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Edible insects: Understanding benzo(*a*)pyrene toxicokinetics in yellow mealworms for safe and sustainable consumption

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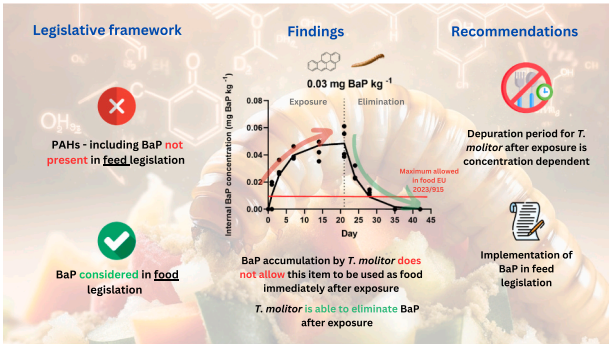
HIGHLIGHTS

- Toxicokinetics of Benzo(*a*)pyrene was studied in edible insect larvae.
- Dose-dependent accumulation in yellow mealworms was observed.
- Introducing a depuration phase to the practice of insect rearing as solution.
- The study results support the improvement of using insects as feed and food.

GRAPHICAL ABSTRACT



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ABSTRACT

The global interest in edible insects as sustainable protein sources raises concerns about the bioaccumulation of contaminants, including polycyclic aromatic hydrocarbons (PAHs), to problematic levels. Understanding the accumulation dynamics of PAHs in edible insects is highly relevant due to the widespread sources and toxicological profiles; however, the bioaccumulative potential of PAHs in edible insects is unexplored. This study examined the uptake and elimination dynamics of benzo(*a*)pyrene (B(*a*)P), a representative and carcinogenic PAH, in yellow mealworm larvae (YMW, *Tenebrio molitor*). Larvae were exposed to feeding substrate with varying B(*a*)P concentrations (0.03, 0.3, and 3 mg kg^{-1}), and uptake (21 days in B(*a*)P-contaminated substrate) and elimination (21 days in B(*a*)P-free substrate) kinetics were subsequently assessed. The results showed that YMW can eliminate B(*a*)P, revealing dose-dependent B(*a*)P bioaccumulation in these insects. Larvae fed on a substrate with 0.03 mg kg^{-1} accumulated B(*a*)P over 21 days, presenting values of 0.049 (Standard deviation - 0.011) mg kg^{-1} and a kinetic-based ($BAF_{kinetic}$) of 1.93 g $_{substrate}$ g $^{-1}_{organism}$, exceeding the EU regulatory limits for food. However, with a B(*a*)P half-life (DT_{50}) of 4.19 days in the larvae, an EU legislation safety criterion was met after

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a 13-day depuration period in clean substrate. Larvae exposed to substrates with 0.3 and 3 mg kg^{-1} showed B(*a*)P accumulation, with $BAF_{kinetic}$ values of 3.27 and 2.09 g $_{substrate}$ g $^{-1}_{organism}$, respectively, not meeting the current legal standards for food consumption at the end of the exposure to B(*a*)P. Although the B(*a*)P half-life values after 35 days were 4.30 and 10.22 days (DT_{50s}), the larvae retained B(*a*)P levels exceeding permitted food safety limits. These findings highlight a significant oversight in regulating PAHs in animal feed and the need for comprehensive safety evaluations of PAH hazards in edible insects for improved PAH feeding guidelines.

1. Introduction

Growth of the global population is escalating, which requires identifying and developing more sustainable, efficient, and nutritious food sources (Mancini et al., 2019). Current challenges center around procuring novel protein sources, considering that traditional sources such as livestock and dairy products are resource-intensive and significant contributors to environmental issues, including greenhouse gas emissions, land degradation, and water pollution and scarcity (Parodi et al., 2018; van Huis, 2013). Coupled with increasing global demands, these sources alone can no longer sustain our nutritional needs, thus creating a pressing need for innovative dietary solutions that promote environmental sustainability (Dobermann et al., 2017; van Huis and Oonincx, 2017). Insects, such as *Tenebrio molitor* (yellow mealworm beetle - YMW), have shown significant potential in this regard. This insect can efficiently convert organic materials such as agri-food waste into protein- and fat-rich biomass that could be further used as animal feed or human food, closing production loops in the food supply chain and improving its circularity and sustainability. Moreover, rearing insects offer a balanced nutritional profile, rich in essential amino acids and other nutrients that reflect their high quality as feed (e.g., Beller et al., 2024; Noyens et al., 2023; Ringseis et al., 2023; Zhang et al., 2019).

Understanding and evaluating the chemical hazards when rearing YMW is a critical component of environmental safety and health research, particularly given the increasing interest in using this insect as an alternative protein source (Poma et al., 2022; Poma et al., 2019; van Huis et al., 2021; Belluco et al., 2018; Belluco et al., 2013; Van der Fels-Klerx et al., 2018). As with any potential food source, assessing the associated chemical risks is crucial (EFSA, 2015). Given the ability of YMW to be reared in different organic side streams and the capacity to bioaccumulate contaminants from various substrates, developing an understanding of chemical uptake and potential elimination should be monitored closely to ensure the safety and quality of the marketable product, whether it is for human consumption or animal feed (Cardoso et al., 2023). There is, in particular, a growing body of studies evaluating the safety of insects as feed, including those on the capacity of rearing insects to bioaccumulate different contaminants from substrates. Metals (e.g., Cardoso et al., 2023; Diener et al., 2015) and mycotoxins (e.g., Bosch et al., 2017; Camenzuli et al., 2018; Purschke et al., 2017) are among the frequently studied contaminants. Bioaccumulation of polychlorobiphenyls (PCBs) by *T. molitor* larvae was recently studied by Ratel and coauthors, confirming the transfer of PCBs to the larvae via food after 20 days of incubation (Ratel et al., 2023). As for polycyclic aromatic hydrocarbons (PAHs), various adverse effects on insect larvae have been reported. B(*a*)P induced oxidative stress, larvae development and mass, and the relative growth rate in *Lymantria dispar* (Ilijin et al., 2015), and the inhibition of acetylcholinesterase activity (Ilijin et al., 2017). An integrative study on the microbial and chemical safety of farming insect *Bombyx mori* confirmed the presence of various groups of contaminants – toxic metals, fungicides, dioxins, PCBs, mycotoxins, along with PAHs (Marzoli et al., 2023). For example, Fan et al. (2020) investigated the potential use of *Hermetia illucens* larvae in PAH-contaminated waste treatment, exposing them to a range of concentrations of naphthalene, fluorene, phenanthrene, and pyrene (1–100 mg kg^{-1}), resulting in longer larval development time and reduction in relative growth rate (Fan et al., 2020). A knowledge gap exists, however, regarding the potential accumulation of PAHs in edible insects, underscoring the necessity for focused research related to use for feed and food (Meyer et al., 2021).

The main sources of PAHs are thermically treated food and contamination of the environment - air, soil, and water - due to various processes and activities such as industry, wildfires, and volcanic eruptions (Sampaio et al., 2021). While recent research is proving the benefits of the use of insects in the feed, both as sources of protein and lipids, understanding PAHs bioaccumulation by edible insects through substrate ingestion is essential to manage potential risks of xenobiotics (including PAHs) entering the food chain and to protect product safety, animal and human health (Beller et al., 2024; Ringseis et al., 2023; Van der Fels-Klerx et al., 2020). Considerable research has also been focused on human health risks of PAHs through the consumption of edible aquatic organisms, with associated risks determined in some cases. For example, Wang et al. (2023) determined bioconcentration factors >2000 in ten marine fish from coastal areas of Guangdong, China. This study concluded that the recommended acceptable daily intake values without causing carcinogenic risk for populations of the area were far below their actual daily intake. In addition, a recent study found biota-to-sediment bioaccumulation factors (BSAF) of 1.7 (phenanthrene), 1.5 (pyrene) and 1.7 (benzo[*k*]fluoranthene) in periwinkles from the Niger Delta, Nigeria (Saunders et al., 2022).

The rationale of evaluating bioaccumulation through the dietary intake of substrate applies to the entire food safety chain and circular bioeconomy perspectives because insects can be reared in organic streams that are poorly characterised and possibly contain contaminants of concern (Cardoso et al., 2023). This can be a significant step in allowing insects to use different types of substrates under current regulatory policy. For example, YMW was formally accepted in January 2021 by the European Food Safety Authority (EFSA) as a viable source for human consumption in Europe by approving the use of dried YMW. This landmark decision marked the inaugural approval of an insect as a novel food source in the European Union, allowing its potential integration into various food products (EFSA Panel on Nutrition et al., 2021). Currently, PAHs are not considered in the European Union Directive, 2002/32/EC regulating undesirable substances in animal feed. Accordingly, it regulates the substrates that YMW uses for feed (Directive, 2002). However, PAHs are included in the Commission Regulation (EU), 2023/915 on maximum levels for specific contaminants in food and revoking Regulation (EC) No 1881/2006, which considers YMW as food (Commission Regulation 2023/915; Commission Regulation 1881/2006). PAHs are frequently detected in food samples, with B(*a*)P being the most frequently detected (Bansal et al., 2017). Regarding human consumption, maximum B(*a*)P levels should not be higher than 0.001 mg kg^{-1} for infant formulae, follow-on formulae, and young-child formulae, and ranges up to 0.01 mg kg^{-1} for dried spices. In addition, the sum of ∑PAH4 such as benzo(*a*)pyrene, benz(*a*)anthracene, benzo(*b*)fluoranthene and chrysene should be limited to values not higher than 0.001 mg kg^{-1} and 0.05 mg kg^{-1} for the same products, respectively, as indicated in the same EC regulation.

B(*a*)P is a genotoxic carcinogen and has thus often been studied as a model carcinogenic PAH (Pogribny, 2019). The decision to focus on B(*a*)P as a model chemical in this study is an essential first step in understanding PAH accumulation in insects, providing a foundation for future research on a broader range of PAHs. Given the current status of B(*a*)P as the sole PAH regulated by the Commission Regulation, 2023/915 and its distinct properties compared to low-ring PAHs, selecting this

contaminant for study underscores its importance for assessing PAH accumulation in edible insects. Due to the rising consideration of YMW as a food protein source and the legislative gaps surrounding PAHs in animal feed (European Union Directive, 2002/32/EC), our study aimed to understand the uptake and elimination of B(*a*)P by YMW when exposed to substrates with varying B(*a*)P levels through a toxicokinetic approach with edible insects (Cardoso et al., 2023). The results of this study promise to serve as a basis for the development of safety regulations for the use of YMW as a food and feed source while promoting the adoption of circular practices in waste management by providing crucial data regarding PAHs for informing future decisions by policymakers, practices of insect farmers, and the global perception of consumers on the safety of this emerging protein source.

2. Materials and methods

2.1. Test organisms and substrate spiking with benzo(a)pyrene

Yellow mealworm larvae from a colony established in 2021 by Thunder Foods, Santarém, Portugal, were used. Newly hatched larvae were reared on semolina wheat bran (Agriloja®, Portugal) and raw potato slices, and 16-week-old larvae were shipped in a bran container to the applied Ecology and Ecotoxicology laboratory at the University of Aveiro, Portugal. Upon arrival, the larvae underwent a one-week acclimatisation period, followed by adaptation to controlled conditions of 25 ± 2 °C and a 16:8 h light/dark cycle for two days before starting the bioaccumulation experiments. Throughout the experimental procedure, YMW larvae were subjected to exposure conditions involving semolina wheat spiked at three realistic concentrations of B(*a*)P, 0.03, 0.3, and 3 mg kg^{-1} (dry weight). This concentration range was selected to address the absence of established PAH limits in feed, where typically higher values are permitted than food legislation. It aligns closely with the stringent maximum of 0.01 mg kg^{-1} set for food contaminants, thus providing a robust opportunity for study bridges regulatory gaps and account for the unique bioaccumulative properties of PAHs in insect-based products. This contamination was achieved using B(*a*)P ($C_{20}H_{12}$, CAS Number: 50–32-8), procured from Merck Millipore (Darmstadt, Germany), with a purity of ≥96 % (HPLC). A stock solution was prepared using methanol as a solvent, from which the necessary volume of the working solution was drawn to apply to 400 g of semolina wheat for each B(*a*)P concentration. Consequently, each treatment level involved adding 400 mL of methanol containing the appropriate B(*a*)P working solution concentration to the semolina wheat, facilitating proper homogenisation. After obtaining satisfactory homogenisation, 400 g of semolina was left for 48 h for the methanol to evaporate. A control group was established simultaneously, specifically using non-contaminated semolina wheat in the same conditions, including the same volume of methanol (B(*a*)P-free). Semolina wheat from all test containers was subjected to lyophilisation to prevent this fungal growth in the test.

2.2. Bioaccumulation studies

Two-phase bioaccumulation assays were used to evaluate how YMW took up and then eliminated B(*a*)P from their whole bodies, following the same developed methodology using YMW under exposure to Hg (Cardoso et al., 2023). Exposure was conducted using individual glass crystallisers covered with parafilm with needle holes, in which each well received 3 g of semolina wheat (either uncontaminated or contaminated with the determined concentrations). Conditions were maintained at 25 ± 2 °C and in a 16:8 h light/dark regime. The larvae used in the current experiments were, on average, 17 weeks old and at the 12^{th} to 13^{th} stage of development with an approximate mean weight of 15–20 mg.

The experimental study period was divided into two phases (uptake and elimination), each lasting for 21 days. YMW larvae were exposed to B(*a*)P-contaminated semolina wheat during the uptake phase, and individuals were extracted for analysis on days 1, 3, 7, 14, and 21. On study day 21, we moved the organisms to a B(*a*)P-free substrate. Then, subsequent sampling on days 22, 24, 28, 35, and 42 followed the same procedure used in the uptake phase. Three replicates were collected at each sampling time in both phases at each chemical concentration. Three experiments with the different chemical levels in substrates (0.03, 0.3, and 3 mg kg^{-1}) were carried out separately. Exposure to 0.03 mg kg^{-1} contained five organisms per replicate, and exposures to 0.3 and 3 mg kg^{-1} contained only one organism per replicate. The difference in the number of organisms per replicate reflected the tissue mass needed for B(*a*)P measurements, considering analytical detection limits (see section 2.3.2). Therefore, the number of organisms per replicate varied according to the substrate's B(*a*)P levels.

A control group of YMW larvae was kept within each experiment for 42 days, always in B(*a*)P-free semolina. Herein, sampling was conducted on days 0, 21, and 42 under experimental conditions identical to the B(*a*)P-contaminated exposure, and at each sampling event, three organisms per replicate were collected.

After sampling, all organisms followed a 24-h depuration period in glass vessels to facilitate gut content clearance, which allowed us to determine bioaccessible B(*a*)P levels accumulated in tissues. After this depuration time, organisms were freeze-dried for 48-h, and then their body weights were recorded for subsequent B(*a*)P quantification across all sampling events and treatments. Consequently, all the B(*a*)P quantifications in the results section are expressed per kg dry weight.

2.3. Benzo(a)pyrene analysis

2.3.1. Extraction of B(a)P from wheat bran and YMW

The mealworm samples were freeze-dried and subsequently cut into small pieces to powder as much as possible, and 2 mL of n-hexane (PanReac AppliChem) was added. Subsequently, 12 μL of 5.0 ppm anthracene (ANT, here used as internal standard) was added to each vial and taken to the sonicator for 10 min. The supernatant was removed and filtered into a test tube through Whatman syringe filters (regenerated cellulose of 0.2 μm pore size). Afterwards, 2 mL of n-hexane was added to each sample, and the extraction procedure was repeated twice.

For the extraction of B(*a*)P from the wheat bran samples (for both B(*a*)P-free control samples and B(*a*)P-contaminated treatments), 2 g of each sample was extracted with 20 mL of n-hexane after the addition of 120 μL of 5.0 ppm ANT and taken to the sonicator for 10 min. The supernatant was removed and filtered through Whatman syringe filters (PVDF of 0.2 μm pore size). Again, 20 mL of n-hexane was added to the samples, and the procedure was repeated twice. For each sample (wheat bran and YMW), the assembled n-hexane extracts were transferred into 1 g of silica (Supelclean LC-Si Bulk Packing, Supelco), packed into glass cartridges, and previously washed with 20 mL of dichloromethane and conditioned with 20 mL of n-hexane. Elution was then performed using 20 mL of n-hexane/dichloromethane (70:30) (*v*/v) mixture. Each collected fraction was evaporated to dryness in a rotary evaporator at 40 °C, and the obtained residues were redissolved in 2 mL of acetonitrile (ACN) and placed in glass vials. Finally, each extract was filtered through Whatman syringe filters (regenerated cellulose of 0.22 μm pore size) before the analysis using high-performance liquid chromatography with fluorescence detection (HPLC-FD). HPLC-FD instrumentation and chromatographic conditions are described in the Supplementary Information file.

2.4. Toxicokinetics and data analysis

Given that YMW grew considerably during the study, growth was accounted for in our estimations for uptake and elimination kinetics. For this, a growth dilution element was incorporated into the toxicokinetics models using a growth rate constant (K_{growth}), as represented by an exponential growth equation (eq. 1) for the organismal biomass data (Ardestani et al., 2014; Cardoso et al., 2023; Van Den Brink et al., 2019):

$$Bt = B0 \times e^{(K_{growth} * t)} \quad (1)$$

where Bt denotes the biomass (mg) at time t (days), B0 is the starting biomass of the larvae (mg), and K_{growth} is the constant growth rate (day^{-1}).

For the toxicokinetic analysis, the uptake and elimination kinetics of B(a)P by YMW were captured through a single-compartment model, treating the animal as a unified compartment with individual uptake and elimination rates and including the K_{growth} in the model in SPSS (version 28).

For the uptake phase, the below equation was utilised (eq. 2):

$$Q_{(t)} = C_0 + \frac{K_1}{K_2 + K_{growth}} \times C_{exp} \times \left(1 - e^{-(K_2 + K_{growth}) \times t}\right) \quad (2)$$

Here, Q(t) is the concentration of B(a)P in the organism at t days (mg B(a)P $kg^{-1}_{organism}$); C_0 is the basal internal B(a)P concentration (mg B(a)P $kg^{-1}_{organism}$) calculated from the mean measured B(a)P body concentration at $t = 0$; K_1 the uptake rate constant ($g_{substrate}\ g^{-1}_{organism}\ day^{-1}$); K_2 the elimination rate constant (day^{-1}); C_{exp} the measured B(a)P concentration in the exposure medium (mg B(a)P $kg^{-1}_{substrate}$); K_{growth} is the growth rate estimated using an exponential growth model (day^{-1}), and t is the time (days).

The equation for the elimination phase (eq. 3) followed a similar structure, also incorporating the growth rate constant:

$$Q_{(t)} = C_0 + \frac{K_1}{K_2 + K_{growth}} \times C_{exp} \times \left(e^{-(K_2 + K_{growth}) \times (t - t_c)} - e^{-(K_2 + K_{growth}) \times t}\right) \quad (3)$$

with t_c as the final day of the uptake phase and when larvae were transferred to a clean substrate, other variables are defined similarly to the previous section. The toxicokinetic parameters were derived using non-linear regression, and differences in K_1 and K_2 values across B(a)P treatments were examined through Generalised Likelihood Ratio Tests using SPSS (IBM Corp, version 29.0.0) The Kinetic Bioaccumulation Factor ($BAF_{kinetic}$) ($g_{substrate}\ g^{-1}_{organism}$) was determined as the ratio between the uptake (K_1) and elimination (K_2) rate constants (Eq. 4):

$$BAF_{kinetic} = \frac{K_1}{K_2} \quad (4)$$

The Biota-Substrate Accumulation Factor (BSAF) ($g_{substrate}\ g^{-1}_{organism}$) was calculated by dividing the internal B(a)P concentration in insects at the end of the exposure period (21d) (C_{org}) by the B(a)P concentrations in the substrate (C_{subst}) (Eq. 5):

$$BSAF = \frac{C_{org}}{C_{subst}} \quad (5)$$

The half-life time values of B(a)P (DT_{50}) in YMW larvae were computed (Eq. 6) as:

$$DT_{50} = \frac{ln(2)}{K_2} \quad (6)$$

The depuration time (1 day) of YMW before sampling processing was included in the calculated DT_{50s} for different B(a)P concentrations.

3. Results

3.1. B(a)P analysis in the substrate

Semolina wheat substrates were contaminated to obtain concentrations of 0.03, 0.3, and 3 mg kg^{-1}. The concentrations obtained upon measurement were 0.028 (92.29 % of the nominal concentration), 0.34 (113.41 %) and 3.42 mg kg^{-1} (113.92 %), respectively, after contamination. The observed differences between the nominal and obtained concentrations are <14 %, making them acceptable for the scope of our study and for using the nominal concentrations in the presentation and discussion of the results. However, it is worth mentioning that all the toxicokinetic analyses were conducted with the measured concentration. Baseline B(a)P concentrations in the substrate were below the LOD (0.29 μg L^{-1}).

3.2. Bioaccumulation studies

3.2.1. B(a)P bioaccumulation in T. molitor

As introduced above, we examined three distinct concentrations of B(a)P in the substrate (0.03, 0.3 and 3 mg kg^{-1}) during 42 days of each experiment, which were subdivided into an uptake phase (days 0–21) and an elimination phase (days 22–42). Baseline B(a)P concentrations in YMW were below the LOD (0.29 μg L^{-1}) in all controls at days 0, 21 and 42. Due to unforeseen events, a sample was lost on sampling day 22 of the exposure to 0.03 mg kg^{-1}. Therefore, the analysis did not consider the sampling time for that concentration.

Uptake Phase (0–21 days) – YMW exposed to the 0.03 mg kg^{-1} exhibited consistent uptake over the 21 days, culminating in a mean concentration of 0.049 (±SD 0.011) mg kg^{-1} dw at day 21 (Table 1, Fig. 1A). Body concentrations of YMW exposed to 0.3 mg kg^{-1} were below the detection limit from day 0 to 7 (Table 1, Fig. 1B). This was likely due to insufficient tissue for analysis since YMW larvae have relatively small sizes/weights during the first several days of the experiment. However, by day 14, there was a pronounced increase, with body burden concentrations reaching an average of 1.20 (±0.075) mg kg^{-1} (Table 1, Fig. 1B). Insects collected on the last day of exposure (day 21) presented a lower amount of B(a)P, with 0.75 (±0.67) mg kg^{-1}, compared with organisms sampled on day 14 (Table 1). The most substantial accumulation was observed when YMW was exposed to 3 mg kg^{-1}. By day 14, the mean concentration reached 4.45 (±0.50) mg kg^{-1}, followed by a slightly lower value measured on the last day of the exposure, presenting 4.10 (±1.20) mg kg^{-1} (Table 1, Fig. 1C).

Elimination Phase (22–42 days) – After the uptake phase, first order elimination trends were evident across all concentrations. For exposure to 0.03 mg kg^{-1}, B(a)P body burdens of exposed organisms rapidly declined, reaching undetectable levels by day 35 (Table 1, Fig. 1A). After exposure to 0.3 mg kg^{-1}, YMW experienced a more gradual decline, registering a mean body burden level of 0.088 (±0.054) mg kg^{-1} when the experiment concluded on day 42 (Table 1, Fig. 1B). Similarly, after exposure to 3 mg kg^{-1} B(a)P content in insects declined, averaging at 0.73 (±0.097) mg kg^{-1} by day 42 (Table 1, Fig. 1C).

3.2.2. Toxicokinetic modelling

Toxicokinetic evaluations of B(a)P in YMW were conducted across the concentrations of 0.03, 0.3, and 3 mg kg^{-1}. The growth constants

Table 1
Measured total Benzo(a)pyrene concentrations (B(a)P; mg kg^{-1}) in *Tenebrio molitor* larvae exposed to contaminated substrates with 0.03, 0.3 and 3 mg kg^{-1} substrate (dry weight), using high-performance liquid chromatography with fluorescence detection (HPLC-FD). Data are shown as average ± SD of three replicates of five organisms each for exposure to 0.03 mg kg^{-1} substrate and three replicates of one organism for exposures to 0.3 and 3 mg kg^{-1} substrate, collected on each sampling day. Baseline BaP concentrations in *T. molitor* were below the LOD (0.29 μg L^{-1}) in all controls at days 0, 21 and 42. Abbreviation n. d. stands for "not determined".

Time (days)	0.03 BaP (mg kg^{-1})	0.3 BaP (mg kg^{-1})	3 BaP (mg kg^{-1})
0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
1	0.013 (0.011)	0.000 (0.000)	1.331 (0.314)
3	0.030 (0.006)	0.000 (0.000)	1.033 (1.790)
7	0.041 (0.005)	0.000 (0.000)	1.799 (1.360)
14	0.042 (0.006)	1.196 (0.075)	4.454 (0.499)
21	0.049 (0.011)	0.748 (0.673)	4.101 (1.202)
22	n.d.	0.823 (0.586)	4.428 (1.250)
24	0.026 (0.005)	0.495 (0.119)	2.913 (1.085)
28	0.012 (0.002)	0.140 (0.059)	1.741 (0.722)
35	0.000 (0.000)	0.075 (0.010)	0.675 (0.291)
42	0.000 (0.000)	0.088 (0.054)	0.728 (0.097)

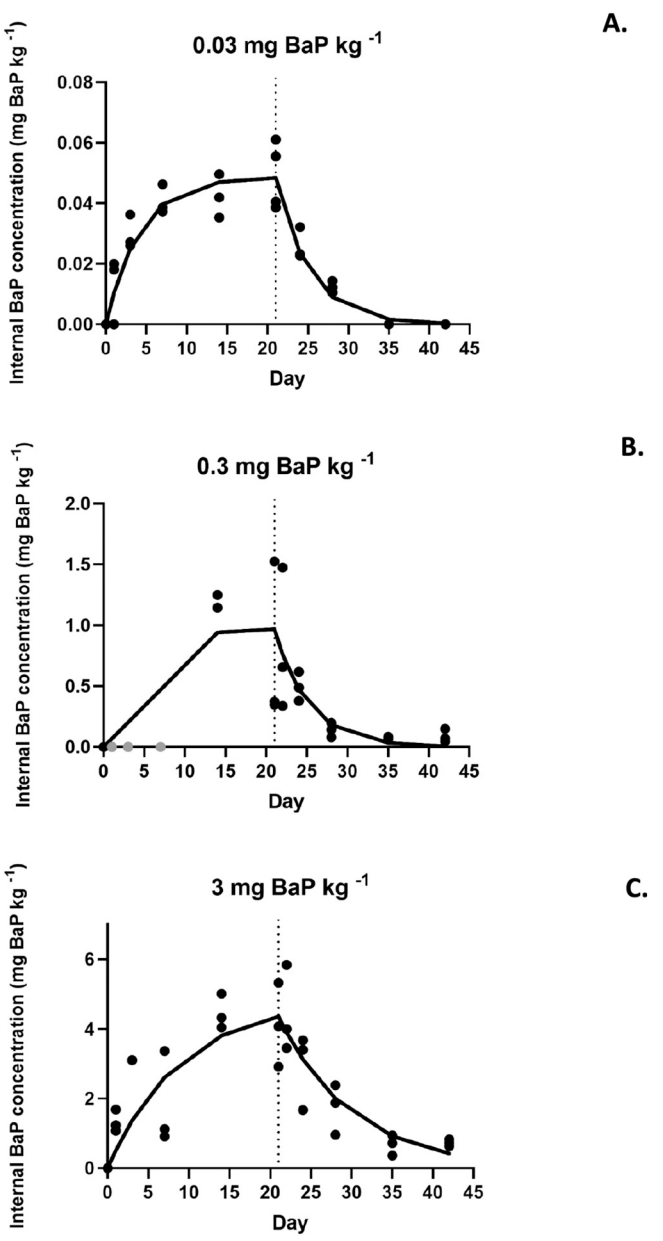

Fig. 1. Uptake and elimination kinetics of BaP in *Tenebrio molitor* (YMW) exposed to 0.03 (A), 0.3 (B) and 3 mg kg^{-1} (C) for 21 days plus 21 days in a clean substrate. Points show measured values ($n = 3$), and grey points represent data not considered in the toxicokinetic modelling. Solid lines show the fit of a one-compartment model with growth dilution (Eqs.2 and 3). The dashed vertical line defines the last day of exposure to the contaminated substrate, ending the uptake and starting the elimination phase of the experiment.

K_{growth}, which reflect an organism's metabolic activity, were observed as 0.024 (±0.003), 0.03 (±0.003), and 0.036 (±0.003) day^{-1} when fed with substrates containing concentrations of 0.03, 0.3, and 3 mg kg^{-1} (Table 2). The uptake constants (K_1) for these concentrations were determined to be 0.42 (±0.044), 0.69 (±0.30), and 0.16 (±0.025) $g_{substrate}\ g^{-1}_{organism}\ day^{-1}$, respectively (Table 2). No statistically significant differences were observed when comparing K_1 obtained in the exposure to 0.03 mg kg^{-1} s with 0.3 mg kg^{-1} ($X^2_{(1)} = 0.03$; $p > 0.05$), as well as between 0.03 and 3 mg kg^{-1} ($X^2_{(1)} = 0.01$; $p > 0.05$), and 0.3 with 3 mg kg^{-1} ($X^2_{(1)} = 2.18$; $p > 0.05$), which indicated that B(*a*)P levels examined in the present study did not affect YMW growth. The toxicokinetic modelling did not include the body concentrations measured below the detection limit from timepoints day 0 to day 7 in exposure to 0.3 mg kg-1. Nevertheless, the values are displayed (in grey) in Fig. 1B. The reason for this was the higher underestimation of uptake and elimination kinetics when accounting for these (zero) values, while a better fit was obtained when excluding these values.

Regarding elimination dynamics, the elimination constants (K_2) at concentrations of 0.03, 0.3, and 3 mg kg^{-1} were 0.217 (0.026), 0.210 (0.090), and 0.075 (0.020) day^{-1}, respectively (Table 2). The obtained elimination constant rates were not statistically different between the multiple comparisons of treatment levels (0.03 vs 0.3 mg kg^{-1} - $X^2_{(1)} = 0$; $p > 0.05$; 0.03 vs 3 mg kg^{-1} - $X^2_{(1)} = 0$; $p > 0.05$; 0.3 vs 3 mg kg^{-1} - $X^2_{(1)} = 0.82$; $p > 0.05$).

The corresponding half life (DT_{50}) was defined as 4.19, 4.30, and 10.22 days. The $BAF_{Kinetic}$ were modelled at 1.93, 3.27 and 2.08 $g_{substrate}\ g^{-1}_{organism}$, and 21d-BSAF were determined as 1.69, 2.19 and 1.20 $g_{substrate}\ g^{-1}_{organism}$ for 0.03, 0.3, and 3 mg kg^{-1} (Table 2), respectively.

4. Discussion

An increasing interest in edible insects as a sustainable protein source has come with concerns regarding product safety, particularly given the potential to accumulate chemicals to harmful levels (EFSA, 2015; Van der Fels-Klerx et al., 2020; van Huis and Oonincx, 2017). Among these contamination concerns, PAHs have recently received significant interest due to their potential carcinogenic adverse outcomes (Van der Fels-Klerx et al., 2020; Meyer et al., 2021). Using B(*a*)P as a representative carcinogenic PAH that can contaminate food substrates, this study intended to comprehensively evaluate how much and to which extent B(*a*)P accumulates in one of the most reared edible insects and whether elimination kinetics could be helpful to inform future insect rearing practices aimed at reducing product safety concerns related to the accumulation of PAHs. To our knowledge, only two studies have considered whether edible insects can appreciably accumulate PAHs (by studying ∑PAH4 - benzo(*a*)pyrene, benz(*a*)anthracene, benzo(*b*)fluoranthene and chrysene), suggesting minimal or no accumulation of PAHs after insect exposure to a contaminated substrate (Charlton et al., 2015; Van der Fels-Klerx et al., 2020).

The current study's findings demonstrate the importance of understanding each phase of the bioaccumulation process. By studying the uptake and elimination of B(*a*)P in insects through time, our observations highlight the use of toxicokinetics studies for more robustly evaluating contaminant accumulation and product safety. A previous

Table 2
Uptake and elimination kinetic parameters for Benzo(*a*)pyrene in *Tenebrio molitor* exposed to 0.03, 0.3 and 3 mg kg^{-1} substrate (dry weight). K_1 (uptake rate constant) and K_2 (elimination rate constant) were estimated, considering a one-compartment model assuming growth dilution. K_{growth} (growth rate constant) was derived using an exponential growth curve (Eq. 1) for each exposure to B(*a*)P concentrations. DT_{50} (half-life time for BaP elimination + depuration period) and $BAF_{kinetic}$ (kinetic Bioaccumulation Factor) were derived according to Eqs.4 and 5, respectively. Biota-Substrate Accumulation Factor (BSAF) were derived according to Eq. 6. Values in brackets represent the standard error for each parameter.

Treatment	K_{growth} (day^{-1})	K_1 ($g_{substrate}\ g^{-1}_{organism}\ day^{-1}$)	K_2 (day^{-1})	DT_{50} (days)	$BAF_{kinetic}$ ($g_{substrate}\ g^{-1}_{organism}$)	BSAF ($g_{substrate}\ g^{-1}_{organism}$)
0.03 mg BaP kg^{-1}	0.024 (0.003)	0.420 (0.044)	0.217 (0.026)	4.191	1.932	1.690
0.3 mg BaP kg^{-1}	0.030 (0.003)	0.687 (0.295)	0.210 (0.090)	4.303	3.273	2.198
3 mg BaP kg^{-1}	0.036 (0.003)	0.157 (0.025)	0.075 (0.020)	10.221	2.088	1.200

investigation assessing larvae of different species, including the house fly (*Musca domestica*), blue bottle fly (*Calliphora vomitoria*), blow fly (*Chrysomya* spp.), and black soldier fly (*Hermetia illucens*) – all reared on diverse waste substrates – demonstrated ∑PAH4 (benzo(*a*)pyrene, benz(*a*)anthracene, benzo(*b*)fluoranthene and chrysene) values ranging between 0.003 and 0.098 mg kg^{-1} in larvae (Charlton et al., 2015). However, the lack of information regarding the PAH content in the substrate used did not allow for estimating a BAF or understanding the accumulation dynamics of the ∑PAH4 in the larvae, limiting understanding of the bioaccumulation process, contextualising the risks, and considering such findings within the current regulatory framework.

A more integrative study recently evaluated the PAH content in *H. illucens* fed on supermarket waste (Van der Fels-Klerx et al., 2020), considering the source of the former foodstuff meal (animal or vegetable) and the packaging material (plastic or paperboard carton). The reported ∑PAH16 in *H. illucens* larvae were up to 0.002 mg kg^{-1}, and the feed material ranged between 0.002 and 0.007 mg kg^{-1}. BAF values of PAH16 ranged between 0.5 ± 0.1 and 1.2 ± 0.0 $g_{substrate}\ g^{-1}_{organism}$ depending on the type of substrate, which led the authors to conclude an absence of or minimal accumulation by BSF larvae (Van der Fels-Klerx et al., 2020). Given the current lack of established regulations concerning PAHs in feed, these findings warrant additional study, especially when matching with the European Commission's regulatory limits on food-based PAH4. These limits (PAH4) span from 0.001 mg kg^{-1} (for baby food) to 0.050 mg kg^{-1} (for food supplements containing botanicals and their preparations) as stipulated by Commission Regulation (EU), 2023/915. Charlton et al. (2015) presented PAH4 levels above the maximum allowed for food, which could raise concerns about the safety of those insects for food, even though specific details on which species and which substrates those values were found were not provided. When the PAH16 were evaluated in *H. illucens* by Van der Fels-Klerx et al. (2020), their higher BAF value (1.2 ± 0.0 $g_{substrate}\ g^{-1}_{organism}$) was very close to the lowest (1.93 $g_{substrate}\ g^{-1}_{organism}$) obtained in this study. These two studies emphasised the need for a more profound evaluation of PAH contamination in the substrates, including how B(*a*)P, as a representative of carcinogenic PAH, can be bioaccessible in substrates and then taken up and eliminated from insect bodies.

Our novel findings demonstrate that B(*a*)P can clearly accumulate in edible insects, namely in YMW larvae. The interaction between YMW larvae and B(*a*)P and subsequent accumulation involves direct substrate uptake and metabolic transformations within the larvae. However, these mechanisms have still not been elucidated for YMW. It is known that PAHs undergo enzymatic modifications in insect species, potentially involving cytochrome P450 enzymes (Lu et al., 2021). Such metabolic processes can result in an array of B(*a*)P-derived metabolites, some of which present toxicological effects, with the capability to form DNA adducts (Reed et al., 2018). The rate of these transformations likely can influence the overall bioaccumulation profile of B(*a*)P and its metabolites in insect larvae. While specific biotransformation can decrease toxic potential by converting B(*a*)P into less harmful metabolites, other metabolites can amplify risks (Patel et al., 2020; Vondráček and Machala, 2021).

In our investigation of B(*a*)P uptake and elimination in YMW larvae, we found varying levels of accumulation based on substrate exposure concentrations. Upon exposure to a substrate concentration of 0.03 mg $kg_{,}^{-1}$ larvae B(*a*)P body burden concentrations peaked at 0.049 mg kg^{-1}. Interestingly, during the elimination phase, where larvae were transferred to a clean substrate, the B(*a*)P levels diminished to or below the European Commission's feed safety threshold of 0.01 mg kg^{-1} by day 35 (corresponding to 14 days in clean substrate). In contrast, larvae exposed to higher substrate concentrations of 0.3 mg kg^{-1} and 3 mg kg^{-1} retained B(*a*)P at body burden levels that exceeded this threshold through day 42 (corresponding to 21 days of elimination in clean substrate, the last day of our experimental observation). The higher internal concentrations achieved at higher levels of substrate exposure may indicate that larvae clearance rates of B(*a*)P were influenced by this level of exposure, indicated by a lower K_2 value in the case of 3 mg kg^{-1}. However, this should be carefully interpreted since statistically significant differences were not found between kinetic rate constants (Table 1). Besides, a deeper mechanistic analysis of PAH metabolic pathways in YMW was outside the scope of the current study.

Furthermore, the toxicokinetic parameters derived from modelling without the initial body concentration values (days 0 to 7) from exposure to 0.3 mg kg^{-1} require cautious interpretation. We acknowledge that this is a limitation of our study, emphasising the need for future studies with enhanced statistical power. Levels of B(*a*)P in insects corresponded to the substrate concentrations; however, current feeding legislation does not presently limit values for concentrations of contaminants in substrates. While larvae exposed to lower B(*a*)P concentrations can potentially be deemed acceptable for consumption if the practice adopts a specific elimination period to protect product safety, those insects exposed to higher concentrations may require extended periods in a clean substrate or alternative detoxification measures to ensure their safety for consumption.

The fate of PAHs, such as B(*a*)P, after a prolonged depuration period, was also not in the scope of the present study. After larvae partially or totally excrete B(*a*)P, there could also be an accumulation of PAHs in the feed substrate left and (insect) frass mixture. Insect frass is one of the byproducts generated in insect rearing, rich in plant nutrients and bioactive compounds of great interest in agriculture (Poveda et al., 2019). There is a lack of knowledge on transferring contaminants from insect feed substrate to the frass, which results from insect biodigestion. Truzzi et al. (2019) studied metal concentrations in the larvae and the frass, comparing them against European Union regulations to assess safety after insect exposure to metal-contaminated substrates. These results indicated that the risk of exposure to metals from consumption of mealworm larvae and handling of frass is relatively low, with all metal concentrations being within safe limits (Truzzi et al., 2019). However, these types of studies are scarce, and to our knowledge, there are no studies on PAH-contaminated insect frass and possible management issues related to its reuse application in cropland. In addition, for the insect larvae being used as feed or food, there is no regulatory guidance on the presence of PAHs in fertiliser, which should be the focus of subsequent studies.

A pressing issue emerges from our research: the lack of regulatory guidance for insect feeding substrate. Our results illustrate a clear link between substrate B(*a*)P concentrations and the level of accumulation by the insect larvae. Despite this relationship, there is an absence of regulatory standards for B(*a*)P in substrates, which results in an unpredictable safety scenario, as the unregulated substrate directly challenges the safety profile of insects as potential food sources. Considering these findings, it is imperative to establish more comprehensive regulatory guidance, guided by robust scientific findings, that encompasses insects and their rearing substrates to ensure the product safety of insect-based foods.

The observed accumulation of B(*a*)P in YMW larvae underscores the need for effective predictive strategies. Introducing a depuration phase to insect rearing could be a promising solution for the insect industry to protect product safety, as we recently proposed (Cardoso et al., 2023) when YMW and *H. illucens* were exposed to Hg in the feeding substrate. The toxicokinetic parameters between both studies showed differences between YMW responses to B(*a*)P and Hg exposures. Our study revealed that YMW generally exhibited higher K_1 values for B(*a*)P (0.42–0.16 $g_{substrate}\ g^{-1}_{organism}\ day^{-1}$) than for Hg (0.096–0.17 $g_{substrate}\ g^{-1}_{organism}\ day^{-1}$), suggesting a more efficient uptake of B(*a*)P. Values of K_2 were generally higher for B(*a*)P, suggesting a faster depuration process for this compound. However, both chemicals' half-life times (DT_{50}) were comparable, except for larvae exposed to 3 mg kg^{-1}, revealing a DT_{50} value around two times higher than those obtained in Hg exposure. $BAF_{kinetic}$ and BSAF values were higher for B(*a*)P exposure, implying a greater propensity for B(*a*)P bioaccumulation and substrate-organism accumulation in these insect larvae, which is likely explained by lipid-

based partitioning of this non-ionisable organic contaminant. These findings underscore differential bioaccumulation patterns of YMW for model organic and inorganic contaminants, highlighting the need to target a broader range of representative contaminants of concern and apply the toxicokinetics approach to different species of edible insects in future studies.

In the present work, moving larvae to a contaminant-free substrate before processing allowed them to expel body residues, as observed by the significant reductions in B(*a*)P body burden levels. Specifically, larvae exposed to the lowest concentration of B(*a*)P (0.03 mg kg^{-1}) in the substrate successfully diminished B(*a*)P tissue concentrations by 14 days under clean conditions. This depuration process can be used in practice as a robust safety measure if it is standardised and incorporated into insect mass-rearing production, addressing the variability in B(*a*)P substrate levels and enhancing the overall safety profile of insect larvae for consumption. Further exploration is needed to optimise depuration conditions, ensuring the comprehensive safety and nutritional integrity of YMW and other edible insects. Hence, to holistically assess the implications of B(*a*)P presence in insect-derived food sources, it is also imperative to consider both the direct uptake and elimination and the metabolites of B(*a*)P that are produced and either retained or eliminated within these organisms. To fully understand the effects of PAHs on the metabolism of *T. molitor*. Future studies should involve biomarkers testing (oxidative stress, cellular energy reserves and allocation) and state-of-the-art omics tools. The findings of this paper should be complemented with such studies to develop thorough mechanistic knowledge, ensure a more accurate product safety assessment, and provide a scientifically sound basis for future legislative improvements.

5. Conclusions

Our research emphasises the influence of feed quality – considering the presence of contaminants - on the safety parameters of insect-based foods, as highlighted by YMW larvae's capability to accumulate and eliminate a model carcinogenic PAH. The data provided in the current study revealed dose-dependent B(*a*)P accumulation in these insects and that these larvae have mechanisms to eliminate B(*a*)P. Knowing how insects might take up and eliminate harmful substances is crucial as the interest in edible insects grows, given the direct implications for food safety. Our study highlights the need for more consistent feed and food regulatory standards. This becomes even more critical as the global industry looks to use various feed sources, some of which might not be well-studied. We suggest that while expanding feed options can grow the food/feed industry, safety must remain the priority to ensure product sustainability and consumer trust. Our novel observations reported here support the improvement of using insects as feed and food, ensuring with elimination kinetics that insects can be safe for consumption following B(*a*)P exposure.

Abbreviations

ANT	anthracene
B(*a*)P	Benzo(*a*)pyrene
$BAF_{kinetic}$	Kinetic Bioaccumulation Factor
BSAF	Biota-Substrate Accumulation Factor
DT_{50}	Half-life BaP in the organism
EFSA	European Food Safety Authority
EU	European Union
HPLC-FD	high-performance liquid chromatography with fluorescence detection
K_1	Uptake rate constant
K_2	Elimination rate constant
K_{growth}	Growth rate
PAHs	polycyclic aromatic hydrocarbons
PVDF	polyvinylidene fluoride
YMW	yellow mealworm *Tenebrio molitor*

CRediT authorship contribution statement

Diogo N. Cardoso: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Regina M.B.O. Duarte:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Ana Rita R. Silva:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Marija Prodana:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana Góis:** Writing – original draft, Visualization, Investigation, Formal analysis. **Patricia V. Silva:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Amid Mostafaie:** Methodology, Investigation, Formal analysis. **José Pinto:** Methodology, Investigation, Formal analysis. **Pedro F. Brandão:** Formal analysis. **Ivã G. Lopes:** Writing – review & editing, Investigation, Conceptualization. **Bryan W. Brooks:** Writing – review & editing, Investigation, Conceptualization. **Susana Loureiro:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data available at Zenodo. https://doi.org/10.5281/zenodo.11197345

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Appendix A. Suppplementary data

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