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Evaluating heavy metal risks to biomass valorization during black soldier fly larva bioconversion of municipal sewage sludge: speciation, bioaccumulation, and safety assessment

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Evaluating heavy metal risks to biomass valorization during black soldier fly larva bioconversion of municipal sewage sludge: speciation, bioaccumulation, and safety assessment

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Abstract

Municipal sewage sludge can potentially be valorized using black soldier fly larvae (BSFL), but heavy metal contamination may pose risks to the safety and circularity of the resulting biomass and residue. This study evaluated the distribution, operational fractionation, and larval accumulation of Cu, Zn, Mn, Ni, Cd, Pb, and Cr during BSFL treatment of an artificial-diet control (S0) and sludge-incorporated substrates prepared at wet-weight ratios of 1:1 (S50) and 1:3 (S75). BSFL processed all substrates, with S50 providing the best overall balance between larval growth and substrate reduction. Metal-loading analysis identified the endpoint residue as the principal sink for most metals. BCR (Community Bureau of Reference) sequential extraction showed that Zn, Mn, Cd, and Ni occurred largely in the more readily extractable F1-F2 fractions, whereas Cu, Pb, and Cr were distributed mainly in the less extractable F3-F4 fractions. Increased F4 proportions of Pb and Cr in sludge-incorporated residue indicated enhanced stabilization. Larval accumulation was assessed using the bioaccumulation factor (BAF), background-corrected bioaccumulation factor (BAF_{net}), and net metal retention efficiency (NRE). Cd showed the clearest accumulation pattern, with BAF values above unity under all treatments and consistently positive NRE. Regulatory comparison identified Cd as the principal constraint on using sludge-reared larvae as feed, while Cu, Zn, and Ni constrained residue use as fertilizer. Integrating metal loading, fractionation, accumulation indices, and regulatory thresholds provides an environmental risk assessment of BSFL-based sludge valorization.

Keywords: black soldier fly; municipal sewage sludge; heavy metal; BCR sequential extraction; bioaccumulation; ecotoxicological risk.

1. Introduction

Municipal sewage sludge is an inevitable byproduct of wastewater treatment, generated in substantial quantities as suspended solids and dissolved pollutants are concentrated and transferred into the solid phase during treatment processes. Sludge composition is highly variable, reflecting differences in influent characteristics, treatment configurations, and operational conditions, and typically contains organic matter, nutrients, and a range of inorganic constituents including heavy metals [1, 2]. The latter accumulate in sludge through adsorption, co-precipitation, and complexation with organic matter and metal oxides as industrial effluents, stormwater runoff, and other anthropogenic inputs enter the sewage network [3]. Given the non-biodegradable and bioaccumulative nature of heavy metals, their presence in sewage sludge constitutes a central challenge for downstream management and resource recovery.

Under the growing pressure of circular economy principles and tightening landfill restrictions, sewage sludge is increasingly viewed as a resource rather than a waste. Potential reuse pathways include agricultural land application, composting, energy recovery, and material production, each subject to regulatory constraints on heavy metal content [4]. Among these, agricultural application is particularly sensitive to heavy metal loading, as it introduces contaminants directly into the soil-plant system and the broader food chain [5]. Consequently, the feasibility of sludge reuse is critically dependent on the concentration and chemical speciation of heavy metals, which determine their environmental mobility, bioavailability, and potential ecological risk [3].

Black soldier fly larva (BSFL, *Hermetia illucens*) treatment has emerged as a promising biotechnological approach for the valorization of organic waste streams [6]. BSFL exhibit high feeding capacity, rapid growth, and broad substrate adaptability, converting organic substrates into larval biomass rich in protein and lipid while simultaneously reducing waste volume [7, 8]. The resulting products, larval biomass and the post-processing residue, are of considerable interest for downstream applications including animal feed, fertilizer, and bioenergy production [9, 10]. The application of BSFL to municipal sewage sludge treatment has attracted growing research interest owing to the large volumes of sludge generated globally, its high organic content, and the potential for simultaneous waste reduction and resource recovery [11].

Nevertheless, when heavy metal-laden substrates such as sewage sludge are used as BSFL feedstocks, the fate of heavy metals across system phases becomes a critical concern [12]. Heavy metals may be redistributed between larval biomass and the processing residue during treatment, with element-specific accumulation patterns governed by intestinal uptake mechanisms, intracellular detoxification pathways, and excretion behavior [13]. For instance, Cd^{2+} has been reported to exploit ionic mimicry to enter cells via Ca^{2+} ion channels [14], while Zn is actively regulated through intestinal transporter systems such as ZnuCBA [15]. Metallothionein proteins expressed in BSFL serve as intracellular metal-binding agents that

sequester Cu, Cd, and other metals, modulating their intracellular reactivity and toxicity [16, 17]. These element-specific mechanisms result in markedly different accumulation tendencies across metals, which in turn affect the safety and regulatory compliance of the resulting products for reuse.

Heavy metal speciation, rather than total concentration alone, is increasingly recognized as a more informative basis for environmental risk assessment [18]. The Community Bureau of Reference (BCR) sequential extraction procedure, which partitions metals into exchangeable and acid-soluble (F1), reducible (F2), oxidizable (F3), and residual (F4) fractions, provides a widely accepted framework for characterizing metal mobility and potential bioavailability across different environmental phases [19]. Application of BCR speciation to BSFL-treated materials can reveal whether processing promotes stabilization or mobilization of metals within the residue and larval biomass, complementing total concentration data for more comprehensive risk interpretation [14].

Despite growing research on BSFL-based organic waste treatment, studies specifically addressing the system-level mass balance and chemical speciation of heavy metals across all product phases during sludge treatment remain limited. Most existing studies report total metal concentrations in larvae or residues without establishing a comprehensive phase-level distribution framework or evaluating speciation changes relative to the input substrate [11, 12, 19]. Furthermore, bioaccumulation assessment in BSFL systems has commonly relied on the bioaccumulation factor (BAF) alone, which does not account for the initial larval background concentration and may overestimate actual net accumulation for certain elements [20].

This study addresses these gaps by establishing a system-level mass balance for seven heavy metals (Cu, Zn, Mn, Ni, Cd, Pb, and Cr) across all phases of a BSFL treatment system processing municipal sewage sludge at three sludge incorporation ratios. BCR sequential extraction was applied to characterize speciation changes from substrate to endpoint larvae and residue; the Risk Assessment Code (RAC) and Mobility Factor (MF) were additionally derived from BCR results to quantify potential metal mobility in the feed and endpoint residue. Bioaccumulation was assessed using Bioaccumulation Factor (BAF), a net BAF (BAF_{net}) corrected for initial larval background, and Net Metal Retention Efficiency (NRE) based on metal loading. The feasibility of downstream reuse of larval biomass and residue was further evaluated against applicable Taiwanese regulatory limits for feed and fertilizer applications. Together, these analyses provide an integrated framework for characterizing heavy metal behavior and risk in BSFL-based sludge treatment systems, offering quantitative risk-informed guidelines for safe biomass valorization in circular sludge management.

2. Materials and Methods

2.1 Feeding substrates and black soldier fly larvae (BSFL)

The BSFL colony used in this study was obtained from a continuously maintained

population at the Department of Entomology, National Chung Hsing University, Taiwan. Eggs were incubated at 27°C under a 12L:12D photoperiod at 70-80% relative humidity. Newly hatched larvae were reared on an artificial diet composed of wheat bran, commercial chick feed, and water (1:3:10, w/w/w) until the pre-pupal stage. Pre-pupae were transferred to 30×30×30 cm cages for pupation and eclosion; eclosed adults were maintained in the same cages and provided cotton pads saturated with sucrose solution for oviposition. The colony has been maintained under these conditions for more than ten consecutive generations, providing a genetically stable population with minimized intergenerational variation.

Municipal sewage sludge was collected from a water resource recovery facility in Taichung City, Taiwan, within one week prior to each experiment and stored at 4°C until use. Three feeding substrate conditions were formulated by mixing the artificial diet and sludge at different wet-weight ratios: pure artificial diet (S0, control), 1:1 (S50), and 1:3 (S75), to establish a gradient in sludge loading and substrate nutritional characteristics. All substrates were prepared and thoroughly homogenized on the day of the experiment; reserve portions were retained for moisture content determination and metal analysis. The moisture contents of the artificial diet and municipal sewage sludge were 71.8% and 80.3% (wet weight basis), respectively, yielding dry matter contents of 28.2% and 19.7%. These values were applied to convert substrate wet weights to dry weights for all subsequent mass balance and metal concentration calculations.

2.2 Feeding experiment design

Larvae were pre-reared on an artificial diet for nine days, and 10-day-old larvae were used as the experimental inoculum. At the start of each trial, 500 larvae from the same cohort were randomly selected, rinsed with RO water, blotted dry, and transferred to rectangular plastic containers (24×16×10 cm) pre-loaded with the assigned substrate. Mesh covers were fitted to maintain ventilation. Three replicates were established per substrate condition, yielding nine experimental units in total.

A single-dose feeding design was employed, with 350 g (wet weight) of substrate provided at the outset and no replenishment during the 10-day rearing period. This loading, approximately half of the estimated larval consumption based on prior rearing experience (~700 g over 10 days for 500 larvae), was intended to promote near-complete substrate consumption and minimize unconsumed material at endpoint. All endpoint residue was nevertheless collected and incorporated into mass balance calculations regardless of its origin. All rearing

trials were conducted at 27°C under a 12L:12D photoperiod at 70-80% relative humidity. Larvae and residues were collected simultaneously at day 10 and oven-dried at 105°C to constant weight for dry weight determination and subsequent metal analysis.

2.3 System performance indicators

To assess substrate condition changes and treatment performance across the three substrate conditions, the pH of each substrate was measured at both the start and endpoint of the experiment. Measurements were made by mixing 3 g of the sample with 15 mL of ultrapure water, agitating the suspension for 1 hour, and recording the pH of the homogenized mixture. Two dry-weight-based performance indicators were calculated. The waste reduction rate (WR) represents the actual decrease in substrate dry mass over the rearing period, calculated as Eq. (1)

$$WR(\%) = \frac{\mathrm{W}_{\mathrm{feed},0} - \mathrm{W}_{\mathrm{residue},10}}{\mathrm{W}_{\mathrm{feed},0}} \times 100\% \qquad \text{Eq. (1)}$$

where $W_{feed,0}$ is the initial substrate dry weight (g) and $Wr_{esidue,10}$ is the endpoint residue dry weight (g). A higher WR value indicates greater dry matter reduction of the substrate.

The substrate-reduction-to-biomass-gain ratio (SRB) describes the dry substrate mass reduced per unit increase in larval dry biomass. It was calculated as Eq. (2)

$$SRB(\%) = \frac{W_{feed,0} - W_{residue,10}}{W_{larvae,10} - W_{larvae,0}} \times 100\% \qquad \text{Eq. (2)}$$

where $W_{larvae,0}$ and $W_{larvae,10}$ are the initial and endpoint larval dry weights (g), respectively. A higher SRB indicates that less substrate reduction was associated with each unit of larval biomass gain. Because this inverse ratio is not a direct conversion efficiency, it was interpreted together with larval biomass gain and WR rather than as an independent measure for which higher values are necessarily preferable.

2.4 Heavy metal analysis

2.4.1 Total metal digestion

Total metal concentrations were determined for all sample types collected before and after the feeding trial, including BSFL, feeding substrates, and endpoint residues. The target elements were Cu, Zn, Mn, Ni, Cd, Pb, and Cr. Following oven-drying to constant weight, all samples were homogenized prior to digestion. A 0.5 g aliquot of each sample was placed into a digestion

vessel, treated with 30 mL of a 3:1 volumetric ratio of HNO_3 (69-70%; J.T.Baker) and HCl (37%; Fisher), and digested at 160°C. The resulting digest was filtered through filter paper to remove suspended solids, then diluted to volume with ultrapure water prior to instrumental analysis.

Metal concentrations in the digests were quantified by inductively coupled plasma mass spectrometry (ICP-MS; NexION 2200G, PerkinElmer Inc., USA). All analyses incorporated a certified reference material (CRM) for sewage sludge and procedural blanks to monitor accuracy and reproducibility throughout the digestion and measurement processes. All treatment conditions were conducted in triplicate (n = 3), and results are reported as mean values. To support system-level assessment of metal phase distribution and speciation trends, metal loading in each phase was calculated by multiplying the measured metal concentration by the corresponding sample dry weight. These loading values served as the basis for mass balance construction and subsequent bioaccumulation evaluation.

2.4.2 BCR sequential extraction and operational metal fractions

Chemical speciation of heavy metals was determined using the Community Bureau of Reference (BCR) sequential extraction procedure, in which metals were operationally divided into exchangeable and acid-soluble (F1), reducible (F2), oxidizable (F3), and residual (F4) fractions [21].

Briefly, 0.5 g of each sample was sequentially extracted: F1 with 20 mL of 0.11 M acetic acid (16 h, room temperature); F2 with 20 mL of 0.5 M hydroxylamine hydrochloride (pH 2, 16 h); F3 with 8.8 M hydrogen peroxide (85°C) followed by 25 mL of 1.0 M ammonium acetate (pH 2, 16 h). Phases were separated by centrifugation and residues washed with ultrapure water between steps. The final residue (F4) was acid-digested as in Section 2.4.1. Extracts were analyzed by ICP-MS, and results were expressed as concentration and percentage of each fraction.

2.5 Metal fate, larval accumulation, and mobility indicators

2.5.1 Bioaccumulation Factor (BAF)

The bioaccumulation factor describes the enrichment of an element in larvae relative to the feeding substrate based on a concentration ratio. BAF was defined as Eq. (3)

$$BAF_j = \frac{C_{larvae,10,j}}{C_{feed,0,j}} \quad \text{Eq. (3)}$$

where $C_{larvae,10,j}$ is the concentration of element j in endpoint larvae (mg/kg) and $C_{feed,0,j}$ is the initial concentration in the feeding substrate (mg/kg). A BAF value >1 indicates that larval concentrations exceed those of the substrate, suggesting a tendency toward bioaccumulation; conversely, a BAF value ≤ 1 indicates no apparent enrichment[20].

Conventional BAF does not account for the metal concentration already present in larvae at the start of the experiment. To describe the change in larval concentration after correction for this background, an operational background-corrected net concentration enrichment index (BAF_{net}) was calculated using Eq. (4). Unlike a mass-balance metric, BAF_{net} does not account for larval biomass change and was therefore interpreted only as a concentration-based index.

$$BAF_{net,j} = \frac{C_{larvae,10,j} - C_{larvae,0,j}}{C_{feed,0,j}} \quad \text{Eq. (4)}$$

where $C_{larvae,0,j}$ is the initial larval concentration of element j (mg/kg). A BAF_{net} value of zero indicates no change relative to the initial larval background; negative values indicate a decline in larval concentration. Values between 0 and 1 reflect a positive but sub-feed-level concentration change, while values above 1 indicate enrichment exceeding the initial feed concentration. BAF_{net} describes concentration change and does not, by itself, establish net gain or loss in total larval metal mass.

2.5.2 Net metal retention efficiency (NRE)

To complement the concentration-based BAF and BAF_{net} indices, net metal retention efficiency (NRE) was used to evaluate the change in larval metal burden on a mass basis. NRE was defined as the net change in larval metal mass divided by the metal mass initially introduced with the substrate (Eq. (5)). The index was reported as a dimensionless fraction; multiplication by 100 converts the value to a percentage. NRE is an operational mass-balance metric rather than conventional assimilation efficiency, because the amount of substrate actually ingested and the dietary origin of individual metal atoms were not determined.

$$NRE = \frac{\Delta M_b}{M_i} \quad \text{Eq. (5)}$$

where ΔM_b is the difference between endpoint and initial larval metal mass, calculated from the corresponding concentration and larval dry biomass, and M_i is the initial metal mass in the

substrate. A positive NRE indicates a net increase in larval metal burden, zero indicates no net change, and a negative value indicates a net decrease during the experimental period. NRE was interpreted only when the corresponding system mass-balance closure was acceptable.

2.5.3 Risk Assessment Code (RAC) and Mobility Factor (MF)

RAC and MF were calculated from the operational BCR fractions. RAC represents the proportion of F1, the most readily extractable fraction, relative to the sum of all fractions (Eq. (6)). Conventional RAC classes were used to describe potential mobility risk in the feed and endpoint residue. For larval tissue, F1 was reported as an operationally labile proportion rather than treated as a direct environmental or toxicological risk classification.

$$\text{RAC (\%)} = \frac{F_1}{F_1 + F_2 + F_3 + F_4} \times 100\% \qquad \text{Eq. (6)}$$

For feed and residue, RAC was classified as no risk (<1%), low risk (1-10%), medium risk (11-30%), high risk (31-50%), and very high risk (>50%). MF was additionally calculated as the combined proportion of F1 and F2 (Eq. (7)). Because RAC is nested within MF, the two indices were not treated as independent lines of evidence: RAC provides the conventional F1-based classification, whereas MF broadens the description to include metals that may become mobilized under reducing conditions.

$$\text{MF (\%)} = \frac{F_1 + F_2}{F_1 + F_2 + F_3 + F_4} \times 100\% \qquad \text{Eq. (7)}$$

2.6 Quality control

2.6.1 System mass balance

Mass balance closure was assessed to evaluate the degree of metal recovery within the defined system boundary. Closure was defined as the ratio of total endpoint metal loading to total initial metal loading. The formula is shown in Eq. (8):

$$Closure = \frac{\mathrm{M_{larvae,10,j}} + \mathrm{M_{residue,10,j}}}{\mathrm{M_{feed,0,j}} + \mathrm{M_{larvae,0,j}}} \times 100\ \% \qquad \text{Eq. (8)}$$

where $M_{larvae,0,j}$ and $M_{larvae,10,j}$ are the initial and endpoint metal loading (μg) in larvae,

respectively. $M_{feed,0,j}$ and $M_{residue,10,j}$ are the initial and endpoint metal loading (μg) in substrate and residue, respectively.

A closure value approaching 100% indicates that the metal distribution within the system can be adequately described by the defined phases [22]. Deviations from 100% may reflect uncertainties arising from sample homogeneity, instrument sensitivity, container wall adsorption during pre-treatment, or analytical measurement error. A closure range of 80-120% was adopted as the acceptable criterion in this study [23]; results within this range were considered to demonstrate reasonable consistency in the mass balance, and closure values were used as the basis for interpreting phase-level metal distribution.

2.6.2 Method Validation with Standard Reference Material

To confirm the accuracy and traceability of both the total metal digestion procedure and the BCR sequential extraction protocol, Standard Reference Material® SRM 2781 (Domestic Sludge) was processed using the same pre-treatment workflows applied to experimental samples. Quality verification was conducted at two levels: total digestion accuracy assessed against certified reference values, and BCR recovery evaluated by comparing the sum of all four fractions against the total digestion result obtained from the same sample. The BCR recovery rate for each element j was calculated as Eq. (9)

$$RR_j(\%) = \frac{\sum_{k=1}^{4} M_{Fk,j}}{M_{total,j}} \times 100\% \qquad \text{Eq. (9)}$$

where $M_{Fk,j}$ is the metal mass fraction of element j in fraction k (F1 through F4, mg/kg), and Mtotal,j is the total metal mass fraction of the same sample determined by 3:1 (v/v) HNO_3/HCl acid digestion (mg/kg). A recovery rate closer to 100% indicates better agreement between the summed fractions and the total digestion result [19]. A target range of 80-120% was applied as the acceptability criterion for both verification levels; results falling within this range were considered to confirm the reliability of the speciation distribution data.

The validation results are summarized in Table 1. For total metal digestion, recovery rates ranged from 86.0% (Cr) to 103% (Cd), confirming acceptable accuracy. For the BCR extraction, the summed F1-F4 concentrations also fell within the 80-120% acceptable range for all elements. Both workflows thus yielded consistent, traceable measurements supporting their application to phase-level distribution and risk assessment.

Table 1. Quality control of total metal digestion and BCR sequential extraction.

Unit: mg/kg	Total metal digestion	BCR sequential extraction

Element	SRM 2781 reference value	Measured value[a]	RR[b] (%)	F1[a]	F2[a]	F3[a]	F4[a]	RR[b] (%)
Cd	11 ± 2	11.4 ± 0.1	103	2.73	6.17	1.68	0.44	96.7
Cr	143 ± 14	123 ± 1	86.0	1.27	0	81.0	32.5	92.4
Cu	601 ± 16	590 ± 6	98.2	59.0	11.4	434	58.7	95.3
Pb	183 ± 15	162 ± 3	88.5	0	0.69	16.9	130	90.8
Mn	745 ± 33	736 ± 12	98.8	423	193	74.4	46.1	100
Ni	72.3 ± 6.3	72.6 ± 1.6	100	31.1	14.8	17.1	8.75	98.1
Zn	1120 ± 34	1027 ± 15	91.7	414	449	131	39.4	101

a.Average of triplicate results;b.Ratio of the measured value to the reference value.

3. Results and Discussion

3.1 BSFL growth performance and system efficiency

Treatment performance was evaluated using larval biomass gain, substrate dry-mass reduction, SRB, and substrate pH. These measures were interpreted jointly because they describe different aspects of system behavior; biomass gain reflects larval production, WR reflects substrate reduction, and SRB expresses the amount of dry substrate reduction associated with one unit of larval biomass gain. The corresponding treatment-performance results are summarized in Fig. 1, and the initial and endpoint system parameters are provided in the appendix (Table S1).

Larval biomass increased under all three conditions, confirming that BSFL retained viable feeding and bioconversion capacity across the full range of sludge proportions tested. The net larval dry weight gain was 7.00, 8.31, and 5.12 g for S0, S50, and S75, respectively, based on initial larval dry weights of 10.1, 9.79, and 9.98 g. The WR for S0, S50, and S75 were 66.8%, 65.2%, and 59.2%, respectively. The comparable WR values between S0 and S50 indicate that incorporating sludge at a 1:1 wet-weight ratio did not meaningfully compromise the waste reduction capacity of the system. A more pronounced decline in WR was observed at the higher sludge proportion of S75, reflecting reduced substrate utilization efficiency under elevated sludge loading.

The SRB values were 9.41, 6.57, and 8.81 for S0, S50, and S75, respectively. The lower value under S50 reflects its larger larval biomass gain relative to substrate dry mass reduction, consistent with the favorable growth response under S50. Substrate pH was initially acidic across all treatments (4.44, 4.85, and 4.95 for S0, S50, and S75; Table S1) and increased to 6.22, 5.44, and 5.28 after 10 days. This alkalizing trend is consistent with previous findings [24, 25] and is attributable to ammonium release through larval nitrogen metabolism and microbial

decomposition of organic nitrogen [26].

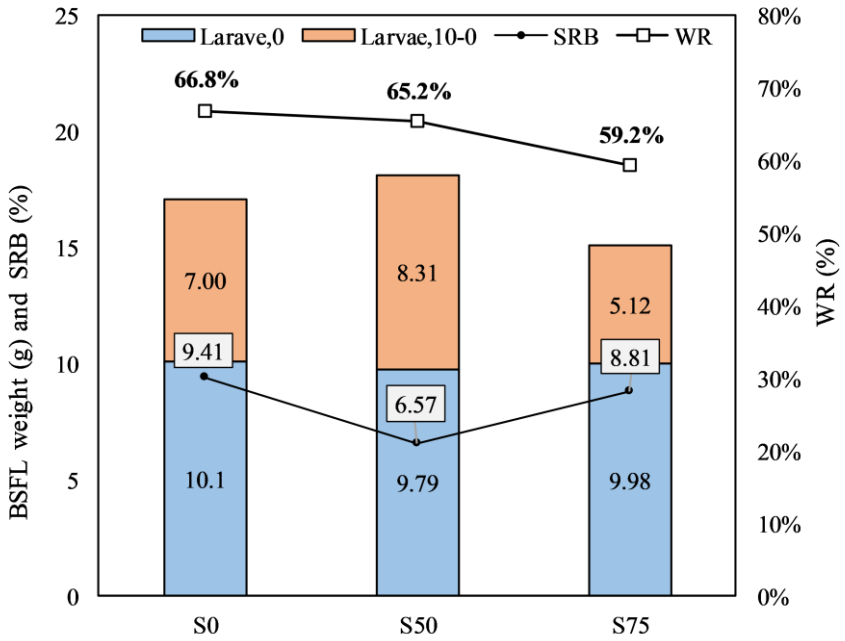


Fig. 1. Larval dry weight, substrate-reduction-to-biomass-gain ratio (SRB), and waste reduction rate (WR) under three substrate conditions.

3.2 Heavy metal mass balance and phase distribution

The system-level mass balance of heavy metals across the three substrate conditions is illustrated in Fig. 2, which presents the metal loading in the endpoint residue (Residue,10) and larval accumulation alongside the initial feed input (Feed,0) for each element. The measured heavy metal concentrations in the feed (Cfeed,0), initial larvae (Clarvae,0), endpoint larvae (Clarvae,10), and endpoint residue (Cresidue,10) across all three substrate conditions are summarized in the appendix Table S2. The mass balance details and closure values for all elements and conditions are provided in the appendix Table S3. Closure values for most element-condition combinations fell within the 80-120% acceptable range, supporting phase-level interpretation for those conditions. Three exceptions were observed under the S0 (pure artificial diet) condition: Ni (66.5%), Pb (144%), and Cr (52.7%). In S0, absolute metal inputs for these elements were at or near background levels (Table S2), making their mass balance disproportionately sensitive to measurement uncertainty, container-wall adsorption, and analytical variability at low analyte concentrations. These outlier closures do not affect the mass balance results for S50 and S75, where all three elements fell within the acceptable range. Accordingly, phase-level distribution data and NRE for Ni, Pb, and Cr under S0 are presented

for completeness.

As expected, the total metal input introduced through the feed increased substantially and progressively from S0 to S50 and S75 across all seven elements, directly reflecting the increasing sludge content in the substrate, given that municipal sewage sludge is the primary source of heavy metal loading in this system [1, 2]. Across all conditions, the majority of the total metal mass at the endpoint was retained in the residue phase rather than accumulated in larval biomass, indicating that the residue is the primary sink for heavy metals within this system. This partitioning pattern was consistently observed across all seven elements and all three substrate conditions and is in agreement with findings from previous BSFL-based organic waste treatment studies, which have similarly reported that the bulk of heavy metals introduced through the substrate remain in the residue fraction following larval processing [27].

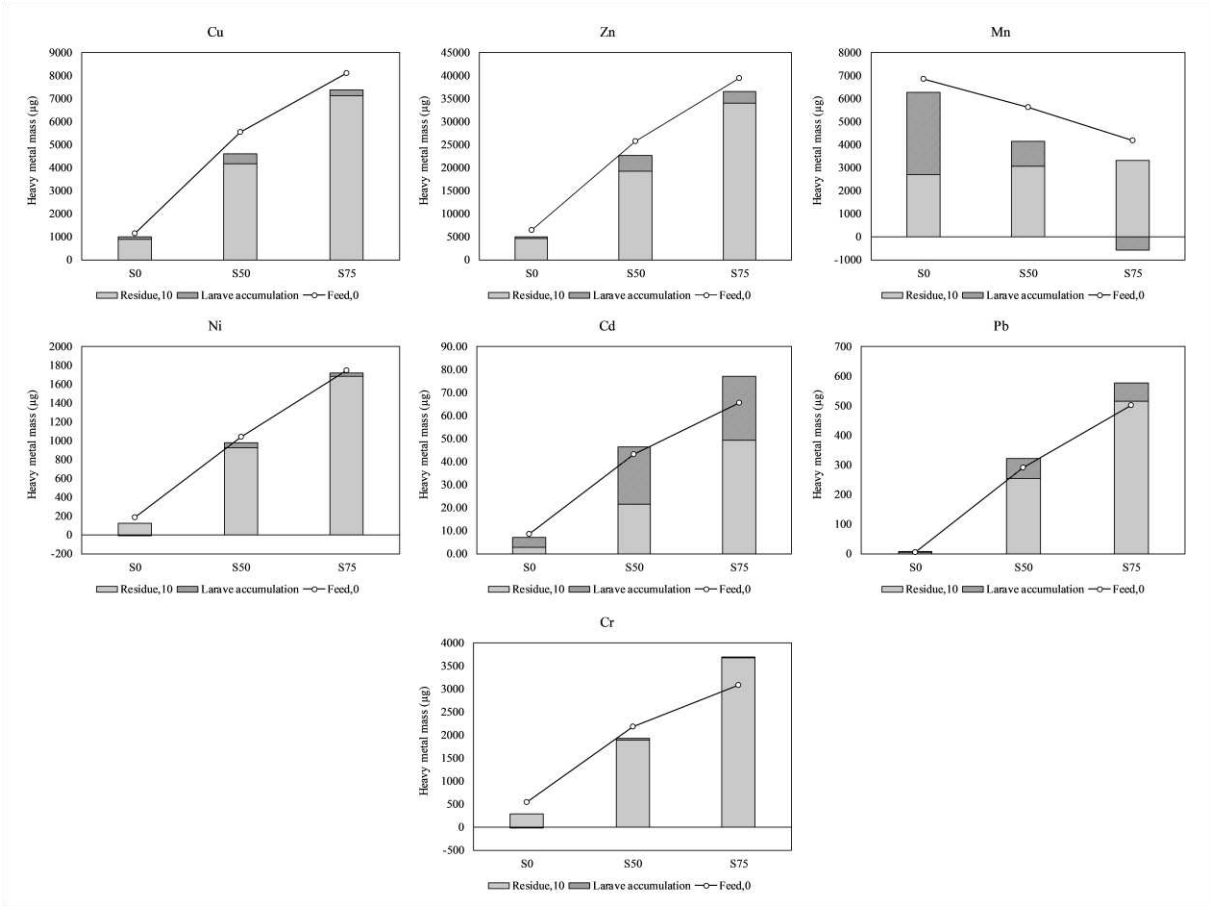


Fig. 2. Heavy metal mass loading in endpoint residue, larval accumulation, and initial feed input across three substrate conditions.

Among the seven elements, larval accumulation behavior varied considerably and is discussed below according to three distinct patterns: low accumulation, notable accumulation, and net excretion.

Low accumulation: Cr, Cu, and Ni. All three elements showed negligible larval accumulation, with endpoint metal mass dominated by the residue fraction. Cr was essentially undetectable in larvae across all conditions, consistent with the poor intestinal uptake of Cr in invertebrates due to its occurrence in stable, low-solubility forms [12]. Cu, although an essential trace element, showed only minimal larval accumulation, likely reflecting active excretion as a homeostatic response to elevated Cu exposure [11]. Ni similarly remained predominantly in the residue phase, reflecting limited transfer efficiency across the larval gut epithelium.

Notable accumulation: Cd, Zn, and Pb. Cd exhibited the most pronounced larval accumulation, contributing a meaningful proportion of total endpoint metal mass across all conditions and showing a clear dose-dependent increase with sludge proportion. Its selective uptake reflects the structural similarity between Cd^{2+} and Ca^{2+}, allowing entry via Ca^{2+} ion channels [28].

Zn also showed meaningful larval accumulation, particularly under S50 and S75, consistent with its active regulation through intestinal transporter systems, including the ZnuCBA pathway in BSFL [15]. Pb showed dose-dependent accumulation under S50 and S75, though absolute levels remained lower than Cd and Zn. Pb transfer was negligible under S0 but became apparent at higher sludge proportions, suggesting accumulation was driven primarily by elevated sludge loading rather than active uptake – consistent with the generally weak Pb accumulation capacity of BSFL [11, 12].

Net excretion: Mn. Mn showed a distinct partitioning pattern: larval accumulation was positive under S0 and S50 but turned negative under S75, meaning endpoint larval Mn loading fell below the initial larval background. This suggests that elevated sludge incorporation actively promoted Mn excretion into the residue. The behavior is consistent with insect sensitivity to excess Mn, which can induce neurotoxic effects and trigger active efflux [29], and with observations that Mn may be eliminated via molting through preferential accumulation in the pupal case [12].

To identify the origin of metal inputs, contributions from the artificial diet and sewage sludge were deconvoluted based on dry weight fractions, with the diet concentration estimated from S0 data (Table S4). Sludge accounted for 87-99% of total metal input for Cu, Zn, Ni, Cd, Pb, and Cr under both S50 and S75. Mn was the exception: sludge contributed only 39% and 59% under S50 and S75, respectively, because the artificial diet contained a higher Mn concentration (69.4 mg/kg DS) than the sludge (48-64 mg/kg DS). This source-composition difference explains both the anomalous decline in total Mn loading from S0 to S75 and the net

399 Mn excretion observed in the mass balance.
400
401 **3.3 Heavy metal speciation by BCR sequential extraction**
402 The BCR fractional distributions of Cu, Zn, Mn, Ni, Cd, Pb, and Cr in the initial feed,
403 endpoint residue, initial larvae, and endpoint larvae are presented in Fig. 3. Clear element-
404 specific differences were observed in the operational partitioning of the target metals. These
405 differences were evaluated using the relative contributions of F1-F4 and further summarized by
406 the RAC and MF to characterize their potential mobility in the feed and endpoint residue.

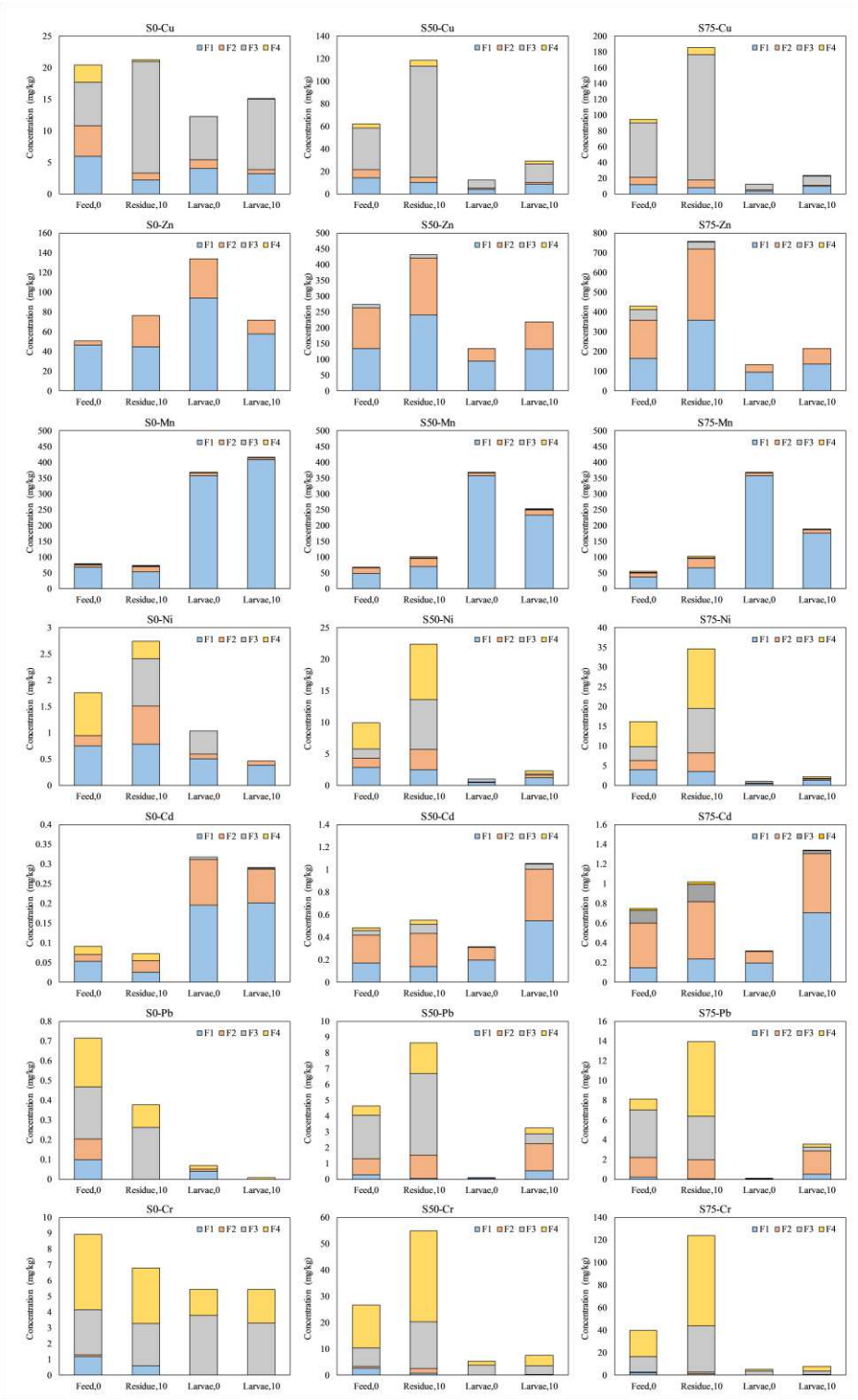


Fig. 3. BCR sequential extraction results of seven heavy metals across all phases and three substrate conditions.

Cu was distributed mainly in the less readily extractable F3 and F4 fractions, dominated by F3 across the feed, endpoint residue, and larval biomass. This pattern is consistent with the binding of Cu to intracellular proteins involved in metal homeostasis and detoxification [16, 17,

30].

Zn, Mn, and Cd contained comparatively large F1 and F2 proportions, indicating that substantial shares of these metals occurred in relatively labile operational fractions. Zn showed substantial F1-F2 proportions in the feed and endpoint residue, while F1 was particularly prominent in endpoint larvae. Mn was strongly dominated by F1 across the measured phases, indicating consistently high operational extractability. The pattern is compatible with the biological regulation of Zn as an essential element and with previously reported Zn-transport systems in BSFL [15, 31]. Cd exhibited a phase-dependent distribution: F1-F2 accounted for most of the Cd recovered from larval tissue, whereas comparatively larger F2-F3 proportions occurred in the endpoint residue. These results indicate that Zn, Mn, and Cd remained relatively extractable, although their biological accumulation patterns differed, as discussed in Section 3.4.

Ni occupied a distribution pattern distinct from both groups described above. F1-F2 accounted for 63.7%, 52.0%, and 59-62% in the feed, endpoint larvae, and endpoint residue, respectively, placing Ni predominantly in the more readily extractable fractions. However, unlike Zn, Mn, and Cd, which exhibited especially high F1 proportions, Ni was concentrated mainly in F2 across all phases, suggesting a preferential association with reducible phases rather than the exchangeable fraction.

Pb and Cr occurred predominantly in the less readily extractable F3 and F4 fractions. The F4 proportion of Pb was larger in the sludge-incorporated endpoint residue than in the corresponding feed, indicating a shift toward lower operational extractability in the residual material. Cr was likewise dominated by F3-F4 across the feed, residue, and larval samples, and the sludge-incorporated residue contained a larger F4 proportion than the feed. These patterns indicate that BSFL treatment was associated with increased stabilization of Pb and Cr in the endpoint residue.

The RAC and MF results are summarized in Table 2. In the feed, RAC classified Zn (38.2%), Mn (42.1%), and Cd (48.3%) as high risk; Ni (22.4%), Pb (14.2%), and Cr (11.8%) as medium risk; and Cu (2.1%) as low risk. The endpoint residue retained high F1 proportions for Zn, Mn, and Cd, indicating that their potential mobility remained relevant to land-application assessment. In contrast, the RAC values of Cu, Pb, and Cr in the endpoint residue were generally low, consistent with their greater allocation to the less readily extractable fractions.

MF followed the same general pattern after inclusion of F2. Zn, Mn, and Cd exhibited

comparatively high MF values in the endpoint residue, whereas Pb and Cr showed substantially lower values. Because RAC is based on F1 and MF includes both F1 and F2, the two indices were interpreted as complementary rather than independent indicators of potential mobility.

Because BCR fractions are operationally defined, the observed distributions represent differences in extractability and potential mobility rather than direct identification of specific molecular species. Conventional RAC risk classes were therefore applied to the feed and endpoint residue. For larval tissue, the BCR fractions were interpreted as operational tissue partitioning rather than direct environmental- or toxicological-risk classes. The practical safety of the larval biomass was evaluated separately using total metal concentrations and applicable feed limits in Section 3.5.

Table 2. Risk Assessment Code (RAC) and Mobility Factor (MF) for Feed, endpoint Larvae (Day 10), and endpoint Residue under each substrate condition.

Metal	Phase	Cond.	F1(%)	F2(%)	F3(%)	F4(%)	RAC(%)	RAC Class	MF(%)
Cu	Feed	All	2.1	27.3	41.8	28.8	2.1	Low	29.4
	Larvae	All	8.3	35.2	29.6	26.9	8.3	Low	43.5
	Residue	S0	1.8	24.1	43.7	30.4	1.8	Low	25.9
	Residue	S50	2.3	26.8	40.2	30.7	2.3	Low	29.1
	Residue	S75	2.0	25.9	41.1	31.0	2.0	Low	27.9
Zn	Feed	All	38.2	31.4	12.8	17.6	38.2	High	69.6
	Larvae	All	55.1	22.3	10.8	11.8	55.1	Very High	77.4
	Residue	S0	36.1	29.7	14.2	20.0	36.1	High	65.8
	Residue	S50	39.8	30.1	12.7	17.4	39.8	High	69.9
	Residue	S75	40.2	30.8	12.3	16.7	40.2	High	71.0
Mn	Feed	All	42.1	36.8	8.4	12.7	42.1	High	78.9
	Larvae	All	58.3	28.4	7.6	5.7	58.3	Very High	86.7
	Residue	S0	43.2	35.1	9.2	12.5	43.2	High	78.3
	Residue	S50	44.7	33.9	8.8	12.6	44.7	High	78.6
	Residue	S75	45.1	34.2	8.5	12.2	45.1	High	79.3
Ni	Feed	All	22.4	41.3	18.6	17.7	22.4	Medium	63.7
	Larvae	All	18.3	33.7	22.4	25.6	18.3	Medium	52.0
	Residue	S0	19.8	39.4	19.7	21.1	19.8	Medium	59.2
	Residue	S50	21.3	40.1	18.4	20.2	21.3	Medium	61.4
	Residue	S75	22.1	39.8	18.1	20.0	22.1	Medium	61.9
Cd	Feed	All	48.3	27.1	12.4	12.2	48.3	High	75.4
	Larvae	All	62.4	19.8	9.7	8.1	62.4	Very High	82.2
	Residue	S0	44.8	25.3	13.9	16.0	44.8	High	70.1
	Residue	S50	49.2	26.8	12.1	11.9	49.2	High	76.0
	Residue	S75	50.1	26.3	11.8	11.8	50.1	Very High	76.4
Pb	Feed	All	14.2	62.8	14.1	8.9	14.2	Medium	77.0
	Larvae	All	0.4	18.7	37.2	43.7	0.4	No risk	19.1
	Residue	S0	0.6	12.1	36.8	50.5	0.6	No risk	12.7
	Residue	S50	1.2	18.4	39.4	41.0	1.2	Low	19.6
	Residue	S75	1.0	16.9	38.7	43.4	1.0	Low	17.9
Cr	Feed	All	11.8	9.7	44.2	34.3	11.8	Medium	21.5

Larvae	All	2.1	7.4	35.6	54.9	2.1	Low	9.5
Residue	S0	1.2	5.8	38.1	54.9	1.2	Low	7.0
Residue	S50	3.4	8.1	41.2	47.3	3.4	Low	11.5
Residue	S75	2.8	7.6	40.3	49.3	2.8	Low	10.4

*RAC classification: <1% No risk; 1-10% Low; 11-30% Medium; 31-50% High; >50% Very High.

*Cond.: substrate condition (S0, S50, or S75). "All" indicates that BCR fractional distributions (%) showed no significant variation across the three substrate conditions for the respective phase; values represent means across all three conditions.

3.4 Larval metal accumulation assessment

Larval metal accumulation was evaluated using three complementary indices: the conventional bioaccumulation factor (BAF), the background-corrected net concentration enrichment index (BAF_{net}), and net metal retention efficiency (NRE). The results are summarized in Table 3. BAF describes endpoint larval concentration relative to the initial feed concentration, BAFnet describes the change in larval concentration after correction for the initial larval background, and NRE describes the net change in total larval metal burden relative to the initial substrate metal input. Accordingly, disagreement among these indices reflects differences between concentration enrichment, background-corrected concentration change, and mass-based net retention rather than contradictory results.

Cd showed the most consistent accumulation signal. Its BAF exceeded unity under S0, S50, and S75, with values of 5.93, 3.12, and 2.46, respectively. NRE was also consistently positive, with corresponding values of 0.50, 0.57, and 0.42. Although BAF_{net} was below unity under S0 (0.75), it increased to 2.25 and 1.94 under S50 and S75. These results demonstrate endpoint concentration enrichment under all conditions, positive net retention throughout the experiment, and particularly strong background-corrected enrichment under the sludge-incorporated conditions. The Cd accumulation results were consistent with its BCR distribution in Section 3.3. The predominance of F1-F2 in endpoint larvae indicates that a large proportion of the retained Cd occurred in relatively extractable operational pools. Uptake through transport pathways for essential divalent ions and subsequent intracellular binding are plausible mechanisms reported in the literature [13, 14, 17]. The agreement among BAF, BAF_{net}, NRE, and the larval BCR distribution identifies Cd as the principal larval accumulation concern.

Mn illustrated most clearly why BAF alone may not reflect net retention. Mn BAF remained above unity under all conditions (5.60, 3.38, and 3.07 for S0, S50, and S75), yet BAFnet declined from 1.16 to -1.20 and -2.54, and NRE fell from 0.52 to 0.19 and -0.14. The

divergence reflects the initially high larval Mn background and the disproportionate contribution of the artificial diet to total Mn loading (Table S4; sludge contributing only 39-59%). Despite dominance of Mn in the labile F1 fraction, this did not translate into positive net retention under elevated sludge loading. The results demonstrate that chemical extractability, concentration enrichment, and mass-based retention are distinct dimensions of metal behavior.

Pb exhibited condition-dependent concentration enrichment. Its BAF exceeded unity under S0 and S50, with values of 2.00 and 1.10, but declined to 0.64 under S75. BAFnet exceeded unity only under S50, whereas positive NRE values were observed under all conditions. These results indicate a modest net increase in total larval Pb burden even when endpoint concentration enrichment was weak. However, the predominance of Pb in the less readily extractable F3-F4 fractions and its comparatively low larval concentrations indicate limited overall transfer into larval biomass.

Cu and Zn showed decreasing BAF values with increasing sludge incorporation. Cu BAF declined from 1.38 under S0 to 0.50 and 0.25 under S50 and S75, while Zn BAF declined from 1.87 to 0.95 and 0.55. Their BAF_{net} values remained below unity, and their NRE values were positive but modest. These results indicate limited net retention relative to the initial substrate input. For Cu, the predominance of F3 in larval biomass suggests that the relatively small amount retained by larvae was mainly associated with less readily extractable operational pools. Zn showed a larger F1 proportion in larvae, but this comparatively labile distribution was not accompanied by substantial mass-based retention.

Ni and Cr showed the weakest accumulation patterns. Their BAF values remained below unity under all conditions; BAFnet values were negative or close to zero, and NRE values were small. Negative NRE values for Ni under S0 and Cr under S0 represent net decreases in larval metal burden within the operational mass-balance framework. The predominance of Ni and Cr in F3-F4 further supports their limited transfer and retention in larval biomass.

Taken together, the three accumulation indices and the BCR results describe complementary aspects of metal behavior. BAF remains useful for comparison with previous studies, but its interpretation may be influenced by initial larval concentrations and differences in substrate metal concentration. BAF_{net} identifies background-corrected changes in larval concentration, whereas NRE determines whether total larval metal burden increased after biomass change was considered. Within this integrated framework, Cd was the most consistent accumulation concern, while Mn demonstrated most clearly why concentration enrichment should not be interpreted as equivalent to net retention.

Table 3. BAF, BAF_{net}, and NRE of heavy metals in endpoint larvae under three substrate conditions.

		BAF	BAF_{net}	NRE
Cu	S0	1.38	0.11	0.11
	S50	0.50	0.27	0.08
	S75	0.25	0.12	0.03
Zn	S0	1.87	-0.86	0.05
	S50	0.95	0.37	0.14
	S75	0.55	0.20	0.06
Mn	S0	5.60	1.16	0.52
	S50	3.38	-1.20	0.19
	S75	3.07	-2.54	-0.14
Ni	S0	0.41	-0.50	-0.02
	S50	0.30	0.16	0.05
	S75	0.15	0.08	0.02
Cd	S0	5.93	0.75	0.50
	S50	3.12	2.25	0.57
	S75	2.46	1.94	0.42
Pb	S0	2.00	-0.63	0.13
	S50	1.10	1.07	0.23
	S75	0.64	0.63	0.13
Cr	S0	0.26	-0.64	-0.05
	S50	0.20	0.01	0.02
	S75	0.11	-0.01	0.01
Number		BAF > 1	BAF_{net} > 1	NRE < 0
		10	4	3

3.5 Feasibility assessment of product reuse

To evaluate the resource-recovery potential of the products generated by BSFL treatment, the measured metal concentrations in endpoint larvae and residue were compared with applicable regulatory limits for animal feed and fertilizer use. The comparison results are presented in Table 4. Regulatory compliance was evaluated using total metal concentrations because BCR, RAC, and MF describe operational fractionation and potential mobility but do not replace product-specific concentration limits.

3.5.1 Larval biomass as a potential feed material

BSFL biomass is widely regarded as a promising protein and lipid source for feed applications [32-34]. Cd and Pb were the two elements subject to regulatory limits under the

applicable feed standard [35, 36].

According to the values reported in Table 4, larval Pb concentrations were 0.091, 3.83, and 4.24 mg/kg under S0, S50, and S75, respectively. These concentrations remained below the 5 mg/kg limit applied in the referenced pet-food standard. The increase from S0 to S75 nevertheless indicates progressively greater Pb transfer to larval biomass as the proportion of sewage sludge increased.

Larval Cd concentrations were 0.513, 1.61, and 2.13 mg/kg under S0, S50, and S75, respectively. Under S0, the larval Cd concentration remained below the 2 mg/kg limit and the 1 mg/kg livestock-feed limit, though it marginally exceeded the most stringent threshold of 0.5 mg/kg applicable to certain animal categories. Under S50, the larval Cd concentration exceeded the livestock-feed limits of 0.5 and 1 mg/kg but remained below the 2 mg/kg limit. Under S75, the larval Cd concentration of 2.13 mg/kg exceeded all applicable thresholds, including the 2 mg/kg limit and the livestock-feed limits. These results are consistent with the accumulation assessment in Section 3.4 and identify Cd as the principal constraint on the use of larvae reared on sludge-incorporated substrates as feed material.

3.5.2 Endpoint residue as a potential fertilizer material

The endpoint residue may potentially be used as an organic amendment if it complies with applicable fertilizer or compost standards. The regulatory limits considered in this study were 100 mg/kg for Cu, 500 mg/kg for Zn, 25 mg/kg for Ni, 2 mg/kg for Cd, 150 mg/kg for Pb, and 150 mg/kg for Cr [37].

Under S0, all measured metals in the endpoint residue remained below their respective regulatory limits. Under S50, Cu, Zn, and Ni concentrations increased to 143, 660, and 31.7 mg/kg, respectively, exceeding their corresponding limits. The exceedances became more pronounced under S75, where Cu, Zn, and Ni reached 229, 1090, and 53.9 mg/kg, respectively. Cd, Pb, and Cr remained below their respective fertilizer limits under all three conditions.

These concentration-based results should be considered together with the BCR findings in Section 3.3. Zn retained high RAC and MF values in the endpoint residue, indicating that its regulatory exceedance was accompanied by a substantial potentially mobile fraction. Cu exceeded its concentration limit despite its predominantly F3 distribution and relatively low RAC. Ni similarly exceeded its regulatory limit under S50 and S75, although much of the recovered Ni occurred in F2-F4. Consequently, low operational mobility does not compensate for an exceedance of the applicable total-concentration limit.

Overall, the endpoint residue obtained under S50 and S75 would not comply with the fertilizer criteria applied in this assessment, with Cu, Zn, and Ni representing the principal limiting elements.

Table 4. Heavy metal concentrations in endpoint larvae and residues compared against applicable regulatory limits for feed and fertilizer reuse.

Element	Feed regulation		Endpoint larvae (mg/kg)			Fertilizer regulation	Endpoint residue (mg/kg)		
	[35]	[36]	S0	S50	S75	[37]	S0	S50	S75
Cu	-	-	-	-	-	100	26.9	143	229
Zn	-	-	-	-	-	500	141	660	1090
Ni	-	-	-	-	-	25	3.81	31.7	53.9
Cd	2	1 or 0.5	0.513	1.61	2.13	2	0.083	0.745	1.58
Pb	5	-	0.091	3.83	4.24	150	0.169	8.67	16.5
Cr	-	-	-	-	-	150	8.74	64.8	118

4. Conclusion

BSFL successfully processed sludge-incorporated substrates, with S50 achieving the best balance between larval growth and substrate reduction. Most metals remained in the endpoint residue, while their fractionation and larval accumulation were strongly element-dependent. Pb and Cr showed increased association with the stable F4 fraction in sludge-incorporated residue, whereas the mobile fractions of Zn, Mn, and Cd remained relevant. Among the examined metals, Cd presented the clearest larval accumulation signal and the principal limitation for feed use, while Cu, Zn, and Ni constrained the potential use of the residue as fertilizer. These findings demonstrate that treatment performance alone is insufficient to determine valorization potential. Integrating metal distribution, BCR fractionation, RAC, MF, BAF, BAF_{net}, NRE, and product-specific regulatory limits provides a coherent basis for selecting sludge incorporation levels and determining appropriate end uses. Under the tested conditions, S50 represented the most balanced operating condition, although metal-specific controls remain necessary before either product can be safely reused.

CRediT authorship contribution statement

Minhsuan Chen: Investigation, Conceptualization, Methodology, Formal analysis, Validation, Data Curation, Writing, Visualization, Supervision, Project administration.

Chiyuan Chu: Investigation, Methodology, Formal analysis, Data curation, Software.

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