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*Article*

# A chemically safe way to produce insect biomass for possible application in feed and food production

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**Abstract:** Among other species, Black Soldier Fly (*Hermetia illucens*, HI, Diptera, Stratiomydae) has the great potential as food and feed ingredient in the EU. The production of insects as livestock feed or as food ingredient requires a strict monitoring of heavy metals content in the growth substrate in order to meet the security requirements. This study aims to investigate the presence of toxic metals like cadmium, lead, mercury, arsenic, and nickel in HI prepupae and in their growth substrates based on coffee roasting by-product and microlagae *Schizochytrium* sp. and *Isochrysis* sp. Analyses were carried out via graphite furnace atomic absorption spectrophotometry for Cd, Pb, Ni, and As, and via Direct Mercury Analyzer for Hg. All metal concentrations found in growth substrates were below the legal limit of undesirable substances in animal feed (2002/32/EC). Metals concentrations in HI prepupae were in the range (mg $kg^{-1}$ wet weight) 0.072–0.084 for Cd, 0.018–0.026 for Pb, 0.010–0.032 for Hg, 0.036–0.047 for As, 0.18–0.76 for Ni. Even if HI prepupae accumulated Cd, Pb and Hg, our results indicated that the risk of exposure to metals from HI prepupae consumption is relatively low and in compliance with European Union regulations.

**Keywords:** *Hermetia illucens* prepupae; Black soldier fly; coffee silverskin; microalgae; toxic metals; bioaccumulation; chemical hazard

## 1. Introduction

In the light of the predicted increase in world population by 2050 and the growing demand for high-quality protein sources for food and feed production, insects culture deserves a special attention [1,2]. In fact, insects show a high protein and fat content, can grow on organic by-products, their rearing is characterized by a low environmental impact [3-5] since they produce low greenhouse gases and ammonia emissions [1,6,7], and show low water and space requirements [4]. Among other species, the Black Soldier Fly (*Hermetia illucens*, HI Diptera, Stratiomydae), has been proposed by the European Food Safety Authority Scientific Committee [8] as one of the main species to have a great potential as food and feed ingredient in the European Union. HI larvae are characterized by a high protein (up to 42%) and fat (up to 30%) content [9-11], a short life cycle [12], reduced environmental footprint [13], and preference for organic by-products as growth substrate [3,14,15]. Therefore, HI is one of the most promising insect species to meet the future lack of conventional feed and food ingredients, the excessive production of agro-food waste [16-18] and the mitigation of climate change [19].

Additionally, it should be underlined that the organic by-products used as feed for the insects can be often contaminated by pollutants. Among several pollutants, heavy metals deserve special attention because of their high degree of toxicity and their wide distribution in the environment [20-22]. Most of them, such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg) rank among the priority metals that are of great public health significance. They are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure [20]. Heavy metals eventually present in growth substrates, can be transferred to the insect larvae and, therefore, enter the feed/food chain. It has been demonstrated that larvae of mealworm (*Tenebrio molitor*, Coleoptera

Tenebrionidae) reared on olive fruits processing by-products can accumulate Pb and Hg [23] while Biancarosa et al. (2018) [24] evidenced that HI grown on seaweed-enriched media accumulated significant amounts of toxic metals such as Cd, lead Pb and mercury (Hg). However, knowledge on chemical hazard associated to insects as potential ingredients for feed and food is scarce [8], and most studies used artificially-contaminated growth substrates to investigate a potential accumulation of toxic metals in insects [25-28], and specifically in HI [19,29-31]. Hence, in order to meet a safe production of insects, as well as the need to look for other substrates compatible with European rules and safe about chemical contaminants, a strict monitoring of heavy metal content is necessary [19]. The EU regulation 2017/893 [32], aside identifying the insect species that can be cultured, poses some limitations on the substrates that can be used as growth substrate (Annex X).

In a previous study performed by our research group, we developed a valid method to improve the PUFA content of HI prepupe by adding to the main growth substrate (coffee silverskin-a coffee roasting by-product) a certain amount of *Schizochytrium* sp. or *Isochrysis* sp. [33]. In this sense, the lack of PUFAs in the insect biomass was by-passed, satisfying at the meantime, the EU regulation 2017/893 [32] that imposes the use of "products of non-animal origin" for culturing insects intended for feed production. However, in the light of feed/food security and in order to meet a safe production the possible chemical hazard of each ingredient should be a priority.

Therefore, the present study aims to investigate the presence of toxic metals such as Cd, Pb, As, Ni and Hg, according to EU Regulations [34,35]. Specifically, analyses have been carried out on the single growth substrate-ingredients (coffee silverskin, *Schizochytrium* sp. and *Isochrysis* sp.), on growth substrates, on HI prepupae reared on these new substrates and on frass (excrement from larvae mixed with substrate residues and exuviae). Bioaccumulation or up-take of contaminants occurring during insect culture has been evaluated in order to better define the safety traits of the final insect biomass produced.

## 2. Materials and Methods

### *2.1. Insect growth substrate preparation*

Nine different growth substrates were tested during the experiment. The basal substrate consisted of by-products obtained from roasting coffee (a mixture of Arabica and Robusta varieties) process (coffee silverskin, CS), provided by Saccaria Caffè S.R.L. (Marina di Montemarciano, AN, Italy). CS (moisture 44%) was collected in plastic bags, frozen at −20 °C, and ground in an Ariete 1769 food processor (De'Longhi Appliances Srl, Italy) to a particle size of 2 ± 0.4mm before the growth substrate preparation. *Schizochytrium* sp. and *Isochrysis* sp. were freeze-dried provided by AlghItaly Società Agricola S.R.L. (Sommacampagna (VR), Italy) and stored at 4 °C. Growth substrates were formulated as follow: substrate E, 100% coffe silverskin (CS); substrates As, Bs, Cs and Ds: CS added with 5%, 10%, 20% and 25% of *Schyzochytrium* sp., respectively; substrates Ai, Bi, Ci and Di: CS added with 5%, 10%, 20% and 25% *Isochrysis* sp., respectively. All substrates were added with water to reach an optimal moisture close to 70% [9], as reported in Truzzi et al. (2020) [33]. Microalgae and growth substrates samples were stored at −20 °C for toxic metals determination.

### *2.2. Rearing of Hermetia illucens larvae*

HI rearing was carried out at the D3A experimental facility (Polytechnic University of Marche) starting from 6 days old larvae purchased from Smart Bugs s.s. (Ponzano Veneto, TV, Italy). Larvae were divided in the following groups (five replicates; each containing 150 larvae) [36]: HI E, prepupae reared on substrate E (100% CS); HI As, HI Bs, HI Cs, HI Ds: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% (W/W) of *Schyzochytrium* sp, respectively; HI Ai, HI Bi, HI Ci, HI Di: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% (W/W) of *Isochrysis* sp., respectively. Each group contained 750 larvae (6 days old, hand counted). Rearing conditions were explained in detail in Truzzi et al. (2020) [33]. About 10 g of single ingredients and growth substrates were stored at -20 °C for further analyses. Larvae were visually inspected every day and

when prepupae were identified by the change in tegument colour from white to black [37], they were manually collected using forceps and brushes and stored at −20 °C for further analyses. At the end of the experiment also frasses were collected and stored at −20 °C. Experiments were performed in compliance with the Italian laws and institutional guidelines. No specific authorization is requested to conduct experiments on invertebrates such as insects.

### 2.3. *Laboratory and apparatus*

A clean room laboratory ISO 14644–1 Class 6, with areas at ISO Class 5 under laminar flow, was used for all laboratory activities. Samples were handled with plastic materials (low density polyethylene 30 mL cylindrical containers, Kartell, Milan, Italy, Mod K912), washed with acid-cleaning procedures and rinsed with Milli-Q water obtained from a two-stage system Midi (Elix and Milli-Q) from Millipore (Bedford, MA, USA), in order to avoid any metal contamination [38]. The laboratory analytical balance was the AT261 Mettler Toledo (Greifensee, Switzerland, readability 0.01 mg, repeatability SD = 0.015 mg). Variable volume micropipettes and neutral tips were from Brand (Wertheim, Germany, Transferpette).

### 2.4. *Chemical analyses and quality control*

Samples of single ingredients (CS and microalgae), growth substrates, HI prepupae and frasses were minced, homogenized (homogenizer MZ 4110, DCG Eltronic), and divided in aliquot of 0.5 g each. To determine the moisture, samples were accurately weighed with the analytical balance AT261 (Mettler Toledo, Greifensee, Switzerland) and freeze-dried (Edwards EF4 modulyo, Crawley, Sussex, England) until constant weight (±0.2 mg). Analyses were carried out on three aliquots *per* sample. For the determination of Cd, Pb, Ni, and As, samples were digested in a high-quality (65% w/v) nitric acid $HNO_3$ and 30% v/v $H_2O_2$ (Merk) mixture in a Microwave Accelerated Reaction System, MARS-X, 1500 W (CEM, Mathews, NC, USA) and the operational parameters were as in Truzzi et al. (2019) [23].

Quantitative determinations of Cd, Pb, Ni, and As were made with an Agilent DUO 240FS atomic absorption spectrometer (Agilent, Santa Clara, CA 95051, USA) equipped with graphite furnace (GTA120 Graphite Tube Atomizer) and with Zeeman-effect background corrector. The analytical methodology and instrumental parameters were described earlier [23].

The total mercury content was quantified by thermal decomposition amalgamation atomic absorption spectrometry (TDA AAS) [39] using a Direct Mercury Analyzer (DMA-1, Milestone, Sorisole, BG, Italy). The homogenised samples were weighed directly into quartz containers. The optimized reading conditions for mercury determination in feed and in insects were as in Truzzi et al (2019) [23]. It was not possible to perform frass analysis with DMA-1 because of the presence of some substances that rapidly altered and destroyed the catalytic tube. Calibration curve technique was used for the quantification of mercury content [40]. To correct for possible mercury contamination during the analysis, the mercury concentration of a blank was subtracted from sample Hg concentrations.

All analyses were carried out in triplicate. Analytical quality control was achieved using the certified reference material, DORM-2 Dogfish muscle (National Research Council of Canada). Table 1 shows the validation parameters for the analytical procedures. Results were in good agreement with the certified values, and the standard deviation were low, proving good repeatability of the methods.

### 2.5. *Bioaccumulation factor*

The bioaccumulation factor (BAF) was calculated on a dry weight (dw) basis [31], as the ratio metal concentration in the organism/metal concentration in the feed provided. Thus, a BAF greater than 1 suggests bioaccumulation of the element from the substrate into the insect.

**Table 1.** Accuracy test using Certified reference material DORM-2 (dog fish muscle), NRC Canada. Data are expressed in mg $kg^{-1}$.

| Element | Analytical method | Analytical result (n=9) | Certified value | Δ (%) |
|---|---|---|---|---|
| Cd | GF-AAS | 0.042±0.005 | 0.043±0.008 | -2 |
| Pb | GF-AAS | 0.068±0.003 | 0.065±0.007 | +5 |
| As | GF-AAS | 17.4±0.6 | 18±1.1 | -3 |
| Ni | GF-AAS | 18±1.2 | 19.4±3.1 | -7 |
| Hg | DMA-1 | 4.21±0.06 | 4.58±0.16 | -8 |

### *2.6. Statistical analysis*

Data are expressed as mean ± standard deviation (SD) of the performed replications. Data were subjected to the one-way analysis of variance (ANOVA), followed by the Multiple Range Test [41], after testing the homogeneity of variance with Levene's test. Significant differences were evaluated at the 95% confidence level. When the ANOVA test gave a P-value equal to 0.0000, in the text it was indicated as P<0.0001. All statistical treatments were performed using STATGRAPHICS 18 Centurion [42].

## 3. Results and Discussion

### *3.1. Metal content in growth substrate ingredients*

Metals content in ingredients used to prepare growth substrates was reported in Table 2. In coffee silverskin the concentrations of Cd, Pb, As and Hg were found to be very low, i.e. less than 0.15 mg $kg^{-1}$ dw. Ni showed the highest content, with 3.5±0.2 mg $kg^{-1}$ dw. Cd, Pb, As and Ni concentration are consistent with literature data or even lower [43-46], whereas Hg showed a content about 3-fold higher than Zarrinbakhsh et al. (2016) [46], but of the same order of magnitude.

**Table 2.** Metals content (mg $kg^{-1}$ dw) in ingredients used to prepare growth substrates.

| Ingredients | Cd | Pb | Hg | As | Ni |
|---|---|---|---|---|---|
| Silverskin | $0.053\pm0.008^{b}$ | $0.032\pm0.002^{a}$ | $0.027\pm0.001^{c}$ | $0.147\pm0.006^{a}$ | $3.5\pm0.2^{b}$ |
| *Schizochytrium* sp. | $0.0025\pm0.0002^{a}$ | $0.065\pm0.003^{b}$ | $0.009\pm0.002^{b}$ | $0.184\pm0.002^{b}$ | $3.6\pm0.1^{b}$ |
| *Isochrysis* sp. | $0.0020\pm0.0005^{a}$ | $0.084\pm0.009^{c}$ | $0.0016\pm0.0004^{a}$ | $0.153\pm0.001^{a}$ | $1.18\pm0.03^{a}$ |
| *p*-value | <0.0001 | 0.0002 | <0.0001 | <0.0001 | <0.0001 |

The content of toxic metals in freeze-dried microalgae *Schizochytrium* sp. and *Isochrysis* sp. was very low, (Ni excluded). Being this micoalgae reared in a company that produces food products, it was expected to find acceptable values of these toxic metals. However, it should be pointed out that no data about heavy metals content in tested microalgae are available in literature. When comparing coffee silverskin to the two microalgae species it was evident that; i) both microalgae showed a significantly lower Cd and Hg concentrations, and a significantly higher Pb content with respect to CS; ii) *Schizochytrium* sp. showed a significantly higher As content with respect to CS and *Isochcysis* sp; ii) *Isochcysis* sp. showed a significantly lower Ni content and a significantly higher content of Pb with respect to CS and *Schizochytrium* sp.

Figures 1 and 2 show metals content in growth substrates, HI prepupae and frasses. Table 3 shows bioaccumulation factor (BAF) for prepupae of HI reared on tested growth substrates, calculated on a dry weight basis.

**Table 3.** Bioaccumulation factor (BAF) of prepupae of *Hermetia illucens* reared on tested growth substrates, calculated on a dry weight basis.

| HI prepupae | Cd | Pb | Hg | As | Ni |
|---|---|---|---|---|---|
| HI E | 4.2±0.9 | 2.3±0.3 | 3.9±0.2 | 0.88±0.05 | 0.57±0.05 |
| HI As | 5.0±0.7 | 2.2±0.3 | 2.9±0.2 | 0.82±0.05 | 0.54±0.03 |
| HI Bs | 4.8±0.5 | 1.9±0.2 | 2.5±0.1 | 0.86±0.03 | 0.31±0.03 |
| HI Cs | 5.7±0.8 | 1.7±0.2 | 1.6±0.1 | 0.83±0.06 | 0.31±0.03 |
| HI Ds | 5.3±0.7 | 1.7±0.2 | 1.4±0.1 | 0.89±0.03 | 0.36±0.04 |
| HI Ai | 5.7±0.9 | 2.0±0.3 | 4.5±0.2 | 0.95±0.08 | 0.52±0.06 |
| HI Bi | 5.6±0.7 | 1.7±0.2 | 4.0±0.2 | 0.91±0.06 | 0.26±0.04 |
| HI Ci | 6.1±0.6 | 1.6±0.2 | 3.2±0.1 | 0.93±0.06 | 0.23±0.02 |
| HI Di | 6.9±0.9 | 1.7±0.2 | 2.8±0.1 | 0.99±0.05 | 0.21±0.03 |

HI E: prepupae reared on substrate E (100% coffe silverskin, CS); HI As, HI Bs, HI Cs, HI Ds: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% of *Schyzochytrium* sp, respectively; HI Ai, HI Bi, HI Ci, HI Di: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% of *Isochrysis* sp., respectively. Data represent mean ± standard deviation (n=9).

### 3.2. *Cadmium*

*Growth substrate* - Considering that Cd content in microalgae was one order of magnitude lower than its concentration in CS (Table 2), the concentration of this metal in feed was mainly influenced by its content in CS, varying from 0.037 to 0.050 mg $kg^{-1}$ dw (Fig. 1). These concentrations were consistent or lower with respect to Cd content recorded in different HI growth substrates, such as plant/macroalgae-based medium [24,29], or cereal processing leftovers [47]. The inclusion of microalgae in the growth substrate led to a reduction of Cd content with respect to the control substrate E, but a statistically significant reduction ($p$ = 0.0046) was evidenced only with the inclusion of 20% and 25% of *Schizochytrium* sp. (substrates Cs and Ds, respectively) or *Isochrysis* sp. (substrates Ci and Di, respectively). Referring to the EC limit 2002/32/EC [48] on undesirable substances in animal feed, the legal limit for Cd in feed materials is 1 mg $kg^{-1}$ (vegetables) or 2 mg $kg^{-1}$ (animals) and for complete feed is 0.5 mg $kg^{-1}$ (maximum content relative to a feedingstuff with a moisture content of 12%). Cd content in tested growth substrates (calculated for a moisture content of 12%) ranged from 0.033 to 0.046 mg $kg^{-1}$ (Table S1), underlying their safety from the point of view of Cd content.

*HI prepupae* - Cd content in prepupae ranged from 0.19 to 0.24 mg $kg^{-1}$ dw (Fig. 1), and no statistically significant differences were evidenced among prepupae reared on different growth substrates ($p$ = 0.07). Data are consistent or lower with respect to literature data [24,29,47]. Referring to the EC limit 2002/32/EC [48] on undesirable substances in animal feed, HI prepupae showed lower Cd concentrations (Table S1).

No statistically significant correlation was found between Cd content in growth substrates and prepupae ($p$ = 0.8745). The BAF for Cd was > 1 for all groups (Table 3), with a mean±SD of 5.6±0.8, indicating that HI prepupae can bioaccumulate this metal. Similar BAF values were reported in literature for HI prepupae reared on different growth substrates, such as 5.8±1.0 [31] and ~2.5 [29] for chicken feed, 5.2 for cereal processing leftovers [47], ~4.2 for wheat bran [30]. The ability to accumulate Cd is typical of various Dipteran species [49,50], and it is explained by the active transport of this metal by means of heat shock proteins and by the capacity of Cd to pass through $Ca^{2+}$ channels [51]. Moreover, due to a very high Ca content, HI larvae can accumulate high quantities of Cd [31].

*Frass* – Cd content ranged from 0.071 to 0.096 mg $kg^{-1}$ dw, and a significant higher level of Cd with respect to control group E were evidenced in frass corresponding to growth substrates included with 10% of microalgae ($p$ = 0.005) (Fig. 1). Cd content in frasses was consistently lower than Cd content in HI prepupae, reinforcing the hypothesis that this metal was incorporated and retained in the insect body [31].

### 3.3. Lead

*Growth substrate* – Lead content in growth substrates was from 0.032 to 0.045 mg $kg^{-1}$ dw (Fig. 1). A statistically significant increase of Pb content with respect to control group E were evidenced in growth substrates included with 20% and 25% of microalgae (i.e. Cs and Ds for *Schizochytrium* sp.; Ci and Di for *Isochrysis* sp.) ($p$ < 0.0001). Pb content in growth substrate was similar or lower than Pb levels found in other substrates, such as chicken pellets [29], by-products of plant processing [47], seeweed-enriched media [24], or in vegetables [52]. Referring to the EC limit 2002/32/EC [48] on undesirable substances in animal feed, the legal limit for Pb in feed materials (animals) is 10 mg $kg^{-1}$ and for complete feed is 5 mg $kg^{-1}$. Pb content in tested growth substrates (calculated for a moisture content of 12%) was in the range 0.028 to 0.040 mg $kg^{-1}$ (Table S1), much lower than the legal limit, suggesting that the growth substrates tested in the present study were safe from the point of view of Pb content.

*HI prepupae* – Pb content in prepupae ranged from 0.063 to 0.075 mg $kg^{-1}$ dw (Fig. 1), and no significant differences were evidenced among prepupae reared on different substrates. These Pb levels are consistent with Pb content found in HI prepupae reared on different substrates, such as processed wheat seaweed-enriched growth substrate [24] or corn semolina [19], but are lower than Pb levels found in larvae reared on by-products of plant processing [47]. Referring to the EC limit (2002/32/EC) [48] on undesirable substances in animal feed, HI prepupae showed lower Pb concentrations (Table S1).

No statistically significant correlation was found Pb content in growth substrates and prepupae (r = 0.04352, $p$ = 0.9115). On the other hand, the BAF value ranged from 1.6 to 2.3, with a mean of 1.9±0.3 (Table 3). This BAF value indicated that HI prepupae bioaccumulate Pb. Similar BAFs were reported in literature for HI prepupae reared on different growth substrates, such as 1.2-1.4 for chicken feed [31], 2.7±0.6 for cereal processing leftovers [47], 2.3 in corn semolina [19], but Diener et al. (2015) [29] reported a BAF value <1. After all, these studies showed that metals bioaccumulation by HI larvae depends on the initial concentration in the growth substrate.

*Frass* – Pb content in frasses (from 0.045 to 0.062 mg $kg^{-1}$ dw, Fig. 1) was always lower than the corresponding content in HI prepupae, suggesting (as proposed also for Cd), that this metal was incorporated and retained in the body of HI prepupae [31]. A statistically significant increase of Pb content with respect to control group E were evidenced in growth substrates included with 20% of *Schizochytrium* sp. (Cs), and with 20% and 25% of *Isochrysis* sp. (Ci and Di) (p = 0.0004). A statistically significant correlation was found between Pb content in frass and growth substrates (r = 0.7485, $p$ = 0.0203), and the ratio of Pb concentration between frass and the corresponding growth substrate ranged from 0.6 to 0.94, as reported by Purschke et al. (2017) [19]. Therfore Pb content in frass was influenced by Pb content in the corresponding growth substrate.

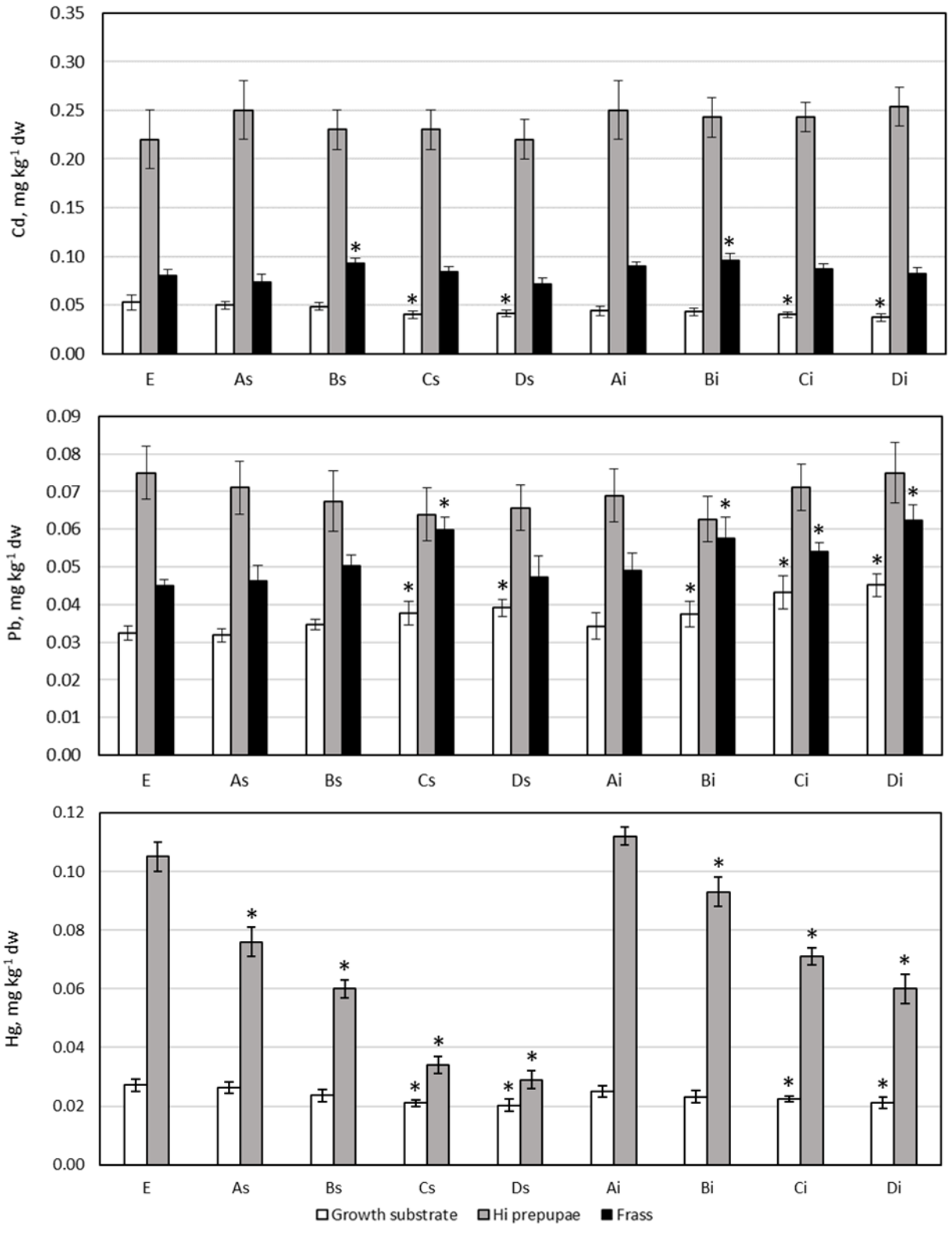


**Figure 1**. Cd, Pb, Hg concentration in feed (white bars), and corresponding HI prepupae (grey bars) and frass (black bars). E: control substrate 100% coffee silverskin; As, Bs, Cs, Ds: substrates enriched with 5%, 10%, 20% and 25% of *Schizochytrium* sp., respectively; Ai, Bi, Ci, Di: substrates enriched with 5%, 10%, 20% and 25% of *Isochrysis* sp., respectively. Each bar represents the mean ± standard deviation (n=9). *: indicates statistically significant differences ($p<0.05$) within the same matrix with respect to control group E.

### 3.4. Mercury

*Growth substrate* - Mercury content in tested growth substrates ranged from 0.027 to 0.020 mg $kg^{-1}$ dw (Fig. 1). Similar Hg content was observed from Biancarosa et al. (2018) [24] in processed wheat seaweed-enriched growth substrate. Showing microalgae a significantly lower Hg content with respect to CS ingredient, the increase of the percentage inclusion of microalgae to 20% and 25% in growth substrates led to a statistically significant reduction of Hg content ($p<0.0001$). Referring to the EC limit (2002/32/EC) [48] on undesirable substances in animal feed, the legal limit for Hg in feed material (animals) is 0.1 mg $kg^{-1}$ and for complete feed is 0.2 mg $kg^{-1}$. Hg content in tested growth substrates (calculated for a moisture content of 12%) was in the range 0.024 to 0.018 mg $kg^{-1}$ (Table S1), about 5 or 10-folds lower than legal limit. Consequently, the tested growth substrates were safe from the point of view of Hg content.

*HI prepupae* – Hg content in prepupae ranged from 0.029 to 0.112 mg $kg^{-1}$ dw (Fig. 1). In general, the microalgae inclusion in growth substrates led to a statistically significant decrease of Hg content in the corresponding HI prepupae with respect to control group E ($p < 0.0001$). Referring to the EC limit (2002/32/EC) [48] on undesirable substances in animal feed, HI prepupae showed lower Hg concentrations (Table S1).

A statistically significant linear correlation was found between Hg content in HI prepupae and corresponding growth substrates ($r = 0.8235$, $p = 0.0034$), indicating a strong relationship between the variables. The r-squared statistic indicates that the model as fitted explains 67.8% of the variability of Hg content in HI prepupae, that was clearly influenced by Hg content in growth substrate. The BAF value for Hg was > 1 for all groups, indicating that HI prepupae bioaccumulated this metal, as reported in literature for HI and for other insect species [23,24,53]. Bioaccumulation and biomagnification of mercury through food webs is well-known [54], and for this reason this metal deserves special monitoring along the feed/food chain. The BAF value was from 4.5 to 1.4, and it decreased with the increase of the microalgae percentage inclusion in the growth substrate. This result suggests that HI larvae could be able to self-select microalgae from feeding substrates [55] leading to a reduction of Hg concentration in prepupae.

### 3.5 Arsenic

*Growth substrate* – As content in tested growth substrates ranged from 0.139 to 0.154 mg $kg^{-1}$ dw (Fig. 2), and no statistically significant differences were evidence among them ($p=0.2345$). These levels are of the same order of magnitude than Arsenic content found in processed wheat used as diet for HI larvae [24]. Referring to the EC limit (2002/32/EC) [48] on undesirable substances in animal feed, the legal limit for arsenic in feed materials and complete feed is 2 mg $kg^{-1}$ (maximum content relative to a feeding stuff with a moisture content of 12%). arsenic content in tested growth substrates (calculated for a moisture content of 12%) was in the range 0.122 to 0.135 mg $kg^{-1}$, about 15-fold lower than legal limit (Table S1), then tested growth substrates were safe from the point of view of Arsenic content.

*HI prepupae* – Arsenic content in prepupae ranged from 0.123 to 0.138 mg $kg^{-1}$ dw (Fig. 2), similar to arsenic levels found in literature [24,28]. HI prepupae reared on substrates containing 25% of microalgae *Schizochytrium* sp. or *Isochrysis* sp. (substrates Ds and Di, respectively) showed higher levels of arsenic than other groups, and this result was well related to the higher content of arsenic in microalgae with respect to CS. In any case, no significant differences were evidenced among groups ($p = 0.071$).

No statistically significant correlation was found between arsenic content in growth substrates and prepupae ($r = -0.3460$, $p = 0.3617$). Referring to the EC limit (2002/32/EC) [48] on undesirable substances in animal feed, HI prepupae showed lower arsenic concentrations (Table S1). Arsenic content in HI larvae reflected metal content of growth substrates, as BAF value was next to 1, ranging between 0.82 to 0.99 (Table 3). Moreover, Arsenic did not accumulate in the body of HI prepupae, as already demonstrated by van der Fels-Klerx (2016) [31].

*Frass* – Arsenic content in frasses (from 0.133 to 0.145 mg $kg^{-1}$ dw, Fig. 2) was generally higher than the corresponding levels in prepupae, confirming that this element was not retained by HI, as already underlined by other studies [31].

### 3.6. Nickel

*Growth substrate* – The addition of different percentages of *Schizochytrium* sp. to CS did not modify Ni content in growth substrates because of the similar concentration of this element in these two ingredients. Conversely, the statistically significant lower Ni content of *Isochrysis* sp. with respect to CS caused a decrease in Ni content in growth substrates in relation to the increase of *Isochrysis* sp. inclusion. In particular, a significant lower content of Ni in growth substrates with the inclusion of 20% (Ci) and 25% (Di) *Isochrysis* sp. was evidenced with respect to control growth substrate E and substrates with the inclusion of *Schizochytrium* sp. (As-Ds) ($p$ = 0.009) (Fig. 2). No legal limits are presently reported for Ni in feed and food.

*HI prepupae* – Ni content in HI prepupae ranged from 0.60 to 2.0 mg $kg^{-1}$ dw, and no significant correlation was found between Ni content in growth substrates and prepupae (r = 0.4498, $p$ = 0.2245). Based on Boyd (2009) [56] they can be classified as low-Ni insect, having a Ni content < 500 mg $kg^{-1}$ dw. However, significant differences were evidenced among HI prepupae reared on different substrates ($p$ < 0.0001) (Fig. 2). HI prepupae reared on control substrate E and on substrates including 5 % of microalgae *Schizochytrium* sp. (As) or *Isochrysis* sp. (Ai) showed the highest Ni concentration, which significantly decreased with the increasing percentage of microalgae inclusion. HI prepupae reared on substrates containing 10%, 20% and 25% of *Isochrysis* sp. showed the lowest concentrations of this metal. If the decrease of Ni content in HI prepupae reared on *Isochrysis* sp.-enriched substrates can be explained by the Ni decrease in the corresponding growth substrates, the same did not happen for prepupae reared on *Schizochytrium* sp.-enriched substrates. In this case, whereas Ni content did not vary significantly in tested substrates, HI prepupae reared on substrates with 10%, 20% and 25% inclusion of microalgae showed a significant lower Ni content with respect to substrates E and As. A different bioavailability of this metal between ingredients of the growth substrates can be supposed. Results about BAF support this hypothesis: the BAF for Ni was < 1 for all groups (Table 3), demonstrating that Ni did not bioaccumulate, but the BAF of HI prepupae reared on control substrate E and on substrates including 5 % of microalgae *Schizochytrium* sp. (As) or *Isochrysis* sp. (Ai) showed a BAF with a mean±SD of 0.55±0.02, about 2-fold higher than BAF value of HI prepupae reared on substrates containing 10%, 20% and 25% of microalgae (from 0.21 to 0.36, mean 0.24±0.06). This last BAF value was consistent with that of HI larvae reared on corn-based substrates [19]. Evidently, Ni uptake of HI depends on the growth substrate, and this result should be further investigated.

*Frass* - The Ni content in frasses varied in the range 3.0-4.6 mg $kg^{-1}$ dw; frasses deriving from growth substrates enriched with 20% and 25% of *Isochrysis* sp. showed a significant lower Ni content with respect to control substrates and growth substrates *Schizochytrium* sp.-enriched ($p$ < 0.0001). Ni content in frasses was always higher than Ni content in corresponding prepupae, as evidenced in literature also for HI larvae reared on corn-based substrates [19]. Therefore, in agreement with the BAF lower than 1, we can affirm that this element is not retained by this insect species, suggesting that Ni present in the growth substrate penetrates in the body of prepupae and was then excreted without bioaccumulation. To further support this hypothesis, a significant correlation has been demonstrated between Ni content in frasses and growth substrates (r = 0.7834, $p$ = 0.0125).

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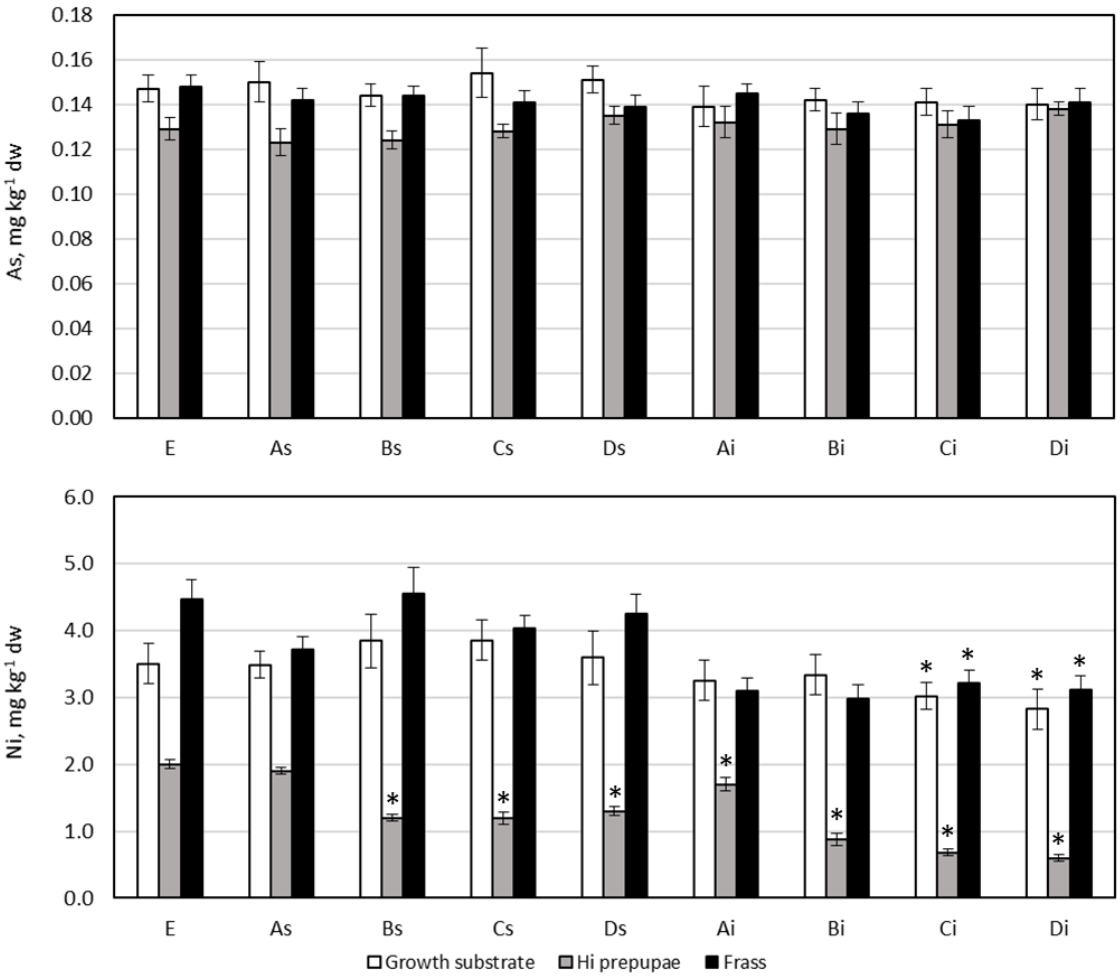


**Figure 2**. As and Ni concentration in feed (white bars), and corresponding HI prepupae (grey bars) and frass (black bars). E: control substrate 100% coffee silverskin; As, Bs, Cs, Ds: substrates enriched with 5%, 10%, 20% and 25% of *Schizochytrium* sp., respectively; Ai, Bi, Ci, Di: substrates enriched with 5%, 10%, 20% and 25% of *Isochrysis* sp., respectively. Each bar represents the mean ± standard deviation of at least three replications. Each bar represents the mean ± standard deviation (n=9). *: indicates statistically significant differences ($p<0.05$) within the same matrix with respect to control group E.

### *3.7. Toxic metals content in HI prepupae and comparison with legal limit for food*

In Table 4 we reported Cd, Pb, Hg, As, and Ni content in HI prepupae, referred to wet weight (ww), to make a comparison with their legal limit for food (EU commission regulation No 1881/2006 of 19 December, 2006 setting maximum levels for certain contaminants in foodstuffs, and amending Regulations No 420/2011 of 29 April, 2011, and No 1006/2015, of 25 June, 2015 as regards maximum levels of inorganic arsenic in foodstuffs) [35]. Cd showed a mean concentration in HI prepupae of 0.076±0.004, that was higher than legal limit of 0.05 referred to meat (excluding offal) of bovine animals, sheep, pig and poultry, but lower than that referred to horsemeat (0.20). Pb, Hg and As showed a mean content of 0.021±0.002, 0.022±0.008, 0.041±0.004, respectively. Their concentration was always lower than respective legal limit (4-5-fold lower for Pb and As, 15-50-fold lower for Hg). No legal limits were reported for Ni in food.

**Table 4.** Concentration (mg $kg^{-1}$ ww) of cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), and nickel (Ni) in HI prepupae, and legal limits for food (Directive 1881/2006/EU and amending regulations 420/2011/EU and 1006/2015/EU).

| HI prepupae | Cd | Pb | Hg | As | Ni |
|---|---|---|---|---|---|
| Legal limit | 0.050-0.20 [a] (meat) | 0.10 (meat) [a] | 0.50 [a] | 0.20 [b] | - |
| HI E | 0.076±0.010 | 0.026±0.002 | 0.030±0.001 | 0.044±0.002 | 0.76±0.02 |
| HI As | 0.072±0.009 | 0.021±0.002 | 0.024±0.002 | 0.036±0.002 | 0.54±0.01 |
| HI Bs | 0.072±0.006 | 0.021±0.002 | 0.020±0.001 | 0.039±0.001 | 0.36±0.02 |
| HI Cs | 0.078±0.007 | 0.022±0.002 | 0.012±0.001 | 0.043±0.001 | 0.39±0.03 |
| HI Ds | 0.076±0.007 | 0.023±0.002 | 0.010±0.001 | 0.047±0.001 | 0.46±0.02 |
| HI Ai | 0.074±0.005 | 0.020±0.002 | 0.032±0.003 | 0.037±0.002 | 0.49±0.03 |
| HI Bi | 0.076±0.008 | 0.018±0.002 | 0.029±0.005 | 0.040±0.002 | 0.27±0.03 |
| HI Ci | 0.084±0.006 | 0.020±0.002 | 0.024±0.003 | 0.045±0.002 | 0.24±0.02 |
| HI Di | 0.076±0.006 | 0.021±0.002 | 0.018±0.005 | 0.041±0.001 | 0.18±0.01 |

[a] 1881/2006/EU and 420/2011/EU

[b] 1006/2015(EU

HI E: prepupae reared on substrate E (100% coffe silverskin, CS); HI As, HI Bs, HI Cs, HI Ds: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% of *Schyzochytrium* sp, respectively; HI Ai, HI Bi, HI Ci, HI Di: prepupae reared on substrate CS enriched with 5%, 10%, 20% and 25% of *Isochrysis* sp., respectively. Data represent mean ± standard deviation (n=9).

## 4. Conclusions

HI prepupae accumulated Cd, Pb and Hg from growth substrates based on coffee roasted by-product and microalgae. This observation underlines that, a safe HI production as ingredient for feed or food needs a strict control of these undesirable contaminants both in the initial substrate as well as in the final product (HI prepupae). In fact, whereas the content of all considered metals in HI prepupae was always lower than the legal limit for feed, a Cd content next to the legal limit for food was detected. Overall, our results indicate that the risk of exposure to metals when using HI prepupae as ingredient for feed and food, is relatively low and in compliance with European Union regulations. However, it should be pointed out that Ni deserves a separate discussion since at present Ni is not considered in the laws that limit toxic metal content in feed and food and only a few studies on its accumulation in insects are available. Considering the level of this metal detected in HI prepupae, and considering its toxicity, authors think that it would be interesting to deepen this topic and suggest the scientific community to identify and fix specific limits for Ni content both in feed and food.

As a final remark, since the content of heavy metals in HI prepupae depends from the growth substrate, the authors suggest to layout, in addition to a list of insect species which may be used for the production of processed animal protein, a specific list of tested growth substrates to be used in a safe way for edible-insects production.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Table S1: Concentration (mg $kg^{-1}$) of cadmium (Cd), Lead (Pb), mercury (Hg), Arsenic (As) and nickel (Ni) converted to moisture content of 12%, in the growth substrates and in HI prepupae. Legal limits for feed material and complete feed (relative to a moisture of 12%), according to Directive 2002/32/EU (and amendments) on undesirable substances in animal feed.

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administration, C.T. and I.O.; resources, C.T., A.A. and P.R.; supervision, C.T. and S.I.; validation, F.G. and L.G.; writing—original draft, C.T.; writing—review and editing, C.T., I.O., P.R., A.A.

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