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Research article

Evaluation of a battery of biotests to improve waste ecotoxicity assessment (HP 14), using incineration bottom ash as a case study

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ABSTRACT

The assessment of waste ecotoxicity (hazardous property HP14 in the European Union) is fundamental for proper waste classification and safe application/disposal. Biotests are relevant for evaluating waste complex matrices, but their efficiency is crucial to encourage their adoption at the industrial level. This work aims at evaluating possibilities of improving the efficiency of a biotest battery previously suggested in the literature, regarding test selection, duration, and/or laboratory resources optimization. Fresh incineration bottom ash (IBA) was the case study. The test battery analysed included standard aquatic (bacteria, microalgae, macrophytes, daphnids, rotifers, fairy shrimp) and terrestrial (bacteria, plants, earthworms, collembolans) organisms. The assessment followed an Extended Limit Test design (three dilutions of eluate or solid IBA) and the Lowest Ineffective Dilution (LID-approach) for ecotoxicity classification. The results emphasize the importance of testing different species. It was also evidenced that tests with daphnids and earthworms may be shortened to 24 h; the miniaturization of tests is suitable as e.g. differential sensitivity of microalgae and macrophytes was captured with low variability; alternative testing kits can be used when methodological difficulties are found. Microalgae were more sensitive than macrophytes. Similar results were found for the Thamnotoxkit and daphnids test for eluates with natural pH, so the former may be used as an alternative. *B. rapa* was the most sensitive organism, suggesting that it may be tested as the only terrestrial plant species and that minimum test duration is appropriate. *F. candida* does not appear to add information to the battery. The differences in sensitivity of *A. fischeri* and *E. fetida* compared to the remaining species were not significant enough to exclude them from the battery. Thus, this work suggests a biotest battery to test IBA comprising aquatic tests - *Aliivibrio fischeri*, *Raphidocelis subcapitata* (miniaturised test), and *Daphnia magna* (24 h when clear deleterious effects are observed) or *Thamnocephalus platyurus* (toxkit) – and terrestrial tests – *Arthrobacter globiformis*, *Brassica rapa* (14 d), and *Eisenia fetida* (24 h). Testing waste with natural pH is also recommended. The Extended Limit Test design considering the LID-approach seems useful in waste testing, particularly for the industry, involving low effort, test material requirements, and few laboratory resources. The LID-approach allowed for differentiating ecotoxic from non-ecotoxic effects and captured different sensitivities between species. Ecotoxicological assessment of other waste may benefit from these recommendations, but caution should be taken given the properties of each waste type.

1. Introduction

The appropriate classification of waste is crucial for its further management. The assessment of the hazardous property HP 14 ("ecotoxic") linked to potential environmental impacts should play a key role in decision-making within this context. However, this is currently one of the main challenges within the waste legislation framework. The revision of EU waste legislation in 2014 aimed to promote a uniform classification of waste (Stiernström et al., 2016). As a result, Commission Regulation 1357/2014 provided the methods for hazard property assessments based on waste substances. Despite its relevance, no method was presented for HP 14, given the recognition that further studies were

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needed to guarantee completeness and representativeness of the information on the potential impacts of an alignment with CLP regulation (EC Regulation No. 1272/2008 on the classification, labelling and packaging of substances and mixtures). Afterwards, Council Regulation (EU) 2017/997 amended Annex III to Directive 2008/98/EC (Waste Framework Directive) regarding HP 14, providing calculation procedures and threshold values for ecotoxicity estimation using waste chemical composition, whereas the results of experimental tests should prevail following Commission Decision 2014/955/EU amending the EU List of Waste (LoW). Still, there are no specific and clear orientations on experimental approaches for waste ecotoxicity assessment at the EU level. In fact, the Commission assigned to the Member States the responsibility of deciding on a case-by-case basis on the use of biotests for ecotoxicological characterization until further EU guidance (Commission notice 2018/C 124/01).

Thus, EU recommendations for HP 14 assessment are still based on CLP legislation for chemicals in general, but there are some challenges in using this methodology. Indeed, CLP is linked to pure chemicals and their stable mixtures, while waste may contain a variety of substances with variable compositions (Hennebert et al., 2014). Furthermore, this approach requires information on substances, but waste composition is often estimated considering elemental content (Hennebert et al., 2014) and the elemental speciation is usually unknown. Hence, the conservative "worst-case scenario" that admits the worst environmentally impacting chemical form for each element is generally considered (Hennebert et al., 2014). This approach often results in the overestimation of ecotoxicity. Another challenge is the unrealistic assumption that the total content contributes to the potential ecotoxicity since not all element content is bioavailable (Manskinen et al., 2011; Nurmesniemi et al., 2005). Finally, it can be difficult to identify specific organic substances in waste, and thus ecotoxicity can be underestimated (BIO by Deloitte, 2015). Different authors acknowledged leaching as the main route for ecotoxicity, in line with CLP regulation, and proposed methodologies for HP 14 assessment built on leaching data and geochemical speciation modelling to determine which specific minerals/substances may be present in waste and their leaching at selected pH (Wahlström et al., 2016; Hjelmar et al., 2013; Klymko et al., 2017; WRc, 2012). However, direct ecotoxicity regarding soil ecosystems should also be evaluated. Ecotoxic effects of substances insoluble in water might be underestimated when considering only the aquatic compartment, and thus the terrestrial compartment is relevant, particularly to evaluate hydrophobic waste (Pandard and Römbke, 2013; BIO by Deloitte, 2015). Also, for some waste, ecotoxicity has been found only for the terrestrial compartment, e.g. some incineration ashes (Römbke et al., 2009).

Biotests measure an effect on a biological parameter. Contrarily to the chemical approach, biotests reflect the effects of all substances, their interactions (additive, antagonistic, and synergistic), and the bioavailable fraction in particular (Pandard and Römbke, 2013; BIO by Deloitte, 2015; Wilke et al., 2008). Thus, the evaluation of ecotoxicity through biotests is a very relevant approach to classify waste since it covers this complex matrix in total. The use of a battery of biotests, including tests both in aquatic and terrestrial compartments with organisms of different trophic/functional levels, and considering different types of effect (e.g., physiological and behavioural) and different scenarios of exposure (e.g., short and long-term, acute and chronic) has been advocated for a more complete HP14 assessment (Moser and Römbke, 2009). Consequently, a battery of biotests was recommended by Pandard and Römbke (2013). Later, Römbke (2018) discussed the classification of 23 representative samples of solid waste by ecotoxicological testing, using the same battery but substituting the luminescent bacteria test (ISO 11348-3, 2007) with a genotoxicity test (Umu test - ISO 13829). Nonetheless, the study recommended following the battery of Pandard and Römbke (2013) given that genotoxicity is a very specific endpoint, making it rarely relevant for waste.

Indeed, discussing the efficiency of biotest batteries is an up-to-date issue that can encourage the adoption of this approach. Namely, the duration of the biotests and the amount of laboratory resources needed are key factors to consider when aiming to make waste ecotoxicological assessment widely adopted. The duration of testing is also an important variable as the industry intends to avoid storing waste for long periods. Furthermore, the amount of laboratory resources needed will define the convenience of applying these tests in different laboratories, and thus the acceptance of that approach.

Incineration bottom ash (IBA) is the main solid waste from municipal solid waste (MSW) incineration. These ashes are the incombustible material remaining from the MSW combustion and present a very heterogeneous composition. IBA contains minerals, ceramics, metallic compounds, and glass (Chimenos et al., 1999). Potentially toxic metals (PTM), e.g., Pb, Cd, Zn, Cu, Cr, and Ni, may be present in relevant concentrations from an environmental viewpoint (Bayuseno and Schmahl, 2010; Forteza et al., 2004; Huber et al., 2019; Hyks et al., 2009; Lindberg et al., 2015; Neuwahl et al., 2019; Rambaldi et al., 2010; Tang and Steenari, 2016; Zhang et al., 2015a). Therefore, there are some environmental concerns about their use (Crillesen et al., 2006; del Valle-zermeño et al., 2014). However, IBA comprises a wide particle size distribution and the PTM composition differs according to particle size (Crillesen et al., 2006; Alam et al., 2019; Dou et al., 2017; Huber et al., 2020), with higher concentrations of these elements being found in the finest fractions (Chimenos et al., 1999; Tang and Steenari, 2016; Alam et al., 2019; Chen and Chiou, 2007; Xia et al., 2017; Luo et al., 2019). Fresh IBA is generally alkaline, with pH 10–13 (Dou et al., 2017; Luo et al., 2019), but after natural weathering for 6–20 weeks, pH decreases to 8–10 due to carbonation and oxidation reactions (Chimenos et al., 2000; Maldonado-Alameda et al., 2020a). This is relevant because the leaching potential of some PTM can be reduced, hence reducing IBA hazardousness (Baciocchi et al., 2010; Cornelis et al., 2006, 2012; Shimaoka et al., 2007; Silva et al., 2019; Wei et al., 2011a, 2011b, 2014). IBA is considered a mirror entry in the LoW (codes 19 01 11* and 19 01 12), resulting in different management procedures among countries. Indeed, the utilisation rate in Europe is in the range 0–100% (or 100-0% landfill) since legal regulations substantially diverge among countries (Blasenbauer et al., 2020; Neuwahl et al., 2019; Dou et al., 2017). IBA contains metals (ferrous and non-ferrous) and minerals that have been recycled in some countries, and different applications have been used (Dou et al., 2017; CEWEP, 2017; Juric et al., 2006; Ginés et al., 2009; Silva et al., 2017; Joseph et al., 2018). Thus, IBA is an ideal candidate for a comprehensive environmental hazard assessment towards contributing to better management harmonization, which necessarily includes ecotoxicity assessment.

Under this rationale, the present study discusses the possibilities of improving the battery of biotests suggested so far in terms of efficiency using IBA as a case study. Focused modifications concern test selection (alternatives to the most frequently used tests), test duration, and/or laboratory resources optimization, these adding significant novelty to the work. In addition, pH correction of eluates was addressed when relevant to allow further discussion on the role of this physicochemical condition in the outcome of HP 14 evaluation. Thus, this work aims at contributing to the improvement of the ecotoxicological testing approaches that have been proposed for HP 14 evaluation, covering both the terrestrial (soil) and the aquatic (water) compartment. Indeed, the efficiency of biotests is key to motivating their adoption in the industry and their inclusion in legislation. Alterations in legislation in this regard would probably lead to a significant impact on waste management, aiming at appropriately tuning related measures for environmental protection. Improvements in ecotoxicological evaluation not only favour waste assessment in the EU, but also contribute with useful information that can be used for ecotoxicity analysis improvement worldwide.

2. Materials and methods

2.1. Materials

A sample of fresh IBA was supplied by a Western European incineration plant with a moving grate technology operating at a temperature above 1000 °C. The sample was collected after ferrous metal recovery through magnetic separation. The sample was dried at room temperature (about 20 °C), sieved to a particle size below 2 mm (around 29% of the initial sample), and stored in a tight container until further use. Eluates from IBA were prepared for the ecotoxicological tests focused on the aquatic compartment and for chemical characterization. The leaching procedure was performed according to the European standard EN 12457-2: agitation in an end-over-end tumbler with distilled water at a liquid-to-solid (L/S) ratio of 10 L/kg for 24 h and subsequent filtration using a 0.45 μm membrane. For terrestrial tests, IBA was dosed directly as solid waste, respecting a geometric range of load in each test substrate according to the guidelines of each biotest and EN 14735.

2.2. Waste characterization

Organic matter content was estimated as volatile solids. Accordingly, IBA was dried at 105 °C for 24 h to obtain the moisture content and then calcined at 550 °C for 2 h to determine volatile solids (EPA Method 1684). The elemental composition of solid IBA was determined by Total Reflection X-Ray Fluorescence (TXRF; Bruker S2 PICOFOX) in a milled sample and flame atomic absorption spectroscopy (FAAS; Analytikjena ContrAA 300 with Aspect CS 2.1.1.0 Tech:Flame software) preceded by acid digestion. Method 3051A was followed for microwave-assisted acid digestion (TRANSFORM 680; Aurora Instruments). Mineralogical characterization was performed in a powdered sample (milled to <75 μm) through X-Ray Diffraction (XRD), using Philips X'Pert equipment (40 kV and 35 mA) with Co ampoule ($\lambda = 1.78897$ Å), with Bragg-Brentano geometry, in a range of angles 2θ between 4° and 70° with a 0.025° step and an acquisition time of 1 s per step. The phase identification was performed using the X'Pert HighScore Plus 2.2 software and the ICDD database version 2000 (PDF-2). The leaching behaviour was analysed in eluates focusing on pH, electrical conductivity (EC), and chemical composition. Chemical elements in aqueous extracts were also quantified through TXRF and FAAS. Chlorides and sulfates were determined by ion chromatography (Thermo Scientific Dionex ICS-5000+). The pH and the EC of all eluates as well as the pH of the solid waste were measured using a Consort C1020 Multiparameter Benchtop Meter. The pH of the solid waste was measured in 0.01 M $CaCl_2$ solution, according to ISO 10390.

2.3. Test methods

All tests were carried out following an Extended Limit Test design (three dilutions of IBA eluate or solid IBA) (Römbke, 2018): control (0%), D8 (12.5%), D4 (25.0%), and D2 (50.0%) for the aquatic compartment; control (0%), D16 (6.25%), D8 (12.5%), and D4 (25.0%) for the soil compartment. Dx represents the dilution step. For example, D2 means that a dilution factor of 2 was used to prepare the dilution and establish the test treatment. Different dilutions were selected for aquatic and terrestrial compartments based on previous studies (Römbke et al., 2009; Römbke, 2018). The biotests and modifications focused herein are summarized in Table 1. All tests were first carried out in natural pH conditions. Only the tests with pH outside the optimal tolerance range of species revealing ecotoxic effects were repeated with pH adjustment to be within the range of culture media produced in the laboratory for those organisms.

2.3.1. Aquatic compartment

The luminescent bacteria test with *Aliivibrio fischeri* was performed according to ISO 11348-2 (2007) using Dr. Lange equipment and LUMIStox 300 kit. Solid NaCl was added to eluates for osmotic adjustment, with two replicates per treatment. Bacteria were thawed immediately preceding the test, reactivated, and exposed to eluates for 30 min. The light output of bacteria in eluates and the nontoxic control solution (2% NaCl solution) was compared to determine the bioluminescence inhibition due to IBA aqueous extracts.

Table 1
Battery of biotests and corresponding modifications evaluated.

Test	Standard	Modifications analysed
Aquatic compartment		
Luminescence inhibition test with bacteria *Aliivibrio fischeri*	ISO 11348-2	–
Growth inhibition test with freshwater microalgae *Raphidocelis subcapitata*	OECD guideline 201	Adaptation to 24-well micro-plates (Geis et al., 2000). Evaluation based on optical density at 96 h
Growth inhibition test with macrophytes *Lemna minor*	OECD guideline 221	Introduction in the battery. Adaptation to 6-well microplates (Kaza et al., 2007). Evaluation of frond number at 3, 5, and 7 d
Mobility inhibition test with *Daphnia magna*	OECD guideline 202	Evaluation at 24 and 48 h
Acute toxicity test with *Brachionus calyciflorus* – Rotoxkit	ISO 19827	Introduction in the battery
Acute toxicity test with *Thamnocephalus platyurus* – Thamnotoxkit	ISO 14380	Introduction in the battery
Terrestrial compartment		
Soil contact test with bacteria *Arthrobacter globiformis*	ISO 18187	–
Effects on emergence and growth of higher plants (*Brassica rapa*)	ISO 11269-2	Use of 1 species rather than 2
Avoidance test with earthworms *Eisenia fetida*	ISO 17512-1	Evaluation at 24 and 48 h
Avoidance test with collembolans *Folsomia candida*	ISO 17512-2	Evaluation at 24 and 48 h

The growth inhibition test with freshwater microalgae was carried out following the OECD guideline 201 (OECD, 2011) with adaptation to 24-well micro-plates (Geis et al., 2000). Exponentially growing organisms were exposed to test eluates diluted in Woods Hole MBL (Stein, 1973) and a full-strength MBL control. All treatments were tested in triplicate with 1-mL test solution per treatment (990 μL of the test solution and 10 μL of microalgae inoculum). An initial cell density of 10^4 cells/mL in each test well was guaranteed through microscopic cell counting with a Neubauer hemocytometer. Microplates were incubated at 23 ± 1 °C with continuous light and their content was resuspended every 12 h. The growth rate was calculated based on cell density, estimated based on absorbance at 440 nm (Shimadzu UV–Vis; UV-1800) at the end (96 h) and at the beginning of the test.

The growth inhibition test with *Lemna minor* was based on the OECD guideline 221 (OECD, 2006) with adaptation to 6-well microplates (Kaza et al., 2007). Plants were exposed to test eluates and control treatments for 7 days. The effects were evaluated on the frond number and the dry weight after the exposure period. Nutrient sufficient conditions were guaranteed since dilutions were prepared with Steinberg culture medium (OECD, 2006). Eluates were tested in triplicate, each replicate containing 10 mL final volume of the dilution or the control (full strength Steinberg), initiated with 9 fronds. The microplates were incubated at 23 ± 1 °C, under continuous illumination. The number of fronds was counted after 3, 5, and 7 days of exposure, and the final biomass (dry weight) was determined after 7 days of exposure.

The immobilization test with *Daphnia magna* was carried out according to the OECD guideline 202 (OECD, 2004). Neonates aged less than 24 h and born between the 3rd and 5th brood in bulk cultures were

exposed to the eluates and control treatments for 48 h. Optimal osmolarity conditions were guaranteed since dilutions were prepared with ASTM culture medium (ASTM, 1980). Four 10-mL replicates were tested holding 5 daphnids each. The test was incubated at 20 ± 2 °C for 48 h, under a photoperiod of $16h^L$:$8h^D$. The immobilized organisms were recorded after 24 h and 48 h.

The acute toxicity test with *Thamnocephalus platyurus* was evaluated according to the procedure supplied in the THAMNOTOXKIT F™ kit (MicroBioTests Inc, 1999). The eluate was diluted with standard (artificial) freshwater. Larvae of *T. platyurus* (20–22h) obtained from cysts hatching were exposed to the dilutions tested in triplicate in 24-well microplates. Each replicate contained 1 mL of test dilution and ten crustaceans. The microplates were incubated at 25 °C for 24 h in the dark and no food was added during the exposure period. The number of dead organisms was recorded at the end of the test.

The toxicity test with *Brachionus calyciflorus* was performed using the ROTOXKIT F™ (MicroBioTests Inc, 2000), according to the kit test protocol. The dilutions were prepared with standard (artificial) freshwater and six replicates were tested for each dilution in 24-well microplates. Each replicate consisted of 1 mL test solution or blank medium (control) and 5 organisms (16–18 h post-hatching). The microplates were incubated at 25 °C in the dark and the organisms were not fed. The number of immobilized *B. calyciflorus* after 24 h of exposure was counted.

2.3.2. Soil compartment

The soil contact test with the bacteria *Arthrobacter globiformis* was conducted according to the international standard ISO 18187 (2016). The endpoint measured in this test is the inhibition of dehydrogenase activity. Test dilutions and control (quartz sand) were moistened to 20% with deionized water. Each dilution was tested in four replicates with 0.6 g wet weight each, using 24-well microplates. In each well, 0.6 mL of deionized water was added, and the microplates were pasteurized at 80 ± 2 °C for 10 min, then cooled in an ice bath for 15 ± 5 min, twice. The inoculum (only nutrient solution for control) was added to each well and the microplates were incubated at 30 ± 2 °C in the dark for 2 h. The redox dye resazurin was added per well and the formation of resorufin was measured fluorometrically (excitation at 535 nm, emission at 590 nm), every 15 min for 1 h. The rate of resorufin increase in the sample and the control was compared to evaluate the inhibition of the dehydrogenase activity.

Emergence and growth tests with the monocotyledonous plant *Brassica rapa* were performed following ISO 11269-2 (2012). Tests were carried out in plastic pots using 4 replicates for each test dilution and control soil (LUFA 2.3 standard soil). The pots were filled with about 450 g of the moistened solid mixture, and 10 seeds were placed in each pot. The pots were incubated in a plant growth chamber under a photoperiod of $16h^L$:$8h^D$ at 23 ± 3 °C. When at least 50% of the seedlings in the control soil emerged, the emergence rates were recorded, and plants were thinned out to a total of five (where possible) evenly spaced representative specimens in each pot. Distilled water was used to maintain soil moisture through capillarity whenever needed. Following seedling emergence, a dilution of a liquid fertilizer was used to provide nutrients. After 14 days, the remaining plants were harvested, the biomass and the shoot length of each plant were measured.

The earthworm avoidance test with *Eisenia fetida* was conducted following ISO 17512-1 (2008). The two-section test design was followed using plastic containers vertically divided into two equal sections. In each container, one section was filled with 250 g (dry weight equivalent) of moistened OECD Artificial Soil (50% of the maximum water holding capacity - WHC_{max}) and the other section with the same amount of each test dilution. Then, the divider was removed and ten adult earthworms (300–600 mg each) were placed in the separating line. Five replicates were tested for each test mixture. The containers were incubated in a climatic chamber under 16 h light (400–800 Lux) and 8 h dark at 20 ± 2 °C for 48 h. Afterwards, the two sections of each container were divided. Finally, the number of organisms in each section was counted and the percentage of organisms avoiding the test substrate was determined.

The avoidance behaviour test with *Folsomia Candida* followed ISO 17512-2 (2011). Plastic containers vertically divided into two equal sections were used. In this case, one section was filled with 30 g (dry weight equivalent) of moistened OECD Artificial Soil (50% of the WHC_{max}), and the other section with the same amount of each test dilution. After the removal of the divider, 20 organisms were placed in the separating line. Five replicates were run for each test mixture. The containers were covered by a transparent lid and incubated in a climatic chamber under a photoperiod of $16h^L$:$8h^D$ at 20 ± 2 °C for 48 h. After incubation, the two sections of each container were divided, contents were separated for flooding with inked water, and organisms were counted.

2.4. Test outcome interpretation and integration

The effect benchmark for each test depends on the natural variability of the test and has been recommended in the literature (Pandard and Römbke, 2013; Moser and Römbke, 2009; Römbke, 2018) and in test standards (e.g., ISO 17512–1:2008). This benchmark refers to the value of the measured parameter for the test substrate compared to the control that can be considered a negative effect following exposure. For the biotests added to the battery in the present study, effect benchmarks are proposed based on recommendations of test standards (*F. candida*) and on values recommended for comparable organisms (value proposed for Rotoxkit and Thamnotoxkit based on the one defined for *D. magna*, and the value defined for *L. minor* based on the one proposed for microalgae). Furthermore, results were analysed according to threshold values proposed by Römbke et al. (Römbke et al., 2009; Römbke, 2018) for the LID (lowest ineffective dilution) approach (Table 2). The LID value refers to the test dilution immediately before the first dilution denoting an ecotoxicologically relevant effect (ISO 17616). For example, LID = 4 means that a mixture of 25% eluate/solid IBA and 75% control water/control soil does not induce a negative effect (e.g., mobility inhibition >20% for daphnids). According to Pandard and Römbke (2013), these values indicate the onset of harmful effects on the chosen organisms, considering the endpoints used, following gathered experimental evidence.

Some modifications of the batteries and test protocols that have been suggested for HP 14 evaluation were made in the present study and other organisms were included (*L. minor*, Rotoxkit, Thamnotoxkit, *F. candida*) (Table 1) to analyse the feasibility and usefulness of these modifications.

Table 2
Effect benchmarks for each test and threshold values used as a reference in this work for waste ecotoxicological assessment.

Test	Effect benchmark	LID threshold value considered
Aquatic compartment		
A. fischeri test	Light emission inhibition >20%	LID >4
R. subcapitata test	Population growth inhibition >25%	LID >4
L. minor test	Growth inhibition >25%[a]	LID >4
D. magna test	Mobility inhibition >20%	LID >4
Rotoxkit (*B. calyciflorus* test)	Mobility inhibition >20%[a]	LID >4
Thamnotoxkit (*T. platyurus* test)	Mobility inhibition >20%[a]	LID >4
Terrestrial compartment		
A. globiformis test	Enzyme activity inhibition >30%	LID >8
B. rapa test	Growth inhibition >30%	LID >8
E. fetida test	Behaviour impact >80%	LID >8
F. candida test	Behaviour impact >70%[a]	LID >8

[a] Proposed in the current study.

3. Results and discussion

3.1. Chemical and physical characterization

In general, the results found for the chemical properties of fresh IBA (Table 3) and its aqueous extracts (Table 4) were in line with the literature. Furthermore, solid IBA presented elemental concentrations within the common range found for soils, with some exceptions (Table 3): IBA presented higher concentrations of Mg and Na regarding major elements, as well as higher concentrations of Cu and Zn concerning trace/minor elements. Mg is an essential element with numerous biological functions, and negative effects on the biota are usually related to its deficiency (Scarpa, 1974; Huber and Jones, 2013; Jahnen-Dechent and Ketteler, 2012). On the other hand, Na may play a beneficial biological role, but high concentrations become toxic (Pilon-Smits et al., 2009; Maathuis, 2014). Likewise, Cu and Zn are essential metals with known biological functions (Karna et al., 2017; Festa and Thiele, 2011), but only when available at levels below toxic concentrations (Karna et al., 2017). Fresh IBA and its eluate are alkaline, showing a high natural pH and a low organic matter (OM) content as expected (Tables 3 and 4). Most of the PTM were found in low concentrations in IBA eluate, while Cu was the one found in higher load (Table 4). The X-ray diffractogram of IBA (Fig. S1, supplementary information) revealed quartz (SiO_2), calcite ($CaCO_3$), ettringite ($Ca_6Al_2(SO_4)_3(OH_{12})$. $26H_2O$), anhydrite ($CaSO_4$) and plagioclases (albite - $(Na_{0.84}Ca_{0.16})Al_{1.16}Si_{2.84}O_8$ - and anorthite - $Na_{0.25}Ca_{0.71}(Al_2Si_2O_8)$ as the main mineral phases, which is in agreement with the literature (Alam et al., 2019; Loginova et al., 2019; Maldonado-Alameda et al., 2020b; Alam et al., 2020; Abramov et al., 2018). Quartz was found to be the major mineral in the sample and is also one of the primary minerals of the lithosphere. Accordingly, Si has been mentioned as one of the major elements in IBA (Klymko et al., 2017; Lindberg et al., 2015; Chandler et al., 1997) as well as in soil (Chandler et al., 1997).

Table 3
Chemical properties of fresh IBA solid sample.

Parameter (mg/kg)	IBA (mg/kg)	Literature (mg/kg)	Common range for soils (mg/kg)[h]
Moisture (%)	13.7 ± 0.30	7–30[d]	-
OM (%)	3.38 ± 0.09	0.2–5[e]	–
pH[a]	10.9 ± 0.07	10.8–11.3[f]	–
Major elements			
Ca	81,172 ± 8[b]	56,300–306,429[g]	7000–500,000
Fe	34,720 ± 412[b]	14,210–158,704[g]	7000–550,000
Mg	12,070 ± 85[b]	7080–30,660[g]	600–6000
Na	12,128 ± 301[b]	11,352–57,723[g]	750–7500
K	10,497 ± 1115[b]	6887–17,600[g]	400–30,000
P	4457 ± 992[c]	2401–24,014[g]	200–5000
Ti	2205 ± 328[c]	2600–18,600[g]	1000–10,000
Mn	326 ± 11[b]	83–3408[g]	20–3000
Minor or trace elements			
Ba	155 ± 7.5[c]	400–3920[g]	100–3000
Cd	<3.6[b]	0.3–146[g]	0.01–0.70
Co	12.8 ± 2.1[c]	6–350[g]	1–40
Cr	59.5 ± 14.5[c]	23–3170[g]	1–1000
Cu	824 ± 21[b]	190–12,000[g]	2–100
Ni	22.3 ± 2.9[c]	7–4280[g]	5–500
Pb	120 ± 8[c]	98–13,700[g]	2–200
Sr	140 ± 9[c]	85–1,000g[g]	50–1000
Rb	20.5 ± 0.93[c]	29–50[g]	50–500
V	14.6 ± 4.68[c]	20–122[g]	20–500
Zn	1624 ± 23[b]	613–13,600[g]	10–300

[a] In 0.01 M $CaCl_2$ solution.
[b] FAAS.
[c] TXRF.
[d] (Wahlström et al., 2016; Römbke et al., 2009; Ginés et al., 2009; Chandler et al., 1997; Andreola et al., 2008; Ashraf et al., 2019; Monteiro et al., 2008; Rendek et al., 2007; Tang et al., 2015; Zhang et al., 2015b; Bunge et al., 2018; Bayuseno and Schmahl, 2010; Forteza et al., 2004; Huber et al., 2019; Hyks et al., 2009; Lindberg et al., 2015; Neuwahl et al., 2019; Rambaldi et al., 2010; Tang and Steenari, 2016).
[e] (Joseph et al., 2018; Lynn et al., 2017).
[f] Fresh IBA (Römbke et al., 2009).
[g] (Chimenos et al., 1999; Astrup et al., 2016).
[h] (Chandler et al., 1997).

Table 4
Chemical properties of the fresh IBA eluate.

Parameter (mg/kg)	IBA eluate (mg/kg)	Literature (mg/kg)
pH[a]	11.1 ± 0.13	9.5–12. 7[e]
EC (mS/cm)[a]	3.52 ± 0.09	4.49[f]
Major elements		
Ca	1254 ± 814[b]	400–4000[g]
Fe	<0.8[b]	0.06–0.6[g]
Mg	0.31 ± 0.11[b]	0.01–1[g]
Na	4398 ± 905[b]	1000–2000[g]
K	640.87 ± 32.13[c]	200–700[g]
Mn	<0.23[b]	<0.6[g]
Minor or trace elements		
Cd	<0.18[b]	<0.001[g]
Cr	<0.56[b]	0.02–2.1[g]
Cu	19 ± 0.6[b]	0.20–14.85[g]
Ni	<0.85[b]	0.004–0.02[g]
Pb	<1.53[b]	0.001–10.2[g]
Sn	2.06 ± 0.39[c]	<2.5[g]
Sr	1.38 ± 0.00[c]	1.54–4.355[g]
Zn	<0.13[b]	<0.02–14.32[g]
Ions		
Chlorides	7149 ± 700[d]	900–42,500[g]
Sulphates	3988 ± 26[d]	66–9980[g]

[a] L/S = 10 L/kg.
[b] FAAS.
[c] TXRF.
[d] Ion chromatography.
[e] Fresh IBA (Moser and Römbke, 2009; Huber et al., 2019; Astrup et al., 2016; Chimenos et al., 2003).
[f] Fresh IBA (Forteza et al., 2004).
[g] (Römbke et al., 2009; Forteza et al., 2004; Chandler et al., 1997; Lapa et al., 2002; Di et al., 2018; Dijkstra et al., 2008; Kalbe and Simon, 2020; Feng et al., 2007).

3.2. Ecotoxicity tests

3.2.1. Aquatic compartment

An inhibition of bioluminescence of *A. fischeri* above 20% was only found for D2 (50% dilution) both with natural pH and with adjusted pH (Fig. 1a). Although the natural pH could be a negative factor for bacteria activity according to the standard, inhibitions were slightly higher for solutions with pH adjustment (Table S1, supplementary material), suggesting that this procedure may have a negative impact on this organism. Nonetheless, low ecotoxicity was found for bacteria. The results were consistent with what was found in the study by Römbke et al. (2009) for fresh IBA samples (1 sample led to inhibitions above 20% for D2 and 2 samples to inhibitions below 20% even for D1), where the luminescent bacteria were the least sensitive organism in the aquatic compartment. This was also true for IBA in the international ring test, although the same observation could not be made for all three types of waste tested (Moser and Römbke, 2009).

Regarding *R. subcapitata*, a yield of 3,099,493 ± 301,359 cells/mL (mean ± standard error) was determined in the control. This organism was clearly more sensitive to IBA since growth rate inhibitions were over 64% for the 3 dilution steps, which are well above the 25% effect benchmark (Fig. 1b). These results agree with the ones reported by Römbke et al. (2009) for growth rate inhibition of microalgae following exposure to a fresh IBA sample.

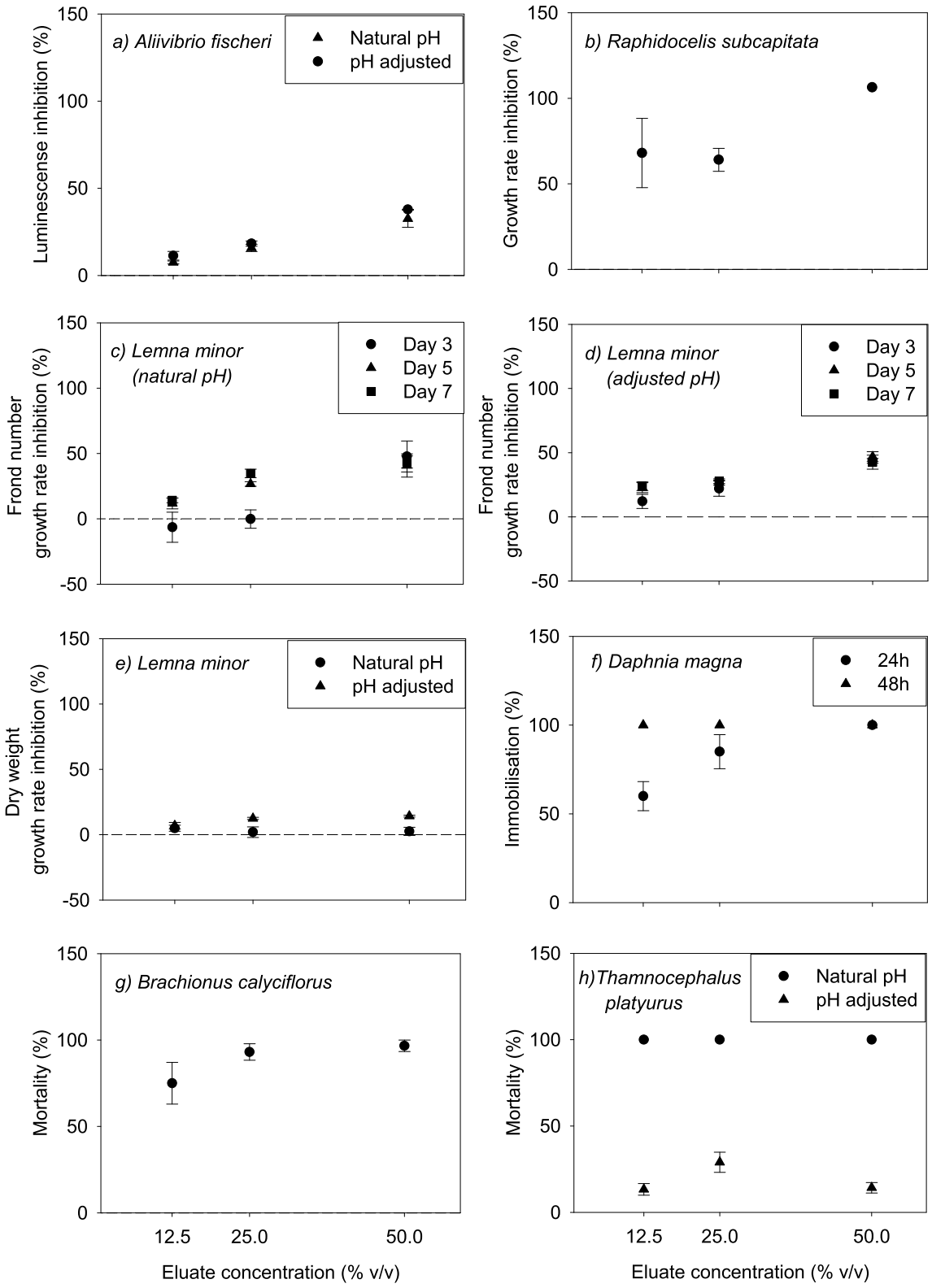

Fig. 1. Effects (mean ± standard error) of IBA eluate dilutions in the tested species from the aquatic compartment, namely: a) bioluminescence inhibition of *A. fischeri*; b) growth rate inhibition of *R. subcapitata*; c) growth rate inhibition of *L. minor* considering frond number in natural pH conditions and d) with pH adjustment to culture media as well as e) growth rate inhibition of *L. minor* considering dry weight; f) immobilization of *D. magna*; g) mortality of *B. calyciflorus*; h) mortality of *T. platyurus*.

The number of fronds in control used for comparison with solutions with natural pH was 7.3 ± 0.9 on day 3, 15.3 ± 0.3 on day 5, and 39 ± 3 on day 7; while for solutions with pH adjustment, these records were 8.3 ± 0.9, 26 ± 4 and 44 ± 5, respectively. *L. minor* responded differently regarding frond number growth after 3, 5, and 7 days of exposure to IBA eluate dilutions, both with natural pH and pH adjusted to culture media, showing enhanced effects the higher the test duration (Fig. 1c-d). Nonetheless, results after 5 and 7 days are similar. Like *A. fischeri*, pH adjustment induced slightly higher inhibitions, showing pH-related effects. After 7 days of exposure to test solutions, inhibitions over 14.1% (natural pH) and 23.9% (pH adjusted) were found (Fig. 1c-d), the latter being above the 20% effect benchmark. The dry weight yield in control was similar under natural pH and adjusted pH (0.0037 ± 0.0003 mg and 0.0039 ± 0.0004 mg, respectively). Growth rate inhibition decreased with lower dilution for solutions with natural pH (maximum inhibition of 4.9%) and, on the other hand, the inhibition increased with lower dilution for solutions adjusted to the pH of culture media (inhibitions above 14.2%) (Fig. 1e). The dry weight was more sensitive to IBA as an endpoint than the frond number. This is likely related to the strategies of the plant to deal with toxic challenges; by producing fewer fronds with a higher biomass per frond, plants can reduce surface contact with waterborne contaminants, thus indirectly reducing uptake. Other studies with inorganic waste found greater sensitivity of *L. minor* compared to microalgae (Bandarra et al., 2019, 2020). However, the results from the present study show that IBA eluate had a higher negative impact on microalgae than on *L. minor*. This observation was also made in the international ring test, where a limited set of tests with *L. minor* were conducted (Moser and Römbke, 2009). Considering the pH of the culture media and the accepted variation of pH throughout the test, the pH of solutions tested with *L. minor* and microalgae would not need to be adjusted (Tables S1 and S2 in the supplementary information, respectively).

In the case of *D. magna*, the control exhibited 10 ± 6% immobilization (for both test durations) and a clear distinction could be observed between results after 24 h and 48 h of exposure: all three dilution steps resulted in 100% immobilization after 48 h, while a dose-response relationship was found after 24 h (Fig. 1f). Nonetheless, all inhibitions were above the 20% benchmark (60% for D8) after 24 h of exposure. Thus, daphnids were very sensitive to the IBA sample tested herein. The same was found by Römbke et al. (2009). Likewise, the mortality of *B. calyciflorus* was above 75% following contact with the 3 dilution steps of IBA eluate (Fig. 1g), demonstrating high sensitivity. Mortality in the control was null. Similarly, null mortality was found for *T. platyurus* in the control at the end of the test. An evident contrasting response to test solutions was found for eluates with natural pH and pH adjusted to control (Table S1, supplementary material). Indeed, 100% mortality was observed for all dilution steps with natural pH, whereas mortality was below 29% (D4) when pH was adjusted to culture media values (Fig. 1h), suggesting that pH caused itself harmful effects to these test organisms.

Therefore, microcrustaceans were the most sensitive organisms, particularly *D. magna*. According to Postma et al. (2002), the values of pH, EC, and chlorides obtained in this work are not toxic for this organism (Table S2, supplementary material; Table 4). From the PTM, Cu was the one in higher concentration in IBA eluate. The study from Okamoto et al. (2014) on the acute toxicity of 50 metals to *D. magna* concluded that Cu was highly toxic for this organism with an EC_{50} (median effect concentration) of 0.013 mg/L, two orders of magnitude below the concentration from the present study (1.9 mg/L). The concentrations of all the other elements analysed (Table 4) were well below EC_{50} values determined by Okamoto et al. (2014) and are probably not leading to such high ecotoxicity. Although individual metal concentrations were generally low, metals present in complex mixtures can interact causing antagonistic or synergetic effects (Altenburger et al., 2000, 2013; Franklin et al., 2002; Horvat et al., 2007; Ince et al., 1999; Wu et al., 2016), thus potentially leading to waste ecotoxicity. For example, Walter et al. (2002) showed that a mixture of substances under their NOEC (no observed effect concentration) caused higher toxicity to algae than the one expected assuming that there is no interaction between added substances.

3.2.2. Terrestrial compartment

A. globiformis showed a clear concentration-response curve when exposed to different concentrations of waste in soil mixture (Fig. 2a), and only D16 (6.35% eluate concentration) caused inhibition below the 30% effect benchmark. The slope of the relative fluorescence (mean ± standard error) obtained for control was 168 ± 6. This organism was the one presenting a lower variability of results in the terrestrial compartment, and our results are consistent with those found for a fresh IBA sample tested by Römbke et al. (2009). The pH of the solid mixtures was within the range (6–9) indicated as suitable for this test in the standard (Table S3, supplementary information).

B. rapa presented similar responses to both endpoints tested (Fig. 2b-c) and a clear concentration-response curve was also observed in this case. The control showed a shoot growth length of 169 ± 6 mm and biomass growth of 3.15 ± 0.07. A low concentration of IBA eluate (6.25%, D16) promoted plant growth regarding both shoot length and biomass, while higher concentrations lead to growth inhibitions above 30%, similar to *A. globiformis*. Even though, biomass was more sensitive to the intermediate dilution (D8).

Considering that the effects on different soil invertebrates may vary due to different sensitivity to contaminants and exposure routes (ISO 17512–1:2008), the avoidance behaviour of *F. candida* was tested in addition to *E. fetida* to evaluate the usefulness of including a second invertebrate in the test battery. The international standards for the avoidance tests of these organisms (Table 1) establish a test duration of 48 h. However, Natal-da-Luz et al. (Natal-da-Luz et al., 2008) concluded that an exposure of 24 h seemed to be sufficient to observe a clear avoidance response. In this context, herein the response of organisms was recorded after 24 h and 48 h of incubation, this approach having a two-way utility: (i) if similar records are obtained, the use of a 24-h exposure decreases the effort allocated to the test, which is favourable to its inclusion in a standard battery designed to rapidly and efficiently assess waste hazardous potential; (ii) waste are complex matrices and their chemical composition may not be entirely known, thus effect reading bias can arise from a longer exposure period in these tests, e.g. concerning neurotoxicity caused by unmonitored organic and inorganic compounds that may affect chemical detection capacity and/or the mobility of organisms. Avoidance of −10.2 ± 22.3% and −12.0 ± 18.5% was found for control after 24 h and 48 h, respectively, for *E. fetida*. The 80% inhibition was surpassed only for the lower dilution (D4), but a substantial difference was found between the effects observed after 24 h and 48 h of exposure to IBA eluate for higher dilutions. *E. fetida* showed higher avoidance with a higher concentration of IBA in the soil mixture after 24 h of exposure, while after 48 h this response was not so clear, and higher variability of results was observed (Fig. 2d). This inconsistency between records taken following 24 h and 48 h of exposure may be related to the presence of elements that may have neurotoxic effects, such as Mn and Pb, as well as elevated concentrations of elements that have been shown to induce neurotoxic effects, such as Zn and Cu, affecting the mobility of earthworms and contributing to results after 48 h. Different studies have indeed shown the (potential) neurotoxicity of Mn (Racette et al., 2012; Neumann et al., 2020; Martins et al., 2022; Chen et al., 2015; Ruszkiewicz et al., 2018), Pb (Ruszkiewicz et al., 2018; Anderson et al., 2004; Chen et al., 2013), Zn (Martinez-Finley et al., 2011; Wang et al., 2007; Li et al., 2022), and Cu (Martinez-Finley et al., 2011; Du and Wang, 2009; Zhang et al., 2021) using the nematode *Caenorhabditis elegans* as a model organism.

In the case of *F. candida*, avoidance values of 5.30 ± 16.7% and −25.7 ± 16.7% were observed in the control, respectively after 24 h and 48 h. *F. candida* seemed to have similar responses after 24 h and 48 h of incubation (Fig. 2e). However, higher variability of results was also

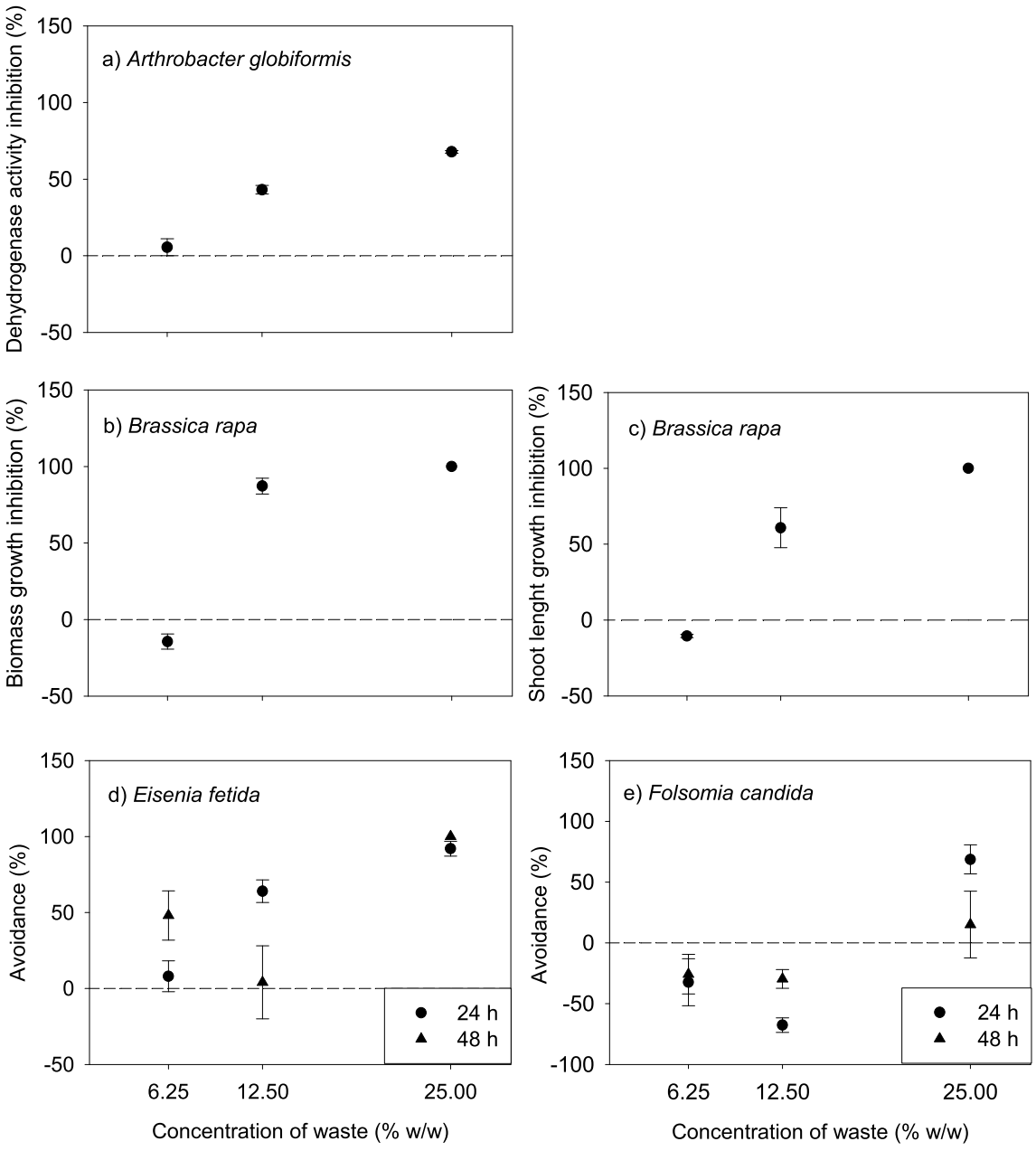

Fig. 2. Effects (mean ± standard error) of the solid dilutions of IBA in the tested species from the terrestrial compartment, namely: a) inhibition in dehydrogenase activity of *A. globiformis*; b) inhibition in growth of *B. rapa* considering shoot length growth and c) biomass growth; d) avoidance of *E. fetida*; e) avoidance of *F. candida*.

found after 48 h in this case. The effect benchmark of 70% of avoidance was not found but D4 resulted in lower avoidance after 48 h (15.1 ± 27.5) than 24 h (68.7 ± 11.9).

Generally, *B. rapa* was the most sensitive species in the terrestrial compartment, particularly regarding biomass growth endpoint, while collembolans seemed to be the least sensitive species. Stronger effects may have been caused by the high concentrations of the potentially toxic metals Cu and Zn, above the common range for soils. Zn is an essential element for plant growth, but high levels of Zn in soils may be phytotoxic, leading to negative effects such as increased production of reactive oxygen species, imbalanced mineral nutrition, and decreased growth, photosynthetic and respiratory rate. The most usual physiological responses to Zn seem to be the decrease in germination vigour and biomass (Kaur and Garg, 2021). Likewise, Cu is a vital trace metal for plants, e.g. is needed as co-factor of numerous proteins, but in higher concentrations may negatively impact the growth and productivity of plants through nutritional imbalance, changes in the root system design, increase of reactive oxygen species, and oxidative stress (Kumar et al., 2021; Moreira et al., 2015). Furthermore, sodium ions may be beneficial at lower uptake to develop osmotic potential, absorb water and maintain turgor, but in excessive concentrations, it may be toxic (Pardo and Quintero, 2002). Sodium may cause osmotic stress, potentially disrupting the integrity of the membrane of roots or shoots, and also ionic stress, leading to potassium deficiency due to the competition of ions (Pardo and Quintero, 2002; Kronzucker et al., 2013; Kronzucker and Britto, 2011). Deleterious effects impact processes such as transpiration, photosynthesis, production of reactive oxygen species, and, consequently, growth and yield (Kronzucker et al., 2013). Furthermore, pH-related effects were probably caused since pH is higher than the optimum range recommended in the standard (5–7.5). However, it should be noted that the standard indicates that pH should not be corrected since it may influence the bioavailability of soil contaminants.

Moreover, the environmental hazardousness of waste derives not only from its chemical composition, but also from other physicochemical properties such as pH. Thus, ecotoxicity should be evaluated as a whole providing a more realistic assessment of potential environmental impacts, while pH modifications may induce changes in bioavailability, leading to a biased conclusion on waste ecotoxicity. Thus, pH adjustment should not be recommended.

3.3. Ecotoxicity assessment based on the responses of aquatic and terrestrial organisms

Due to the lack of specific recommendations for HP 14 assessment using biotests in the EU and consensus in the scientific community on the ones that should be performed, different protocols are often followed to get extracts from waste and test them, including among the EU Member States (Pandard and Römbke, 2013; BIO by Deloitte, 2015; Römbke et al., 2009; Wilke et al., 2008; Ferrari et al., 1999; Pandard et al., 2006; Huguier et al., 2015; Tsiridis et al., 2012), limiting comparability. The interpretation of the results is also constrained by the lack of harmonized regulatory threshold values, although some have been proposed by the scientific community (Pandard and Römbke, 2013; BIO by Deloitte, 2015; Römbke et al., 2009; Lapa et al., 2002; Barbosa et al., 2009; Hennebert et al., 2013; Hennebert, 2018; Pandard, 2016).

The tested IBA triggered effects in different degrees on the species selected (Table 5), highlighting the importance of including diverse species in a battery of biotests for HP 14 assessment. Considering the results from the LID approach (Table 5), all tests in the aquatic compartment were generally very responsive, leading to the conclusion that the sample is ecotoxic (LID >4). The exception was the test with the luminescent bacteria, which was the least sensitive organism, and the test with *L. minor* considering dry weight growth, which was the least sensitive endpoint. On the other hand, *A. globiformis* and *B. rapa* were the most sensitive species in the terrestrial compartment, leading to an ecotoxic classification (LID >16), while earthworms were less sensitive and collembolans were largely unresponsive.

Table 5
- Summary of results of the LID approach.

Test	Duration	LID
Aquatic compartment		
A. fischeri luminescence inhibition		
Natural pH	30 min	4
Adjusted pH	30 min	4
R. subcapitata growth inhibition	96 h	>8
L. minor growth inhibition		
Frond number		
Natural pH	3 d	4
Adjusted pH	3 d	4
Frond number		
Natural pH	5 d	8
Adjusted pH	5 d	8
Frond number		
Natural pH	7 d	8
Adjusted pH	7 d	8
Dry weight		
Natural pH	7 d	<2
Adjusted pH	7 d	<2
D. magna mobility inhibition	24 h	>8
	48 h	>8
Rotoxkit (*B. calyciflorus*)	24 h	>8
Thamnotoxkit (T. platyurus)		
Natural pH	24 h	>8
Adjusted pH	24 h	8
Terrestrial compartment		
A. globiformis soil contact test	6 h	16
B. rapa effects on emergence and growth		
Shoot length	14 d	16
Biomass growth	14 d	16
E. fetida avoidance test	24 h	8
	48 h	8
F. candida avoidance test	24 h	<4
	48 h	<4

Modifications to the test batteries that have been proposed were appraised in the present study. *A. fischeri* does not require culture maintenance in the laboratory, allows a short-duration test, with relatively low cost; this test involves a low amount of sample as well as few laboratory resources, presents relatively easiness of application, and has previously shown high sensitivity to different contaminants (Abbas et al., 2018), which makes it an appealing test to integrate a biotest battery. The immobilization of *D. magna* and the avoidance behaviour of *E. fetida* were evaluated after two periods of time to compare the response of the organisms, focusing on possibly reducing test duration. In this study, the same conclusions regarding ecotoxicity were withdrawn from both periods of time for each of those 2 species, following the LID approach. Remarkable impairment of the tested parameter was found for daphnids, and thus the results led to the same conclusion regardless of the test length, suggesting that a shorter duration may be adequate for this matrix. The same may not occur for less hazardous waste samples. In the terrestrial compartment, a dose-response relationship was found for earthworms after 24 h but not 48 h, with a high variability found for the latter test length, possibly related to neurotoxic effects. For this reason and considering that this is a test of non-forced exposure based on a very sensitive behavioural endpoint, a shorter duration seems suitable in this case. Nevertheless, caution should be taken in generalising for other waste. The experience in waste testing with *A. globiformis* is still limited, but its high sensitivity, short duration, and low cost make it a practical and useful test to include in the test battery. The growth of higher plants was only evaluated for one plant (*B. rapa*) instead of two, considering the one that has been showing higher sensitivity (Moser and Römbke, 2009). The results were evaluated after 14 days, which is the minimum test duration referred to in the ISO guideline. The greatest sensitivity of this species in the terrestrial compartment suggests that this modification may be suitable and relevant, allowing for lowering the amount of laboratory resources (relatively high amounts of soil and duplicate laboratory equipment), the amount of waste handled, and the cost of the test. For the same reason, the miniaturization of the growth inhibition test with *R. subcapitata* was included rather than the apparatus described in the guideline, and sensitive results were also found, suggesting this modification is suitable to improve the test battery regarding practicability.

Organisms other than the most traditionally tested in the field were included in this study. The growth inhibition of *L. minor* was analysed to cover the complementarity of the systemic and the surface contact pathways of chemical uptake, targeting the comparison of these results with the ones from the test with freshwater microalgae, which only cover the latter uptake pathway. In this test, two endpoints were evaluated, namely frond number and dry weight. Furthermore, two modifications were analysed: the miniaturization of the test, and the evaluation of the frond number endpoint after 3, 5, and 7 days for comparison of the responses for a different test duration. The frond number endpoint was clearly more sensitive than the dry weight endpoint, with a same LID obtained after 5 and 7 days of exposure to test solutions indicating that the duration of the test could be reduced. The miniaturised test allowed to observe the sensitive response of this endpoint and distinguish differential sensitivities between endpoints as well as test durations, suggesting this modification can enhance the practicability of the test while ensuring reliable results. The same classification was obtained for the tested sample with microalgae and *L. minor*. Nonetheless, microalgae were more responsive to exposure to test dilutions. The Rotoxkit and the Thamnotoxkit were added to the battery as alternative aquatic invertebrates, due to the lower cost and exempted need to culture test organisms in the laboratory, being *a priori* easier to implement at the industrial level. However, other aspects need to be reasoned. For example, the response of the Rotoxkit was similar to that of *D. magna*, but the organisms are much smaller and can be more

difficult to count by a less experienced operator, which constrains the practicability of the test. Regarding the Thamnotoxkit, similar results were obtained compared to *D. magna* testing to solutions with natural pH. This was the only test presenting different LID values for solutions with natural pH and adjusted pH. However, it is important to note that in real conditions, the pH of waste is not adjusted and the effects of all properties, including pH-related effects, should be considered for a realistic assessment and informed decision-making. In this context, results suggest that the Thamnotoxkit could be used as an alternative to the acute toxicity test with *D. magna*, particularly at the industrial level. In the terrestrial compartment, considering that the effects on different soil invertebrates may vary due to different sensitivity to contaminants and exposure routes (ISO 17512-2, 2011), the avoidance behaviour of *F. candida* was tested at 24 h and 48 h. The results obtained from the two exposure periods led to the same conclusions, but higher variability of results was observed after 48 h, which suggests that the exposure period may be shortened. However, this species was the least sensitive of the battery and it did not bring additional information to that collected with the earthworms. Accordingly, the advantages of adding it to the test battery are not clear, at least concerning IBA.

In summary, based on the results of this study it is suggested to follow the recommendations of Pandard and Römbke (2013) regarding the test battery to test IBA but, regarding the aquatic compartment, the following modifications are proposed: using the miniaturised test for *R. subcapitata* described in the work; reducing the duration of *D. magna* test for 24 h (if clear harmful effects could be observed); and substituting the *D. magna* test for the Thamnotoxkit, particularly at the industrial level. Regarding the terrestrial compartment, the following modifications are suggested: using 1 species of plant, namely *B. rapa*, instead of two species, and considering the minimum test duration of 14 days; reducing the duration of the *E. fetida* test to 24 h. Furthermore, it is recommended to test waste with their natural pH and not carry out pH correction to evaluate their ecotoxicological potential realistically. The ecotoxicity assessment of other types of waste may benefit from the proposed modifications, but it should be noted that each type of waste may have its particularities and they should be taken into account. The Extended Limit Design considering LID (Römbke, 2018) was used in this study. The LID-approach is widely used in Germany for assessing wastewater and contaminated soils. This approach involves relatively low effort, test material, and laboratory resources since it includes only three dilution steps, which could be advantageous when testing waste in a non-academic context. Regarding waste, limit-values for biotests were not established regulatorily yet, but the values considered in this study have been recommended based on an international ring test. The LID-approach allowed distinguishing ecotoxic from non-ecotoxic responses as well as the most sensitive species from the least sensitive.

4. Conclusions on an improved assessment approach

The need of including species from different trophic levels of the aquatic and terrestrial ecosystems in HP 14 assessment of waste was demonstrated by the broad range of effects observed. The results from the biotest battery lead to an efficient ecotoxic classification of the fresh IBA analysed (LID >4). Methodological issues were only identified for the Rotoxkit test. The results obtained both for daphnids (LID >8) and earthworms (LID = 8) after 24 h and 48 h indicate that a reduction in test duration may be acceptable in this case. The higher sensitivity of *B. rapa* (LID = 16) indicates that using it as the only terrestrial plant species representative in the battery and considering the minimum test duration may be suitable. *L. minor* was sensitive regarding frond number (LID = 8), but less than *R. subcapitata* (LID >8). The Thamnotoxkit presented similar results to *D. magna* test for eluates with natural pH (LID >8), and thus it seems that it could be used alternatively. On the other hand, it does not seem useful to add the *F. candida* test to the battery given the lower sensitivity found (LID <4). The differences in sensitivity observed for *A. fischeri* (regarding eluates) and *E. fetida* (regarding solid IBA) compared to the other species were too little to exclude these tests from the battery (LID differed only in one dilution step), which adds to the representativity they bear of specific groups of the corresponding environmental compartments.

The Extended Limit Test design considering LID allowed to conclude regarding IBA ecotoxic effects on test organisms and it seems useful for ecotoxicity assessment of waste given its practicability. Based on the limited testing of one IBA sample, this study suggests using *A. fischeri*, *R. subcapitata* (miniaturised test herein described), and *D. magna* (reduced to 24 h if clear negative effects are detected) or *T. platyurus* (Thamnotoxkit) as its substitute (especially in the industry) in the aquatic compartment; and *A. globiformis, B. rapa* (as the only plant species, using the 14 days period), and *E. fetida* (reduced to 24 h) in the terrestrial compartment for the ecotoxicity assessment of IBA under HP14. It is also recommended to assess waste with natural pH to evaluate the global ecotoxicity. The biotest battery recommended herein seems suitable, comprising representative species from different trophic levels, and practical, including the consideration of reduced amount of waste, laboratory resources, and duration. This battery may be suitable for the assessment of the ecotoxicity of other types of waste, but their specificities should be considered. The enhancement of the practicability of the biotest battery should assist in better acceptance of this approach.

CRediT authorship contribution statement

B.S. Bandarra: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualisation. **H. Passos:** Investigation, Resources, Writing – review & Editing. **T. Vidal:** Investigation, Validation, Data curation, Writing – review & Editing. **R.C. Martins:** Conceptualization, Resources, Writing - Review & Editing, Supervision. **M.J. Quina:** Conceptualization, Resources, Writing - Review & Editing, Supervision. **J.L. Pereira:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision. **J. Römbke:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision.

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

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.118513.

References

Abbas, M., Adil, M., Ehtisham-ul-Haque, S., Munir, B., Yameen, M., Ghaffar, A., Shar, G. A., Asif Tahir, M., Iqbal, M., 2018. Vibrio fischeri bioluminescence inhibition assay for ecotoxicity assessment: a review. Sci. Total Environ. 626, 1295–1309. https://doi.org/10.1016/j.scitotenv.2018.01.066.

Abramov, S., He, J., Wimmer, D., Lemloh, M.L., Muehe, E.M., Gann, B., Roehm, E., Kirchhof, R., Babechuk, M.G., Schoenberg, R., et al., 2018. Heavy metal mobility and valuable contents of processed municipal solid waste incineration residues from Southwestern Germany. Waste Manag. 79, 735–743. https://doi.org/10.1016/j.wasman.2018.08.010.

Alam, Q., Schollbach, K., van Hoek, C., van der Laan, S., de Wolf, T., Brouwers, H.J.H., 2019. In-depth mineralogical quantification of MSWI bottom ash phases and their association with potentially toxic elements. Waste Manag 87, 1–12. https://doi.org/10.1016/j.wasman.2019.01.031.

Alam, Q., Lazaro, A., Schollbach, K., Brouwers, H.J.H., 2020. Chemical speciation, distribution and leaching behavior of chlorides from municipal solid waste incineration bottom ash. Chemosphere 241, 124985. https://doi.org/10.1016/j.chemosphere.2019.124985.

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: mixtures composed of similarly acting chemicals. Environ. Toxicol. Chem. 19, 2341–2347. https://doi.org/10.1002/etc.5620190926.

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., 2013. Simplifying complexity: mixture toxicity assessment in the last 20 years. Environ. Toxicol. Chem. 32, 1685–1687. https://doi.org/10.1002/etc.2294.

Anderson, G.L., Cole, R.D., Williams, P.L., 2004. Assessing behavioral toxicity with Caenorhabditis elegans. Environ. Toxicol. Chem. 23, 1235–1240. https://doi.org/10.1897/03-264.

Andreola, F., Barbieri, L., Hreglich, S., Lancellotti, I., Morselli, L., Passarini, F., Vassura, I., 2008. Reuse of incinerator bottom and fly ashes to obtain glassy materials. J. Hazard Mater. 153, 1270–1274. https://doi.org/10.1016/j.jhazmat.2007.09.103.

Ashraf, M.S., Ghouleh, Z., Shao, Y., 2019. Production of eco-cement exclusively from municipal solid waste incineration residues. Resour. Conserv. Recycl. 149, 332–342. https://doi.org/10.1016/j.resconrec.2019.06.018.

ASTM. Report E729-80, Standard Practice for Conducting Acute Toxicity Tests with Fishes, 1980. Macroinvertebrates and Amphibians. Materials, Philadelphia, PA.

Astrup, T., Muntoni, A., Polettini, A., Pomi, R., van Gerven, T., van Zomeren, A., 2016. Treatment and reuse of incineration bottom ash. In: Prasad, M.N.V., Shih, K. (Eds.), Environmental Materials and Waste: Resource Recovery and Pollution Prevention. Elsevier, pp. 607–645, 9780128039069.

Baciocchi, R., Costa, G., Lategano, E., Marini, C., Polettini, A., Pomi, R., Postorino, P., Rocca, S., 2010. Accelerated carbonation of different size fractions of bottom ash from RDF incineration. Waste Manag. 30, 1310–1317. https://doi.org/10.1016/j.wasman.2009.11.027.

Bandarra, B.S., Gomes, L.A., Pereira, J.L., Gonçalves, F.J.M., Martins, R.C., Quina, M.J., 2019. Characterization of ecotoxicological effects of green liquor dregs from the pulp and paper industry. ACS Sustain. Chem. Eng. 7, 14707–14715. https://doi.org/10.1021/acssuschemeng.9b02636.

Bandarra, B.S., Gomes, L.A., Pereira, J.L., Gonçalves, F.J.M., Martins, R.C., Quina, M.J., 2020. Assessment of hazardous property HP 14 using ecotoxicological tests: a case study of weathered coal fly ash. Environ. Sci. Pollut. Res. 27, 20972–20983. https://doi.org/10.1007/s11356-020-08515-8.

Barbosa, R., Lapa, N., Boavida, D., Lopes, H., Gulyurtlu, I., Mendes, B., 2009. Co-combustion of coal and sewage sludge: chemical and ecotoxicological properties of ashes. J. Hazard Mater. 170, 902–909. https://doi.org/10.1016/j.jhazmat.2009.05.053.

Bayuseno, A.P., Schmahl, W.W., 2010. Understanding the chemical and mineralogical properties of the inorganic portion of MSWI bottom ash. Waste Manag. 30, 1509–1520. https://doi.org/10.1016/j.wasman.2010.03.010.

BIO by Deloitte, 2015. Study to Assess the Impacts of Different Classification Approaches for Hazard Property "HP 14" on Selected Waste Streams - Final report. Prepared for the European Commission (DG ENV), in collaboration with INERIS.

Blasenbauer, D., Huber, F., Lederer, J., Quina, M.J., Blanc-Biscarat, D., Bogush, A., Bontempi, E., Blondeau, J., Chimenos, J.M., Dahlbo, H., et al., 2020. Legal situation and current practice of waste incineration bottom ash utilisation in Europe. Waste Manag. 102, 868–883. https://doi.org/10.1016/j.wasman.2019.11.031.

Bunge, R., 2018. Recovery of metals from waste incineration bottom ash. In: Holm, O., Thome-Kozmiensky, E. (Eds.), Treatment and Utilisation of Waste Incineration Bottom Ash. Neuruppin, Germany, pp. 63–143.

CEWEP. Confederation of European waste-to-energy plants. Bottom ash Factsheet. http://www.cewep.eu/2017/09/08/bottom-ash-factsheet/. (Accessed 11 March 2021).

Chandler, A.J., Eighmy, T.T., Hartlén, J., Hjelmar, O., Kosson, D.S., Sawell, S.E., van der Sloot, H.A., Vehlow, J., 1997. Municipal Solid Waste Incinerator Residues. In: The International Ash Working Group (IAWG); Studies in Environmental Science, vol. 67. Elsevier, Amsterdam, The Netherlands, 0444825630.

Chen, C.H., Chiou, I.J., 2007. Distribution of chloride ion in MSWI bottom ash and de-chlorination performance. J. Hazard Mater. 148, 346–352. https://doi.org/10.1016/j.jhazmat.2007.02.046.

Chen, P., Martinez-Finley, E.J., Bornhorst, J., Chakraborty, S., Aschner, M., 2013. Metal-induced neurodegeneration in C. elegans. Front. Aging Neurosci. 5, 1–11. https://doi.org/10.3389/fnagi.2013.00018.

Chen, P., Chakraborty, S., Peres, T.V., Bowman, A.B., Aschner, M., 2015. Manganese-induced neurotoxicity: from C. elegans to humans. Toxicol. Res. 4, 191–202. https://doi.org/10.1039/c4tx00127c.

Chimenos, J.M., Segarra, M., Fernández, M.A., Espiell, F., 1999. Characterization of the bottom ash in municipal solid waste incinerator. J. Hazard Mater. 64, 211–222. https://doi.org/10.1016/S0304-3894(98)00246-5.

Chimenos, J.M., Fernández, A.I., Nadal, R., Espiell, F., 2000. Short-term natural weathering of MSWI bottom ash. J. Hazard Mater. 79, 287–299. https://doi.org/10.1016/S0304-3894(00)00270-3.

Chimenos, J.M., Fernández, A.I., Miralles, L., Segarra, M., Espiell, F., 2003. Short-term natural weathering of MSWI bottom ash as a function of particle size. Waste Manag. 23, 887–895. https://doi.org/10.1016/S0956-053X(03)00074-6.

Cornelis, G., Gerven, T. Van, Vandecasteele, C., 2006. Antimony leaching from uncarbonated and carbonated MSWI bottom ash. J. Hazard Mater. 137, 1284–1292. https://doi.org/10.1016/j.jhazmat.2006.04.048.

Cornelis, G., Gerven, T. Van, Vandecasteele, C., 2012. Antimony leaching from MSWI bottom ash : modelling of the effect of pH and carbonation. Waste Manag. 32, 278–286. https://doi.org/10.1016/j.wasman.2011.09.018.

Crillesen, K., Skaarup, J., Bojsen, K., 2006. Management of Bottom Ash from WTE Plants. An overview of management options and treatment methods, Copenhagen, Denmark.

del Valle-zermeño, R., Chimenos, J.M., Giró-paloma, J., Formosa, J., 2014. Use of weathered and fresh bottom ash mix layers as a subbase in road constructions: environmental behavior enhancement by means of a retaining barrier. Chemosphere 117, 402–409. https://doi.org/10.1016/j.chemosphere.2014.07.095.

Di, M., Hyks, J., Verginelli, I., Costa, G., Hjelmar, O., Lombardi, F., 2018. Leaching behaviour of incineration bottom ash in a reuse scenario : 12 years-field data vs . lab test results. Waste Manag. 73, 367–380. https://doi.org/10.1016/j.wasman.2017.08.013.

Dijkstra, J.J., Meeussen, J.C.L., van der Sloot, H.A., Comans, R.N.J., 2008. A consistent geochemical modelling approach for the leaching and reactive transport of major and trace elements in MSWI bottom ash. Appl. Geochemistry 23, 1544–1562. https://doi.org/10.1016/j.apgeochem.2007.12.032.

Dou, X., Ren, F., Nguyen, M.Q., Ahamed, A., Yin, K., Chan, W.P., Chang, V.W.C., 2017. Review of MSWI bottom ash utilization from perspectives of collective characterization, treatment and existing application. Renew. Sustain. Energy Rev. 79, 24–38. https://doi.org/10.1016/j.rser.2017.05.044.

Du, M., Wang, D., 2009. The neurotoxic effects of heavy metal exposure on GABAergic nervous system in nematode Caenorhabditis elegans. Environ. Toxicol. Pharmacol. 27, 314–320. https://doi.org/10.1016/j.etap.2008.11.011.

Feng, S., Wang, X., Wei, G., Peng, P., Yang, Y., Cao, Z., 2007. Leachates of municipal solid waste incineration bottom ash from Macao: heavy metal concentrations and genotoxicity. Chemosphere 67, 1133–1137. https://doi.org/10.1016/j.chemosphere.2006.11.030.

Ferrari, B., Radetski, C.M., Veber, A.M., Ferard, J.F., 1999. Ecotoxicological assessment of solid wastes: a combined liquid- and solid-phase testing approach using a battery of bioassays and biomarkers. Environ. Toxicol. Chem. 18, 1195–1202. https://doi.org/10.1897/1551-5028(1999)018<1195. EAOSWA>2.3.CO;2.

Festa, R.A., Thiele, D.J., 2011. Copper: an essential metal in biology. Curr. Biol. 21, R877–R883. https://doi.org/10.1016/j.cub.2011.09.040.

Forteza, R., Far, M., Seguí, C., Cerdá, V., 2004. Characterization of bottom ash in municipal solid waste incinerators for its use in road base. Waste Manag. 24, 899–909. https://doi.org/10.1016/j.wasman.2004.07.004.

Franklin, N.M., Stauber, J.L., Lim, R.P., Petocz, P., 2002. Toxicity of metal mixtures to a tropical freshwater alga (Chlorella sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environ. Toxicol. Chem. 21, 2412–2422. https://doi.org/10.1002/etc.5620211121.

Geis, S.W., Fleming, K.L., Korthals, E.T., Searle, G., Reynolds, L., Karner, D.A., 2000. Modifications to the algal growth inhibition test for Use as a regulatory assay. Environ. Toxicol. Chem. 19, 36–41. https://doi.org/10.1897/1551-5028(2000)019<0036:mttagi>2.3.co, 2.

Ginés, O., Chimenos, J.M., Vizcarro, A., Formosa, J., Rosell, J.R., 2009. Combined use of MSWI bottom ash and fly ash as aggregate in concrete formulation: environmental and mechanical considerations. J. Hazard Mater. 169, 643–650. https://doi.org/10.1016/j.jhazmat.2009.03.141.

Hennebert, P., 2018. Proposal of concentration limits for determining the hazard property HP 14 for waste using ecotoxicological tests. Waste Manag. 74, 74–85. https://doi.org/10.1016/j.wasman.2017.11.048.

Hennebert, P., Papin, A., Padox, J.M., Hasebrouck, B., 2013. The evaluation of an analytical protocol for the determination of substances in waste for hazard classification. Waste Manag. 33, 1577–1588. https://doi.org/10.1016/j.wasman.2013.03.013.

Hennebert, P., van der Sloot, H.A., Rebischung, F., Weltens, R., Geerts, L., Hjelmar, O., 2014. Hazard property classification of waste according to the recent propositions of the EC using different methods. Waste Manag. 34, 1739–1751. https://doi.org/10.1016/j.wasman.2014.05.021.

Hjelmar, O., Van Der Sloot, H.A., Van Zomeren, A. 2013. Hazard property classification of high temperature waste materials. Proceedings Sardinia 2013, Fourteenth

International Waste Management and Landfill Symposium, S. Margherita di Pula, Cagliari, Italy, 30 September–4 October 2013; CISA Publisher: Cagliari, Italy, 2013.
Horvat, T., Vidaković-Cifrek, Ž., Oreščanin, V., Tkalec, M., Pevalek-Kozlina, B., 2007. Toxicity assessment of heavy metal mixtures by Lemna minor L. Sci. Total Environ. 384, 229–238. https://doi.org/10.1016/j.scitotenv.2007.06.007.
Huber, D.M., Jones, J.B., 2013. The role of magnesium in plant disease. Plant Soil 368, 73–85. https://doi.org/10.1007/s11104-012-1476-0.
Huber, F., Blasenbauer, D., Aschenbrenner, P., Fellner, J., 2019. Chemical composition and leachability of differently sized material fractions of municipal solid waste incineration bottom ash. Waste Manag. 95, 593–603. https://doi.org/10.1016/j.wasman.2019.06.047.
Huber, F., Blasenbauer, D., Aschenbrenner, P., Fellner, J., 2020. Complete determination of the material composition of municipal solid waste incineration bottom ash. Waste Manag. 102, 677–685. https://doi.org/10.1016/j.wasman.2019.11.036.
Huguier, P., Manier, N., Chabot, L., Bauda, P., Pandard, P., 2015. Ecotoxicological assessment of organic wastes spread on land: towards a proposal of a suitable test battery. Ecotoxicol. Environ. Saf. 113, 103–111. https://doi.org/10.1016/j.ecoenv.2014.11.017.
Hyks, J., Astrup, T., Christensen, T.H., 2009. Leaching from MSWI bottom ash: evaluation of non-equilibrium in column percolation experiments. Waste Manag. 29, 522–529. https://doi.org/10.1016/j.wasman.2008.06.011.
Ince, N.H., Dirilgen, N., Apikyan, I.G., Tezcanli, G., Üstün, B., 1999. Assessment of toxic interactions of heavy metals in binary mixtures: a statistical approach. Arch. Environ. Contam. Toxicol. 36, 365–372. https://doi.org/10.1007/PL00006607.
ISO 11348-2. Water quality — Determination of the Inhibitory Effect of Water Samples on the Light Emission of Vibrio Fischeri (Luminescent Bacteria Test) — Part 2: Method Using Liquid-Dried Bacteria, 2017. International Organisation for Standardisation, Geneva.
ISO 17512-1. Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behaviour - Part 1: Test with Earthworms (Eisenia fetida and Eisenia andrei), 2008. International Organisation for Standardisation. Geneva.
ISO 17512-2. Soil Quality — Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behaviour — Part 2: Test with Collembolans (Folsomia candida), 2011. International Organisation for Standardisation. Geneva.
ISO 11269-2. Soil quality — Determination of the effects of pollutants on soil flora — Part 2: effects of contaminated soil on the emergence and early growth of higher plants. International Organisation for Standardisation. Geneva, 2012.
ISO 18187. Soil quality — Contact rest for solid samples using the dehydrogenase activity of Arthrobacter globiformis. International Organisation for Standardisation. Geneva, 2016.
Jahnen-Dechent, W., Ketteler, M., 2012. Magnesium basics. Clin. Kidney J. 5 https://doi.org/10.1093/ndtplus/sfr163 i3–i14.
Joseph, A.M., Snellings, R., van den Heede, P., Matthys, S., de Belie, N., 2018. The use of municipal solid waste incineration ash in various building materials: a Belgian point of view. Materials 11. https://doi.org/10.3390/ma11010141.
Juric, B., Hanzic, L., Ilic, R., Samec, N., 2006. Utilization of municipal solid waste bottom ash and recycled aggregate in concrete. Waste Manag. 26, 1436–1442. https://doi.org/10.1016/j.wasman.2005.10.016.
Kalbe, U., Simon, F.-G., 2020. Potential Use of incineration bottom ash in Construction : evaluation of the environmental impact. Waste and Biomass Valorization 11, 7055–7065. https://doi.org/10.1007/s12649-020-01086-2.
Karna, R.R., Luxton, T., Bronstein, K.E., Hoponick Redmon, J., Scheckel, K.G., 2017. State of the science review: potential for beneficial use of waste by-products for in situ remediation of metal-contaminated soil and sediment. Crit. Rev. Environ. Sci. Technol. 47, 1–65. https://doi.org/10.1080/10643389.2016.1275417.
Kaur, H., Garg, N., 2021. Zinc toxicity in plants: a review. Planta 253, 129. https://doi.org/10.1007/s00425-021-03642-z.
Kaza, M., Mankiewicz-Boczek, J., Izydorczyk, K., Sawicki, J., 2007. Toxicity assessment of water samples from rivers in central Poland using a battery of microbiotests - a pilot study. Polish J. Environ. Stud. 16, 81–89.
Klymko, T., Dijkstra, J.J., van Zomeren, A., 2017. Guidance Document on Hazard Classification of MSWI Bottom Ash. ECN report, Petten, the Netherlands.
Kronzucker, H.J., Britto, D.T., 2011. Sodium transport in plants: a critical review. New Phytol. 189, 54–81. https://doi.org/10.1111/j.1469-8137.2010.03540.x.
Kronzucker, H.J., Coskun, D., Schulze, L.M., Wong, J.R., Britto, D.T., 2013. Sodium as nutrient and toxicant. Plant Soil 369, 1–23. https://doi.org/10.1007/s11104-013-1801-2.
Kumar, V., Pandita, S., Singh Sidhu, G.P., Sharma, A., Khanna, K., Kaur, P., Bali, A.S., Setia, R., 2021. Copper bioavailability, uptake, toxicity and tolerance in plants: a comprehensive review. Chemosphere 262, 127810. https://doi.org/10.1016/j.chemosphere.2020.127810.
Lapa, N., Barbosa, R., Morais, J., Mendes, B., Méhu, J., Santos Oliveira, J.F., 2002. Ecotoxicological assessment of leachates from MSWI bottom ashes. Waste Manag. 22, 583–593. https://doi.org/10.1016/S0956-053X(02)00009-0.
Li, H., Zeng, L., Wang, C., Shi, C., Li, Y., Peng, Y., Chen, H., 2022. Review of the toxicity and potential molecular mechanisms of parental or successive exposure to environmental pollutants in the model organism. Environ. Pollut. 311, 119927 https://doi.org/10.1016/j.envpol.2022.119927.
Lindberg, D., Molin, C., Hupa, M., 2015. Thermal treatment of solid residues from WtE units: a review. Waste Manag. 37, 82–94. https://doi.org/10.1016/j.wasman.2014.12.009.
Loginova, E., Volkov, D.S., van de Wouw, P.M.F., Florea, M.V.A., Brouwers, H.J.H., 2019. Detailed characterization of particle size fractions of municipal solid waste incineration bottom ash. J. Clean. Prod. 207, 866–874. https://doi.org/10.1016/j.jclepro.2018.10.022.
Luo, H., Cheng, Y., He, D., Yang, E.H., 2019. Review of leaching behavior of municipal solid waste incineration (MSWI) ash. Sci. Total Environ. 668, 90–103. https://doi.org/10.1016/j.scitotenv.2019.03.004.
Lynn, C.J., Ghataora, G.S., Dhir Obe, R.K., 2017. Municipal incinerated bottom ash (MIBA) characteristics and potential for use in road pavements. Int. J. Pavement Res. Technol. 10, 185–201. https://doi.org/10.1016/j.ijprt.2016.12.003.
Maathuis, F.J.M., 2014. Sodium in plants: Perception, signalling, and regulation of sodium fluxes. J. Exp. Bot. 65, 849–858. https://doi.org/10.1093/jxb/ert326.
Maldonado-Alameda, A., Giro-Paloma, J., Svobodova-Sedlackova, A., Formosa, J., Chimenos, J.M., 2020a. Municipal solid waste incineration bottom ash as alkali-activated cement precursor depending on particle size. J. Clean. Prod. 242, 118443 https://doi.org/10.1016/j.jclepro.2019.118443.
Maldonado-Alameda, À., Giro-Paloma, J., Alfocea-Roig, A., Formosa, J., Chimenos, J.M., 2020b. Municipal solid waste incineration bottom ash as sole precursor in the alkali-activated binder formulation. Appl. Sci. 10, 4129. https://doi.org/10.3390/APP10124129.
Manskinen, K., Nurmesniemi, H., Pöykiö, R., 2011. Total and extractable non-process elements in green liquor dregs from the chemical recovery circuit of a semi-chemical pulp mill. Chem. Eng. J. 166, 954–961. https://doi.org/10.1016/j.cej.2010.11.082.
Martinez-Finley, E.J., Avila, D.S., Chakraborty, S., Aschner, M., 2011. Insights from Caenorhabditis elegans on the role of metals in neurodegenerative diseases. Metallomics 3, 271–279. https://doi.org/10.1039/c0mt00064g.
Martins, A.C., Gubert, P., Li, J., Ke, T., Nicolai, M.M., Moura, A.V., Bornhorst, J., Bowman, A.B., Aschner, M., 2022. Caenorhabditis elegans as a model to study manganese-induced neurotoxicity. Biomolecules 12, 1396. https://doi.org/10.3390/biom12101396.
MicroBioTests Inc. THAMNOTOXKIT F — Crustacean toxicity screening test for freshwater. Stand. Oper. Proced. Belgium, 1999.
MicroBioTests Inc. Acute ROTOXKIT F - Rotifer toxicity screening test for freshwater. Stand. Oper. Proced. Belgium, 2000.
Monteiro, R.C.C., Figueiredo, C.F., Alendouro, M.S., Ferro, M.C., Davim, E.J.R., Fernandes, M.H.V., 2008. Characterization of MSWI bottom ashes towards utilization as glass raw material. Waste Manag. 28, 1119–1125. https://doi.org/10.1016/j.wasman.2007.05.004.
Moreira, I.N., Mourato, M.P., Reis, R., Martins, L.L., 2015. Oxidative stress induced by cadmium and copper in Brassica rapa Leaves: Indicators of stress, oxidative damage, and Antioxidant mechanisms. Commun. Soil Sci. Plant Anal. 46, 2475–2489. https://doi.org/10.1080/00103624.2015.1085554.
Moser, H., Römbke, J., 2009. Ecotoxicological Characterization of Waste - Results and Experiences of a European Ring Test. Springer Ltd., New York, USA, 9780387889580.
Natal-da-Luz, T., Amorim, M.J.B., Römbke, J., Paulo Sousa, J., 2008. Avoidance tests with earthworms and springtails: Defining the minimum exposure time to observe a significant response. Ecotoxicol. Environ. Saf. 71, 545–551. https://doi.org/10.1016/j.ecoenv.2007.09.005.
Neumann, C., Baesler, J., Steffen, G., Nicolai, M.M., Zubel, T., Aschner, M., Bürkle, A., Mangerich, A., Schwerdtle, T., Bornhorst, J., 2020. The role of poly(ADP-ribose) polymerases in manganese exposed Caenorhabditis elegans. J. Trace Elem. Med. Biol. 57, 21–27. https://doi.org/10.1016/j.jtemb.2019.09.001.
Neuwahl, F., Cusano, G., Benavides, J.G., Holbrook, S., Serge, R., 2019. *Best Available Techniques (BAT) Reference Document for Waste Incineration. Industrial Emissions Directive 2010/75/EU (Integrated Pollution Prevention)*; Science for Policy Report by the Joint Research Centre. European Commission, Luxembourg.
Nurmesniemi, H., Pöykiö, R., Periämäki, P., Kuokkanen, T., 2005. The use of a sequential leaching procedure for heavy metal fractionation in green liquor dregs from a causticizing process at a pulp mill. Chemosphere 61, 1475–1484. https://doi.org/10.1016/j.chemosphere.2005.04.114.
OECD Test No. 202: Daphnia Sp. Acute Immobilisation Test. OECD Guidelines for the Testing of Chemicals, Section 2, 2004. OECD editions, Paris.
OECD Test No. 221: Lemna Sp. Growth Inhibition Test. OECD Guidelines for the Testing of Chemicals, Section 2, 2006. OECD editions, Paris.
OECD Test No. 201: freshwater alga and cyanobacteria, growth inhibition test. OECD Guidelines for the Testing of Chemicals, Section 2, OECD editions, 2011. Paris.
Okamoto, A., Yamamuro, M., Tatarazako, N., 2014. Acute toxicity of 50 metals to Daphnia magna. J. Appl. Toxicol. 35, 824–830. https://doi.org/10.1002/jat.3078.
Pandard, P., 2016. Classification réglementaire des déchets - Guide d'application pour la caractérisation en dangerosité (Regulatory waste classification - application guide for hazardous characterization). In: Report Produced for the Ministry of Ecology. Sustainable Developmen.
Pandard, P., Römbke, J., 2013. Proposal for a "Harmonized" strategy for the assessment of the HP 14 property. Integr. Environ. Assess. Manag. 9, 665–672. https://doi.org/10.1002/ieam.1447.
Pandard, P., Devillers, J., Charissou, A.M., Poulsen, V., Jourdain, M.J., Férard, J.F., Grand, C., Bispo, A., 2006. Selecting a battery of bioassays for ecotoxicological characterization of wastes. Sci. Total Environ. 363, 114–125. https://doi.org/10.1016/j.scitotenv.2005.12.016.
Pardo, J.M., Quintero, F.J., 2002. Plants and sodium ions: Keeping company with the enemy. Genome Biol. 3, 1–4. https://doi.org/10.1186/gb-2002-3-6-reviews1017.
Pilon-Smits, E.A., Quinn, C.F., Tapken, W., Malagoli, M., Schiavon, M., 2009. Physiological functions of beneficial elements. Curr. Opin. Plant Biol. 12, 267–274. https://doi.org/10.1016/j.pbi.2009.04.009.
Postma, J.F., Valk, S. De, Dubbeldam, M., Maas, J.L., Tonkes, M., Schipper, C.A., Kater, B.J., 2002. Confounding factors in bioassays with freshwater and Marine organisms. Ecotoxicol. Environ. Saf. 53, 226–237. https://doi.org/10.1006/eesa.2002.2195.

Racette, B.A., Aschner, M., Guilarte, T.R., Dydak, U., Criswell, S.R., Zheng, W., 2012. Pathophysiology of manganese-associated neurotoxicity. Neurotoxicology 33, 881–886. https://doi.org/10.1016/j.neuro.2011.12.010.

Rambaldi, E., Esposito, L., Andreola, F., Barbieri, L., Lancellotti, I., Vassura, I., 2010. The recycling of MSWI bottom ash in silicate based ceramic. Ceram. Int. 36, 2469–2476. https://doi.org/10.1016/j.ceramint.2010.08.005.

Rendek, E., Ducom, G., Germain, P., 2007. Influence of waste input and combustion technology on MSWI bottom ash quality. Waste Manag. 27, 1403–1407. https://doi.org/10.1016/j.wasman.2007.03.016.

Römbke, J., 2018. Testing of 24 potentially hazardous wastes using 6 ecotoxicological tests. Detritus 4, 4–21. https://doi.org/10.31025/2611-4135/2018.13745.

Römbke, J., Moser, T., Moser, H., 2009. Ecotoxicological characterisation of 12 incineration ashes using 6 laboratory tests. Waste Manag. 29, 2475–2482. https://doi.org/10.1016/j.wasman.2009.03.032.

Ruszkiewicz, J.A., Pinkas, A., Miah, M.R., Weitz, R.L., Lawes, M.J.A., Akinyemi, A.J., Ijomone, O.M., Aschner, M.C., 2018. Elegans as a model in developmental neurotoxicology. Toxicol. Appl. Pharmacol. 354, 126–135. https://doi.org/10.1016/j.taap.2018.03.016.

Scarpa, A., 1974. Indicators of free magnesium in biological systems. Biochemistry 13, 2789–2794. https://doi.org/10.1021/bi00711a001.

Shimaoka, T., Zhang, R., Watanabe, K., 2007. Alterations of municipal solid waste incineration residues in a landfill. Waste Manag. 27, 1444–1451. https://doi.org/10.1016/j.wasman.2007.03.011.

Silva, R.V., de Brito, J., Lynn, C.J., Dhir, R.K., 2017. Use of municipal solid waste incineration bottom ashes in alkali-activated materials, ceramics and granular applications: a review. Waste Manag. 68, 207–220. https://doi.org/10.1016/j.wasman.2017.06.043.

Silva, R.V., de Brito, J., Lynn, C.J., Dhir, R.K., 2019. Environmental impacts of the use of bottom ashes from municipal solid waste incineration: a review. Resour. Conserv. Recycl. 140, 23–35. https://doi.org/10.1016/j.resconrec.2018.09.011.

Stein, J.R., 1973. Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, Cambridge.

Stiernström, S., Wik, O., Bendz, D., 2016. Evaluation of frameworks for ecotoxicological hazard classification of waste. Waste Manag. 58, 14–24. https://doi.org/10.1016/j.wasman.2016.08.030.

Tang, J., Steenari, B.M., 2016. Leaching optimization of municipal solid waste incineration ash for resource recovery: a case study of Cu, Zn, Pb and Cd. Waste Manag. 48, 315–322. https://doi.org/10.1016/j.wasman.2015.10.003.

Tang, P., Florea, M.V.A., Spiesz, P., Brouwers, H.J.H., 2015. Characteristics and application potential of municipal solid waste incineration (MSWI) bottom ashes from two waste-to-energy plants. Constr. Build. Mater. 83, 77–94. https://doi.org/10.1016/j.conbuildmat.2015.02.033.

Tsiridis, V., Petala, M., Samaras, P., Kungolos, A., Sakellaropoulos, G.P., 2012. Environmental hazard assessment of coal fly ashes using leaching and ecotoxicity tests. Ecotoxicol. Environ. Saf. 84, 212–220. https://doi.org/10.1016/j.ecoenv.2012.07.011.

Wahlström, M., Laine-Ylijok, J., Wik, O., Oberender, A., Hjelmar, O., 2016. Hazardous Waste Classification: Amendments to the European Waste Classification Regulation – what Do They Mean and what Are the Consequences? Report for the Nordic Council of Ministers., Copenhagen, Denmark.

Walter, H., Consolaro, F., Gramatica, P., Scholze, M., Altenburger, R., 2002. Mixture toxicity of priority pollutants at no observed effect concentrations (NOECs). Ecotoxicology 11, 299–310. https://doi.org/10.1023/A:1020592802989.

Wang, D., Shen, L., Wang, Y., 2007. The phenotypic and behavioral defects can be transferred from zinc-exposed nematodes to their progeny 24, 223–230. https://doi.org/10.1016/j.etap.2007.05.009.

Wei, Y., Shimaoka, T., Saffarzadeh, A., Takahashi, F., 2011a. Alteration of municipal solid waste incineration bottom ash focusing on the evolution of iron-rich constituents. Waste Manag. 31, 1992–2000. https://doi.org/10.1016/j.wasman.2011.04.021.

Wei, Y., Shimaoka, T., Saffarzadeh, A., Takahashi, F., 2011b. Mineralogical characterization of municipal solid waste incineration bottom ash with an emphasis on heavy metal-bearing phases. J. Hazard Mater. 187, 534–543. https://doi.org/10.1016/j.jhazmat.2011.01.070.

Wei, Y., Saffarzadeh, A., Shimaoka, T., Zhao, C., Peng, C., Gao, J., 2014. Geoenvironmental weathering/deterioration of landfilled MSWI-BA glass. J. Hazard Mater. 278, 610–619. https://doi.org/10.1016/j.jhazmat.2014.05.093.

Wilke, B.M., Riepert, F., Koch, C., Kühne, T., 2008. Ecotoxicological characterization of hazardous wastes. Ecotoxicol. Environ. Saf. 70, 283–293. https://doi.org/10.1016/j.ecoenv.2007.10.003.

WRc. *Assessment of Hazard Classification of UK IBA. Report for the January-June 2011 IBA Dataset. Report for Environmental Services Association. WRc reference: UC8540.06*, 2012.

Wu, X., Cobbina, S.J., Mao, G., Xu, H., Zhang, Z., Yang, L., 2016. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. Environ. Sci. Pollut. Res. 23, 8244–8259. https://doi.org/10.1007/s11356-016-6333-x.

Xia, Y., He, P., Shao, L., Zhang, H., 2017. Metal distribution characteristic of MSWI bottom ash in view of metal recovery. J. Environ. Sci. 52, 178–189. https://doi.org/10.1016/j.jes.2016.04.016.

Zhang, Z., Zhang, L., Li, A., 2015a. Development of a sintering process for recycling oil shale fly ash and municipal solid waste incineration bottom ash into glass ceramic composite. Waste Manag. 38, 185–193. https://doi.org/10.1016/j.wasman.2014.12.028.

Zhang, Z., Zhang, L., Li, A., 2015b. Development of a sintering process for recycling oil shale fly ash and municipal solid waste incineration bottom ash into glass ceramic composite. Waste Manag. 38, 185–193. https://doi.org/10.1016/j.wasman.2014.12.028.

Zhang, Y., Zhao, C., Zhang, H., Lu, Q., Zhou, J., Liu, R., Wang, S., Pu, Y., Yin, L., 2021. Trans-generational effects of copper on nerve damage in Caenorhabditis elegans. Chemosphere 284, 131324. https://doi.org/10.1016/j.chemosphere.2021.131324.