were identified. Knowing that *Tenebrio molitor* larvae are more prone to die by a fungal infection in the presence of soil, the bioaccumulation assays were conducted only providing oat without soil. The mortality rates also decreased for the organisms with 60-80 mg. Considering that larger sizes are more suitable for rearing insects for food and feed, this larval stage was chosen for the bioaccumulation assays.

In the light of the safety evaluation on using insects as food and feed, assessing the accumulation of metals considered dangerous and priority pollutants by the regulation in force (European Parliament and Council of European Union, Directive 2000/60/EC) should be a priority. Up to now, only one study evaluated the influence of feeding substrates on the presence of Hg in larvae of *T. molitor* (Truzzi et al., 2019). Results revealed that Hg content in larvae is clearly influenced by Hg content in the feeding substrate. Also, the authors found the bioaccumulation capacity of mealworms to Hg, with Bioaccumulation Factors (BAFs) from 6.9 (Hg contaminated organic wheat flour) to 1.5 (mixture of Hg contaminated organic wheat flour and organic olive-pomace). The BAF obtained on our exposure to oat contaminated with 0.7 ppm of Hg during 21d (0.134) of uptake and 21d (experiment day 42) of elimination (0.163) in clean oat. Comparing with Truzzi et al., (2019) the BAF determined (only absorption phase) was between 1.5 (in substrate: 75% organic wheatmeal and

25% organic olive-pomace; 50% organic wheatmeal and 50% organic olive-pomace) and 6.2 in 100% organic wheat flour. Truzzi et al. (2019) investigated the presence of Hg in new feeding substrates coming from solid residues generated by olive fruits processing (olive-pomace) and their influence on the metal content in larvae of *T. molitor*. As an example, larvae exposed to a mixture of 25% organic wheat flour and 75% of organic olive-pomace, assimilated 1.6 $\mu g.kg^{-1}$ dw of Hg in their bodies. This set the alarm to the case of exposure to mercury-contaminated food in larvae of *T. molitor* since mercury is known to bioaccumulate in organisms (Kidd et al. 2012). Differences in bioaccumulation rates between our study and Truzzi et al. (2019) could be explained by different larval stages (related to synchronization processes), different types of substrate, test conditions, and Hg values in the substrate. This leads to a need for a more comprehensive analysis on the effects of mercury on dietary exposure to *T. molitor*, with more detailed information for different larval stages, substrates and even different Hg contents. Unfortunately, no studies evaluate the toxicokinetics of Hg in *T. molitor* or even in insects' larvae. However, studies with soil-dwelling organisms exposed to Hg revealed that both collembolans and earthworms took a long time to reach internal mercury steady-state, turning the detoxification a slow process (Burton et al., 2006; Cardoso et al., 2019). Also, the toxicokinetics of exposure of the isopod *Porcellionides pruinosus* to Hg contaminated soil revealed storage of a large fraction of Hg accumulated by isopods, according to previous metal toxicokinetics studies performed with terrestrial isopods exposed to different metals (Morgado et al. 2021).

Bioaccumulation studies in soil invertebrates such, collembolans, isopods, snails, earthworms, and insects have already been thoroughly investigated, focusing on exposure to contaminated soil or food (Vijver et al. 2003; Nahmani et al. 2007; Coelho et al. 2018; Drăghici et al. 2019; Khodaparast et al. 2021). In these bioaccumulation studies, toxicokinetic models were applied due to their usefulness as a measurement of metal bioavailability by considering uptake, elimination, and biodistribution of the toxicant over time. In addition, non-essential metals may be excreted or stored in the body. Based on these strategies, different bioaccumulation models were applied here to estimate the best bioaccumulation kinetics of Hg in the yellow mealworm larvae exposed via food. The model that fitted best our data was the classic one-compartment model, used to describe the uptake and elimination of Hg. Data obtained in this study was somehow challenging to discuss due to the high variability of measured Hg in bodies in each sampling time. Looking at our data, it seems that mealworms quickly uptake Hg from food at the very beginning of the experiment. However, data is somehow scattered, with high differences in Hg content per sampling time, which can be attributed to some mealworms avoidance of food. We did not find any relation between the high variability of Hg in mealworms in each sampling time and their biomass gain. However, a first biomass gain test showed that in tests without soil, the gain of biomass may not a factor to consider.

With this, it is possible that some mealworms avoided food and start eating only when the uptake phase ended, and the elimination phase started with clean food provided.

The AICc results show that the classic one-compartment model fits best our data. This is due to the high variability obtained in our samples and that simpler models are preferable in toxicokinetic evaluation, due to low number of replicates per sampling time. However, it is possible to observe that mealworms uptake mercury from food and do not eliminate it completely. Bednarska and Świątek (2016) investigated the internal compartmentalization of metals in different subcellular fractions of a non-essential (Cd) and essential (Zn) metal in *T. molitor*, observing that Cd accumulated in higher concentrations in the cellular debris (tissue and cell membranes) followed by the cytosolic and organelle fraction. Zn accumulated in similar concentrations across all fractions, cytosolic and organelle, cellular debris and granule fractions. Research on mercury bioaccumulation in soil-dwelling organisms are scarce; however, being a non-essential metal like cadmium, this study could also explain where this metal could be captured in this species. Though it needs to be confirmed, Hg could be concentrated in the cellular debris.

Similar to this study, Khodaparast et al. (2021) used a one-compartment first-order model with inert fraction (Fi) to describe their results. When mealworms were exposed to 50 nm AgNPs contaminated soil, mealworms sequestered greater quantities of Ag, having the highest inert fraction (Fi=0.54) compared to the other pristine AgNPs. However, mealworms exposed to Ag spiked food revealed a similar difficulty in eliminating 3-8 nm and 60 nm AgNPs (Fi=0.69 and 0.72) compared to the other AgNPs.

Considering the aim to consider this species for food and feed, the trophic transfer of Hg to superior levels could lead to its biomagnification. Bednarska and Świątek (2016) observed that the Cd sequestered in *T. molitor* from contaminated food, 30% of it was located in the cytosolic and organelle fraction, in a soluble form, which is essential for the transport of metals to higher trophic levels due to its bioavailability (Bednarska & Świątek, 2016). In a study on heavy metal accumulation through a soil-plant-insect system, in a subsystem of herbivorous insect – carnivorous insect, it was observed different metal concentrations among different carnivorous species feeding on the same prey (Zhang et al. 2009). Predator physiology is a factor in metal transfer in the trophic chain. Research on the mechanisms of Hg sequestration is important to assess the biomagnification in livestock, chicken, fish and, subsequently to humans.

Previous research on the potential of the yellow mealworm as a new protein source has shown their promise as a safe and healthy alternative (FAO, 2013; Finke, 2002; Ramos-Elorduy et al., 2002; Sánchez-Muros et al., 2016). Nevertheless, this study on the accumulation of Hg from food observed that Hg was not totally eliminated from mealworms. Considering the study of Truzzi et al. (2019), the study of Hg in mealworms and other edible insects should be carried out for more in-depth

knowledge. Also, different concentrations of Hg, different larval stages and substrates should be tested for a more comprehensive analysis of the possible deleterious effects of Hg in the trophic chain. This thesis gives the first step for comprehending how Hg can be a problem in terms of accumulation in mealworms, with a possible need for redefining regulations. Future research should study how, where and how much occurs the stored mercury in this species. To conclude, there is of major importance to study the subcellular metal partitioning in *Tenebrio molitor* to comprehend the mechanisms of accumulation and toxicity of mercury.

2.6. Conclusions

Overall, the present study demonstrated that two larval stages of *T. molitor* had different mortality rates and biomass gains when exposed to different substrates. The ones with 60-80 mg had the highest biomass gain and soil induced higher mortality levels. Therefore, we suggest that bioaccumulation studies using *T. molitor* should be conducted without soil, prioritizing the food as a medium of exposure for these type of experiments that aim at evaluating the circularity of (bio)mass, and use learvae as food and fed. This is the first study to report the viability of toxicokinetic models as an approach to understand the uptake and elimination patterns of Hg in yellow mealworm larvae. Our findings suggest that mealworms quickly uptake Hg from food at the very beginning of the experiment and do not entirely eliminate mercury at the end of a 21d elimination period in a clean substrate. This, coupled with previous studies with mercury and mealworms, leads the door open for future research using different conditions to understand how mercury bioaccumulates in mealworm larvae. With the final aim of turning the process of using insects larvae for food and feed, this evaluation is critical to possible redefinitions of the current legislations.

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Chapter 3

3. General Discussion and Conclusion

- A new bioaccumulation experiment was tested with mealworms which could provide a foundation for future experiments with other metals

This study aimed to fill the knowledge gap on the potential to bioaccumulate and eliminate metals, in this case, Hg in larvae of *T. molitor* through the rearing substrate. It showed that the most reliable method for a bioaccumulation experiment using *T. molitor* larvae is without soil due to the presence of fungus during the experiments. With this, it is advised to conduct experiments with contaminated oat only, without soil when aiming at evaluating contamination of larvae that are used for food and fed. Furthermore, the use of *Tenebrio molitor* as a test species for the bioaccumulation tests was chosen due to its short life cycle, easy maintenance, and potential as an alternative protein source. It proved to be a suitable test species for future work.

- The results of this study are relevant to edible insects' industry and the EU Regulation

Through this study, our results show that Hg is sequestered in the yellow mealworm which could affect food safety. Nevertheless, future experiments with a phase of depuration (few days with no/clean substrate after uptake phase) could be considered thus confirming if Hg is eliminated or not. On the other hand, this study design may not be advantageous to the industry due to the expected weight loss and it being time consuming. Heavier larvae originate more biomass to be commercialized, and consequently increased gains for this industry. Maximum levels of mercury didn't exceed the levels set in specific foodstuffs and animal feed by the EU Regulations. However, the new regulation could be updated regarding the absorption of Hg observed in this study and, apply this method with other metals.

4. Supplemental Data

Table 1. Results from the 2-way ANOVA of the effects of exposure/absence of soil and oat on mealworms with a weight range of 60-80 mg.

Source of Variation	df	dd	ms	F	p
	SO vs NSO				
Treatment	1	3.094	3.094	154.64	<0.001
Days	4	5.187	1.297	64.821	<0.001
Treatment x Days	4	0.444	0.111	5.547	<0.001
Residual	54	1.08	0.02		
Total	63	9.489	0.151		
	SO vs SNO				
Treatment	1	0.433	0.433	71.018	<0.001
Days	4	2.747	0.687	112.522	<0.001
Treatment x Days	4	0.0648	0.0162	2.653	0.044
Residual	48	0.293	0.0061		
Total	57	3.589	0.063		
	SNO vs NSNO				
Treatment	1	4.484	4.484	1031.582	<0.001
Days	4	0.41	0.103	23.599	<0.001
Treatment x Days	4	0.615	0.154	35.366	<0.001
Residual	52	0.226	0.00435		
Total	61	6.016	0.0986		
	NSO vs NSNO				
Treatment	1	3.38	3.38	409.273	<0.001
Days	4	1.06	0.265	32.1	<0.001
Treatment x Days	4	1.521	0.38	46.035	<0.001
Residual	58	0.479	0.00826		
Total	67	6.522	0.0973		
	SO vs NSNO				
Treatment	1	11.805	11.805	992.107	<0.001
Days	4	1.449	0.362	30.448	<0.001
Treatment x Days	4	1.87	0.467	39.281	<0.001
Residual	52	0.619	0.0119		
Total	61	14.928	0.245		
	NSO vs SNO				
Treatment	1	0.439	0.439	43.503	<0.001
Days	3	2.7	0.9	89.173	<0.001
Treatment x Days	3	0.258	0.0861	8.527	<0.001
Residual	47	0.474	0.0101		
Total	54	3.891	0.0721		

SO: Soil + Oat; NSO: Oat only; SNO: Soil only; NSNO: No Soil, No Oat.

Table 2. Results from the 2-way ANOVA of the effects of exposure/absence of soil and oat on mealworms with a weight range of 60-80 mg, within days.

	Diff of Means	p	q	P
	SO vs NSO			
Within day 1	0.226	2	4.223	0.004
Within day 3	0.614	2	11.477	<0.001
Within day 7	0.644	2	12.053	<0.001
Within day 14	0.329	2	5.611	<0.001
Within day 21	0.465	2	6.736	<0.001
	SO vs SNO			
Within day 1	0.105	2	3.544	0.016
Within day 3	0.149	2	5.041	<0.001
Within day 7	0.272	2	9.222	<0.001
Within day 14	0.15	2	4.493	0.003
Within day 21	0.268	2	5.318	<0.001
	SNO vs NSNO			
Within day 1	0.223	2	8.966	<0.001
Within day 3	0.547	2	21.938	<0.001
Within day 7	0.619	2	24.824	<0.001
Within day 14	0.805	2	29.911	<0.001
Within day 21	0.692	2	18.178	<0.001
	NSO vs NSNO			
Within day 1	0.0602	2	1.753	0.22
Within day 3	0.15	2	4.362	0.003
Within day 7	0.479	2	13.936	<0.001
Within day 14	0.789	2	22.076	<0.001
Within day 21	0.756	2	21.14	<0.001
	SO vs NSNO			
Within day 1	0.286	2	6.937	<0.001
Within day 3	0.763	2	18.517	<0.001
Within day 7	1.123	2	27.239	<0.001
Within day 14	1.118	2	23.934	<0.001
Within day 21	1.221	2	22.382	<0.001
	NSO vs SNO			
Within day 1	0.163	2	4.298	0.004
Within day 3	0.397	2	10.45	<0.001
Within day 7	0.14	2	3.684	0.012
Within day 14	0.0158	2	0.4	0.779

SO: Soil + Oat; NSO: Oat only; SNO: Soil only; NSNO: No Soil, No Oat.

Table 3. Results from the 2-way ANOVA of the effects of exposure/absence of soil and oat on mealworms with a weight range of 20-40 mg.

Source of Variation	df	dd	ms	F	p
	SO vs NSO				
Treatment	1	6.261	6.261	80.777	<0.001
Days	3	2.55	0.85	10.967	<0.001
Treatment x Days	3	0.864	0.288	3.714	0.018
Residual	46	3.566	0.0775		
Total	53	12.507	0.236		
	SO vs SNO				
Treatment	1	0.217	0.217	9.81	0.003
Days	3	1.139	0.38	17.157	<0.001
Treatment x Days	3	0.129	0.0432	1.95	0.135
Residual	45	0.996	0.0221		
Total	52	2.423	0.0466		
	SNO vs NSNO				
Treatment	1	5.752	5.752	554.259	<0.001
Days	3	0.0742	0.0247	2.382	0.081
Treatment x Days	3	0.298	0.0994	9.578	<0.001
Residual	47	0.488	0.0104		
Total	54	6.527	0.121		
	NSO vs NSNO				
Treatment	1	1.639	1.639	60.753	<0.001
Days	4	0.0652	0.0163	0.605	0.661
Treatment x Days	4	0.415	0.104	3.847	0.008
Residual	58	1.565	0.027		
Total	67	3.664	0.0547		
	SO vs NSNO				
Treatment	1	11.898	11.898	199.075	<0.001
Days	3	1.262	0.421	7.039	<0.001
Treatment x Days	3	1.95	0.65	10.876	<0.001
Residual	46	2.749	0.0598		
Total	53	16.941	0.32		
	NSO vs SNO				
Treatment	1	4.62	4.62	70.958	<0.001
Days	3	1.344	0.448	6.882	<0.001
Treatment x Days	3	0.0968	0.0323	0.496	0.687
Residual	47	3.06	0.0651		
Total	54	8.955	0.166		

SO: Soil + Oat; NSO: Oat only; SNO: Soil only; NSNO: No Soil, No Oat.

Table 4. Results from the 2-way ANOVA of the effects of exposure/absence of soil and oat on mealworms with a weight range of 20-40 mg, within days.

	Diff of Means	p	q	P
	SO vs NSO			
Within day 1	0.317	2	3.01	0.039
Within day 3	0.636	2	6.046	<0.001
Within day 7	0.747	2	7.096	<0.001
Within day 14	1.041	2	9.034	<0.001
	SO vs SNO			
Within day 1	0.0275	2	0.49	0.731
Within day 3	0.122	2	2.166	0.133
Within day 7	0.177	2	3.155	0.031
Within day 14	0.244	2	3.827	0.01
	SNO vs NSNO			
Within day 1	0.441	2	11.446	<0.001
Within day 3	0.602	2	15.644	<0.001
Within day 7	0.697	2	18.102	<0.001
Within day 14	0.85	2	21.22	<0.001
	NSO vs NSNO			
Within day 1	0.0792	2	1.275	0.371
Within day 3	0.19	2	3.061	0.035
Within day 7	0.35	2	5.643	<0.001
Within day 14	0.418	2	6.732	<0.001
Within day 21	0.523	2	7.691	<0.001
	SO vs NSNO			
Within day 1	0.396	2	4.285	0.004
Within day 3	0.826	2	8.942	<0.001
Within day 7	1.097	2	11.873	<0.001
Within day 14	1.459	2	14.417	<0.001
	NSO vs SNO			
Within day 1	0.49	2	5.082	<0.001
Within day 3	0.597	2	6.189	<0.001
Within day 7	0.523	2	5.421	<0.001
Within day 14	0.712	2	7.091	<0.001

SO: Soil + Oat; NSO: Oat only; SNO: Soil only; NSNO: No Soil, No Oat.