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4 # Chemical composition of wildfire ash produced in contrasting

5 # ecosystems and its toxicity on *Daphnia magna.*

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19

20 **Abstract**

21 It is well established in the world's fire-prone regions that wildfires can considerably change

22 the hydrological dynamics of freshwater catchments. Limited research, however, has focused

23 on the potential impacts of wildfire ash toxicity on aquatic biota. Here we assess the chemical

24 composition and toxicity of ash generated from wildfires in six contrasting vegetation types

25 distributed globally (UK grassland, Spanish pine forest, Spanish heathland, USA chaparral,

26 Australian eucalypt forest and Canadian spruce forest). Acute (48 h) immobilisation tests were

27 conducted on the aquatic macroinvertebrate *Daphnia magna,* a species extensively used in

28 ecological and toxicological studies as a sensitive indicator of aquatic contaminants. We found

29 significant differences between the chemical composition and toxicity of these ash types. The

30 UK and Spanish ash types had no detectable toxicity on *Daphnia magna* whereas, the

31 Australian eucalypt (48 h EC_{50}[*]) ([*]9.5 g L^{-1}), USA chaparral ([*]20 g L^{-1}) and Canadian spruce

32 ([*]26 g L^{-1}) ash all caused significant toxicity (immobilisation). The principle characteristics of

33 the latter ash types were their high pH, NO_3^-, Cl^- and conductivity levels. Elevated water-

34 soluble and total concentrations of metals (e.g. Mn, Fe, Zn, Pb, Cu and As) and total polycyclic

35 aromatic hydrocarbons (PAHs) were not linked to toxicity.

2

Introduction

Fires are a natural process in many habitat types worldwide (Bixby *et al.* 2015), but they can be a social and environmental concern, potentially impacting public health, safety, infrastructure, biodiversity, land-use, water and air pollution (Bladon *et al.* 2014; Brito *et al.* 2017). Fire activity is projected to increase in many locations and ecotypes as a result of climate and societal changes, making the full understanding of their impacts crucial (Scholze *et al.* 2006; Chen *et al.* 2018).

During wildland fires, combustion of fuels releases a wide range of organic and inorganic components into the atmosphere, but also concentrates some of them into wildfire ash left on the ground (Bodí *et al.* 2014). Fresh wildfire ash typically consists of mineral materials and charred organic components, is non-cohesive, has a low density, and is not attached to the soil, which facilitates its mobilisation and transportation by post-fire water and wind erosion (Bodí *et al.* 2014; Abraham *et al.* 2017). The release of soluble elements and particulate matter from eroded ash and underlying soil into aquatic systems following fires can cause increases in water turbidity, pH, organic matter, suspended sediment, conductivity and a depletion of dissolved oxygen, among other (Smith *et al.* 2011; Tsai *et al.* 2017). Ash is however, not usually examined as a distinct part of the post-fire sediment and few laboratory studies have characterised the composition of wildfire ash (Bodí *et al.* 2014).

The majority of the general studies into the effects of wildfire on water quality have focused on runoff amounts and nutrient levels and only more recently has increasing research attention been given to pyrolytic substances, chemical elements and biological reactivity (Shakesby and Doerr 2006; Campos *et al.* 2012; Silva *et al.* 2015). Key areas receiving particular attention as a result of their environmental concern are the production and mobilisation of polycyclic aromatic hydrocarbons (PAHs) and heavy metals (e.g. Vila-Escalé *et al.* 2007; Campos *et al.* 2012; Oliveira-Filho *et al.* 2018). Both present major biological concern due to their carcinogenic potential, persistence within ecosystems and tendency to bio-accumulate (Smith *et al.* 2011; Chen *et al.* 2018). These contaminants are thought to have complex impacts on water quality and the biological effects of this in aquatic systems have been observed to persist across long spatial and temporal scales (Earl and Blinn 2003; Costa *et al.* 2014).

Ash has also begun to receive increasing recognition as a source of diffuse contamination in freshwater systems with detrimental impacts on both lake and stream biota, including fish (e.g. Nunes *et al.* 2017; Oliveira-Filho *et al.* 2018; Gonino, Branco, *et al.* 2019), amphibians (Pilliod

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68 *et al.* 2003), macroinvertebrates (Brito *et al.* 2017) and algae (Campos *et al.* 2012) have all

69 been observed. Highly variable impacts of ash contamination on freshwater biota have been

70 reported between different ecosystems, types of ash, fires and species (Smith *et al.* 2011; Silva

71 *et al.* 2015; Oliveira-Filho *et al.* 2018). Campos *et al.* (2012) and Silva *et al.* (2015) for

72 example, found no significant impact of eucalypt ash on the planktonic crustacean *D. magna*

73 reproduction or immobilisation rates over chronic (21 day) and acute (48 h) exposures,

74 respectively. Toxicity was, however, observed on several lower trophic level species in these

75 studies, the bacteria *Vibrio fischeri*, algae *Pseudokirchneriella subcapitata* and the macrophyte

76 *Lemna minor*. A similar study by Brito et al. (2017) tested toxicity over acute exposures (48 h)

77 of three types of ash from the Brazilian *Cerrado* ecoregion on the planktonic crustacean

78 *Ceriodaphnia dubia*, the fish *Danio rerio* and the mollusc *Biomphalaria glabrata* and found

79 that all ash types caused toxicity to *C. dubia*, none impacted *B. glabrata* and only one type was

80 toxic for *D. rerio*. At higher trophic levels negative impacts of Brazilian sugar cane ash have

81 also been observed on several native fish species (*Astyanax lacustris*, *Moankhausia* and *M.*

82 *forestii*) over 24 h acute exposures but not for two non-native fish species (*Oreochromis*

83 *niloticus* and *Poecilia reticulate*) (Gonino *et al.* 2019). These studies demonstrate the

84 variability and complexity of influencing factors in relation to the impacts of ash contamination

85 on aquatic biota, highlighting the limited breadth of available research in this area (Hallema *et*

86 *al.* 2018).

87 To enhance our understanding of the impacts of ash contamination on aquatic biota, this study

88 aimed to (1) determine the chemical composition of wildfire ash produced in six contrasting

89 ecosystems, (2) examine the ecotoxicological effect of these ash types on the freshwater

90 indicator species *Daphnia magna* and (3) evaluate the relationship between chemical

91 composition and observed toxicity and its implications for the relative water contamination

92 potential of ash produced in these differing ecosystems. To the best of our knowledge this

93 constitutes the first ecotoxicology assessment allowing the direct comparison of the

94 composition and toxicity of ash from several globally distributed contrasting ecosystems.

95

96 **Materials and methods**

97 *Ash samples*

98 Six composite ash samples were collected after wildland fires, prior to any rainfall, in each of

99 the selected ecosystems types (Table 1): Australian eucalypt forest (AUS), USA chaparral

4

100   (USA), Canadian spruce forest (CAN), Spanish heathland (URIA), Spanish pine forest (SPA),

101   UK grassland (UK). Fire and vegetation characteristics are summarised in Table 1. Each

102   composite ash samples was sieved through a 1 mm mesh before chemical characterisation or

103   use in the bioassays.

104

Chemical characterisation

106   Chemical characterisation of the six ash types collected was undertaken to determine the total

107   and water-soluble concentrations of major (Ca, Cl$^-$, Mg, Na, Si, SO_4^{2-}, NO_3^-) and trace elements

108   and compounds (Al, B, Cu, F$^-$, Fe, Ni, NH_4^+, As, Cd, Hg, Pb and PO_4^{3-}), in addition to pH,

109   dissolved organic carbon (DOC) and electric conductivity. This characterisation was

110   undertaken using established methods (Plumlee *et al.* 2007; Santín *et al.* 2015, 2018; see

111   supplementary material for full details).

112   The concentrations of 35 PAHs were also determined according to Pérez-Fernández *et al.*

113   (2015) and Viñas *et al.* (2009) with a GC/MS Thermo mod DSQ II (Thermo Electron

114   Corporation, Austin, TX USA). The first step consisted of a 10 h Soxhlet extraction with a 1:3

115   acetone:hexane mixture. The extract was then treated overnight with activated copper for

116   elemental sulphur removal and then cleaned using column chromatography with deactivated

117   alumina (see supplementary material for full details).

118

Daphnia toxicity testing

120   Ecotoxicological assays consisting of acute ash exposures (48 h) were conducted using the

121   planktonic crustacean *Daphnia magna*. This species is extensively used in ecological and

122   toxicological studies as a sensitive indicator of the effects of contaminants on aquatic biota

123   (OECD 2004; USEPA 2016). *Daphnia* sp. are also particularly relevant to freshwater lentic

124   ecosystems (lakes, reservoirs and ponds) and ideal for investigating contamination potential in

125   downstream waterbodies (Robinson and Thorn 2009; Nikinmaa 2014).

126   A monoclonal starter culture of *D. magna* was obtained from a long-term (2 year) rearing

127   program. The new culture was reared and maintained according to recommended guidelines

128   (OECD 2004; USEPA 2016), under controlled temperature (20±2°C), light conditions (uniform

129   illumination of cool-white type, approx. 5000 lux; photoperiod 16^L:8^D) and fed every 2 days

5

130   with a distilled suspension of *Pseudokirchneriella subcapitata* at approximately 0.1-0.2 ml per

131   *Daphnid*/day.

132   To produce the test solutions, each ash sample was combined with a culture medium (synthetic

133   hardwater medium – ASTM 1996) at the ratio 1:10 (mass:volume) (e.g. 100 g of ash in 1 L of

134   medium). The samples were then homogenised in an orbital shaker for 4 h and stored at 4°C

135   (max 24 h) before using in the ecotoxicological assays.

136   The acute toxicity tests were conducted according to the OECD 202 (OECD 2004) guidelines,

137   with the exception of full pH adjustment. pH was not adjusted to control levels (pH 7.2 ± 0.2)

138   in the bioassays to reproduce as close to natural conditions as possible, given pH is one of the

139   most important factors affecting the toxicity and bioavailability of elements to freshwater

140   species (Franklin *et al.* 2000). OECD 202 guidelines acknowledge that tests should be carried

141   out without the adjustment of pH, where values are within pH 6-9 at the highest test

142   concentration (OECD 2004). It is crucially important that pH adjustment does not cause

143   significant changes to the test substances and due to the complex and varying compositions

144   and reactivity of wildfire ash, potential interactions are unclear. Little is known to date on

145   wildfire ash concentrations in water bodies, therefore, a wide range of ash concentrations was

146   tested, trying to represent the potential variability of different natural scenarios. Six different

147   concentrations of the ash-medium solutions were used during testing (3.12, 6.25, 12.5, 25, 50,

148   75 g L^{-1}), plus four controls per concentration.

149   Tests were initiated using new-borns of less than 24 h old, originating from the 3rd – 5th brood

150   of the culture. For each ash type 150 daphnids were used. This sample size was divided into

151   five individuals per test vessel for each concentration with four replicates and one control per

152   concentration. The test was conducted for 48 h and the immobilisation of neonates was

153   documented at 24 h and 48 h. Immobilisation of neonates is defined here as individuals not

154   able to swim within 15 s of gentle agitation of the test vessel. During this period the same

155   temperature (20±2°C) and photoperiod (photoperiod, 16^{L}:8^{D}) conditions as during rearing were

156   maintained. *D. magna* were not fed during the acute exposure (USEPA 2016).

157

158   *Statistical analysis*

159   The water-soluble (leachates) chemical composition results were subjected to principle

160   component analysis (PCA) (RStudio *version 5.4.1*) to identify constituents most strongly

6

161 correlated with the different ash types. This approach to assessing the characteristic

162 components in a given sample is widely used in environmental research when dealing with

163 complex datasets (Brito *et al*. 2017). The leachates data were chosen for this analysis, as

164 opposed to the total elements data, because this is likely the most bioavailable fraction and,

165 therefore, the most likely to have impacted the Daphnia over an acute exposure.

166 To identify thresholds in the *D. magna* toxicity results and in agreement with standard

167 procedures (Musset 2006) the data were subjected to single factor analysis of variance tests

168 (RStudio *version 5.4.1*). Where significant results were identified post-hoc Dunnetts analysis

169 was used to test if the response at each concentration was significantly different to the control

170 groups and therefore, identify critical thresholds (lethal concentrations) in the response

171 relationships. This enables the effect concentrations (EC_{10} = concentration at which 10% of

172 individuals are immobilised and EC_{50} = concentration at which 50% of individuals are

173 immobilised) for each ash to be interpolated, along with the lowest observed effect

174 concentration (LOEC) (Musset 2006). A significance level of 5% (0.05) was used in all

175 statistical tests.

176

Results

Ash chemistry

179 The total elemental composition of the six ash types overall contained a number of potential

180 contaminants, but in highly variable concentrations (Sup. Material Table 1). The most abundant

181 element in all samples was Ca (range = 11,800 – 177,000 mg kg^{-1}) with Al (range = 1320 –

182 22,600 mg kg^{-1}) and Fe (range = 979 – 30,600 mg kg^{-1}) both present in high concentrations

183 throughout. The elements found in the lowest total concentrations were: As (range = 0.46 –

184 9.67 mg kg^{-1}), Cd (range = 0.17 – 1.13 mg kg^{-1}) and Hg (range = 0 – 0.05 mg kg^{-1}) (Sup.

185 Material Table 1).

186 pH and electrical conductivity (EC), measured in the leachates, notably varied across ash

187 samples, with pH levels ranging from moderately alkali in the UK ash (7.9), to strongly alkali

188 in the USA ash (11.2). Equally, EC levels varied greatly from 233 µS cm^{-1} in the SPA ash to

189 3880 µS cm^{-1} in the AUS ash. High pH and EC values were both characteristic features of the

190 ash types producing immobilisation of *D. magna* tested (see *Acute toxicity test* section). pH

191 within the bioassays themselves however, were notably less variable (7.31-9.08, Tables 4 &

7

192   5), likely due to differences in dilution between leachates and the bioassay testing, and the

193   addition of the culture medium in the latter.

194   The water-soluble (leachate) composition of the ash types were also highly variable (Table 2)

195   with the most abundant components being SO_4^{2-} (range = 1203 – 10,180 mg kg^{-1}), Cl$^-$ (range =

196   228 – 1509 mg kg^{-1}) and Na (range = 17 – 3893 mg kg^{-1}). The minor metal and metalloids

197   elements were similarly the components found in the lowest concentration in the leachates; Cd

198   (range = 0 – 7 µg kg^{-1}), Ni (range = 60 – 844 µg kg^{-1}), Zn (range = 0 – 140 µg kg$^-$) and Hg

199   (range = 1 – 2 µg kg^{-1}) (Table 2).

200   Some soluble elements occurred in particularly high levels, highlighting the variation in

201   element content within the ash (Table 2). For example, in the UK sample, PO_4^{3-} (620 mg kg^{-1})

202   and metals such as Fe (4378 µg kg^{-1}) and Mn (9292 µg kg^{-1}) were notably high in comparison

203   to the other ash types. There were also notably high levels of, for example, Ca (5864 mg kg^{-1})

204   and SO_4^{2-} (32,289 mg kg^{-1}) in the CAN sample; B (85 mg kg^{-1}) and Na (3893 mg kg^{-1}) in the

205   AUS sample; and Cu (5158 µg kg^{-1}) and As (329 µg kg^{-1}) in the URIA sample (Table 2).

206   The water-soluble concentrations of each element were relatively low when compared to the

207   total dry concentration within each ash type (Table 2). On average, the proportions of water-

208   soluble Al, Pb, Mn, Fe, Zn were < 1% total dry weight; As, Si, Ca, P, Ni, Cu, Cd were < 5%

209   and Mg was < 10%. The levels of Na (2 – 77%) and Hg (5 – 57%) solubility were highly

210   variable and are clearly the most overall soluble of the components analysed.

211   PCA identified three primary components explaining 79% of the total leachates dataset

212   variance (PC1 = 41%; PC2 = 23%; PC3 = 15%) (Table 3). PC1 is most strongly positively

213   correlated with Mn, Fe, Zn, As, Pb and PO_4^{3-} levels and most strongly negatively correlated

214   with pH, EC, NO_3^-, Cl$^-$, Hg and SO_4^{2-} (Table 3 and Figure 1). A biplot of the standardised PC1

215   and PC2 values (Figure 1) shows which components best characterised each ash type and pH,

216   EC, NO_3^-, Cl$^-$, Hg and SO_4^{2-} were most closely correlated with the three ash types producing

217   significant immobilisation of *D. magna*. Whilst, Al, Cu, Ni, NH_4^+, As, Fe, Mn, PO_4^{3-}, Pb, Cd

218   were more closely correlated with the three non-toxic ash types (Figure 1).

219   Thirty five PAHs were analysed across the ash types including the sixteen United States

220   Environmental Protection Agency (EPA) priority PAHs, which provide the focus of the

221   following discussion (Table 4). The total concentration of these priority contaminants ranged

222   from 1155 – 14,078 ng g^{-1} ash, the highest total being found in the UK ash originating from an

223   upland grassland ecosystem in South Wales ($\sum$16 EPA PAHs: 12,336 ng g^{-1} ash) (Table 4).

8

224   Notably high PAHs concentration were also found in the CAN ($\sum$16 EPA PAHs: 7,486 ng g^{-1})

225   and the SPA ash ($\sum$16 EPA PAHs: 4,393 ng g^{-1} ash) (Table 4).

226   The proportion of the methylated and non-methylated PAHs was very similar in all the samples

227   with around three times more non-methylated PAHs in each ash type, except the USA ash

228   which contained over 15 times the amount of non-methylated PAHs (Table 4). There was also

229   a predominance of 2-ring PAHs in all the samples. Generally, the quantity of each ring type

230   decreases sequentially with the number of rings, 2 > 3 > 4 > 5 & 6 with the exception of the

231   USA sample, which had a relatively similar quantity of 3, 4, 5 & 6 ring PAHs. The predominant

232   2-ring PAHs in all samples was Naphthalene. Phenanthrene was the most common 3-ring

233   PAHs, except in the UK sample where it was Acenaphthylene. All three of these abundant

234   PAHs (naphthalene, phenanthrene and acenaphthylene) are classified as EPA priority

235   contaminants (Table 4).

236

Acute toxicity test

238   High levels of *D. magna* immobilisation were recorded at both 24 and 48 h exposure for three

239   of the six ash types tested: AUS, USA and CAN ($p < 0.001$ for all three ash types) (Figure 2;

240   Table 5 & 6). The response relationships identify the AUS ash as the most toxic, with a 100%

241   immobilisation of *D. magna* individuals at less than 25 g ash L^{-1} within the first 24 hours of

242   exposure (Table 5; Figure 2). The immobilisation effect of both the North American ash

243   samples (USA and CAN) were relatively similar, with 48 h EC$_{50}$ being achieved at 20 and 26

244   g ash L^{-1} respectively, despite the notably different source vegetation (Table 6; Figure 2). In

245   contrast, no significant immobilisation occurred in response to the remaining three ash types

246   (URIA, SPA and UK) (Tables 5 & 6). The UK ash did not produce any observable

247   immobilisation across any of the test concentrations after 48 h of exposure. The Spanish

248   samples (URIA, SPA) only produced small rates of immobilization at the highest

249   concentrations (Tables 5 & 6).

250

251   **Discussion**

252   *Overall ash chemical properties*

9

253   The total concentration of each element within the six ash types showed a wide variability (Sup.

254   Material Table 1). These variations might be explained by the accumulative capacity of the

255   different vegetation types, taking up different levels of elements from the soil and surrounding

256   environment (Peralta-Videa *et al.* 2009; Brito *et al.* 2017). Fire dynamics (e.g. burn

257   temperature) and soil properties are also important features in the composition of elements

258   within ash (Pitman 2006; Bodí *et al.* 2014; Chen *et al.* 2018). In general, oxides and hydroxides

259   of particularly Ca, Mg, Si and P tend to be abundant in wildfire ash (Pereira and Úbeda 2010;

260   Silva *et al.* 2015) as found in the ash tested here (Sup. Material Table 1).

261   Overall, water solubility of the studied elements in all ash types is low (<20% except for Na

262   and Hg). This agrees with previous findings (Khanna *et al.* 1994; Santín *et al.* 2015; Silva *et*

263   *al.* 2015; Brito *et al.* 2017). The most abundant compounds in all leachates were SO_4^{2-}, Cl^- and

264   Na^+ (Table 2), likely due to them forming very soluble salts (i.e. sulphates or chlorides). These

265   components are thus commonly found in high concentrations in the dissolved residuals of ash

266   (Freitas and Rocha 2011; Santín *et al.* 2015) (Table 2). In contrast, heavy metals such as Cd,

267   Ni and Zn showed the lowest concentration in the leachates due to being relatively insoluble in

268   alkaline (pH: 8-10) conditions, precipitating mainly as hydroxides (Brookins 1989; Weiner and

269   Group 2007). These results are similar to those found in other studies assessing post-fire runoff

270   and ash leachates in a range of ecosystem types (Jung *et al.* 2009; Pereira *et al.* 2011) and in

271   agreement with the general trend of alkali (Na, K)>alkaline (Ca, Mg)>>heavy metals (Pb, Cd

272   and Hg) found by Santín *et al.* (2015) in eucalypt forest ash.

273

Ash types and element solubility

275   Despite the overall similarities in ash solubility in the ash leachates, there are also substantial

276   variations among the ash types, making their chemical profiles notably different. Brito *et al.*

277   (2017), assessing Brazilian *Cerrado* ash types, also found there were little qualitative

278   differences in the overall composition of the different ash tested, but large variations in the

279   concentration of the chemical elements between sampling areas.

280   The PCA analysis carried out allowed detection of key differences in the composition of the

281   ash types studied here. The UK ash leachate has a distinctly soluble profile in comparison to

282   the others. PCA analysis shows a number of heavy metals (Mn, Fe, Zn and Pb) and PO_4^{3-} to

283   be characteristic elements of the UK ash leachate (Figure 1). This leachate shows high

284   concentrations of soluble Fe, Mn and PO_4^{3-} in comparison to the other ash types (Table 2). The

10

285   pH (7.9) of the UK leachate was 1 to 3 units lower than the extracts from the other samples

286   (Table 2). These less alkaline conditions favour the solubility of metals and P compared to the

287   other samples where the metals tend to precipitate as hydroxide for values above 8-9 and the

288   phosphate as hydroxyapatite for pH values > 8.5 (for example see: Diaz *et al.* 1994; Stumm

289   and Morgan 2013).

290   A characteristic component of the CAN sample (identified by PCA, Figure 1) was the high

291   levels of soluble Ca, despite the total concentration in dry ash being relatively similar to that

292   of the AUS, SPA and USA ash (Table 2). It is unclear why the solubility of Ca is notably higher

293   in the CAN ash in comparison to the other ash types (Jung *et al.* 2009; Brito *et al.* 2017), but

294   that may be responsible for the reduced PO_4^{3-} levels (1.2 mg kg^{-1}) in the CAN leachate as P has

295   a tendency to precipitate in the presence of Ca (Diaz *et al.* 1994). This P-Ca interaction may

296   influence algal and cyanobacterial growth (and thus, eutrophication) by regulating P levels in

297   freshwater systems (Bladon *et al.* 2008; Blake *et al.* 2009). In the broader context, Ca is not

298   normally considered hazardous, but can significantly influence the overall toxicity of ash

299   eluates (e.g. its strong relationship with SO_4^{2-} leaching) (Mount *et al.* 1997; Tian *et al.* 2018).

300   Stiernström *et al.* (2013) even propose that Ca might be one of the key elements responsible

301   for the ecotoxicity of ash eluates on the crustacean *Nitocra spinipes,* despite Ca not being

302   classified as individually ecotoxic. The high Ca concentration CAN ash tested here also

303   produced significant immobilisation of *D. magna* over the 48 h exposure.

304   For the AUS ash sample, the levels of soluble B and Na are higher than in the other ash types

305   (Figure 1, Table 2). These elements are often found in high concentrations in ash leachates

306   (Jung *et al.* 2009; Pereira *et al.* 2011), particularly B in other eucalyptus ash tested (Freitas and

307   Rocha 2011). High Na$^+$ levels in freshwater systems can present an issue for water purification

308   processes as they cannot be removed using conventional methods (Smith *et al.* 2011). Unlike

309   reported by Silva *et al.* (2015), where the principle potential toxic components of their eucalypt

310   ash were Mn and Zn, neither of these elements were found in the eucalypt (AUS) ash analysed

311   here. This further highlights the differences in ash composition comparing individual fire

312   events and ecosystem types (Bodí *et al.* 2014).

313   In the URIA ash, the most defining components were Cu, Al, Ni, NH$_4^+$ and As (Figure 1). This

314   ash contained comparatively high concentrations of, particularly, soluble Cu (5158 µg kg^{-1})

315   and the carcinogen As (329 µg kg^{-1}). Similar elevated soluble levels of Cu have, however, been

316   found in mixed eucalyptus ash (Cu = 5100 – 6200 µg kg^{-1}) by Santín *et al.* (2015). The reason

11

317   for the significantly higher solubility rate of Cu in this heathland ash (URIA = 12.9%, range

318   excluding URIA = 0.49 – 2.01%) is worth further consideration to identify areas or components

319   likely to increase the risk of Cu contamination. The concentration of As, although elevated in

320   the URIA (and UK) sample here, have been reported in higher quantities in a number of other

321   wildfire ash samples (e.g. 4000 - 7300 μg kg^{-1} in Santin *et al.* (2015); 42000 μg kg^{-1} in Silva *et*

322   *al.* (2015)) and despite being above the 0.01 mg L^{-1} World Health Organisations drinking-water

323   guideline (World Health Organization 2011) it does not appear to cause significant

324   immobilisation of *D. magna* in the URIA or UK ash.

325   The SPA ash has a relatively insoluble overall profile with notably high concentrations of the

326   metals Al, Fe, Zn, Cb, Pb and the metalloid As in the dry ash (Sup. Material 1) but limited, to

327   no, leaching of Al, Fe, Zn and Pb into the water-soluble composition (Table 2). Despite this,

328   Cd presented as a distinct principle component of the SPA ash with a comparatively high

329   soluble concentration (7 μg kg^{-1}) and as the only sample to register a solubility percentage of

330   greater than 1% (2.85%). Similar dry quantities of Cd were recorded by Brito *et al.* (2017)

331   assessing Brazilian *Cerrado* ash types (0.1-0.3 mg kg^{-1}) but Cd solubility was lower in these

332   ash types (<0.01%).

333

334   *PAHs composition*

335   The organic fraction of ash may also contain organic contaminants of biological concern (Vila-

336   Escalé *et al.* 2007; Chen *et al.* 2018). The data available on PAHs release following fire,

337   however, is relatively limited (Vila-Escalé *et al.* 2007; Kim *et al.* 2011; Campos *et al.* 2012;

338   Rey-Salgueiro *et al.* 2018).

339   The concentrations of PAHs found in the ash analysed here are also widely variable, with a

340   range of 1155 ng g^{-1} in the AUS ash to 14,078 ng g^{-1} in the UK ash (16 U.S. Protection Agency

341   (EPA) priority PAHs) (Table 4). The values contained within the ash tested here are

342   substantially higher than those presented by Olivella *et al.* (2006) testing wildfire ash from pine

343   and oak forests ($\sum$12 PAHs: 1- 19 ng g^{-1} ash). The lowest concentration, found in the AUS ash

344   type ($\sum$16 EPA PAHs: 1155 ng g^{-1} ash), was of a comparable level to those found by Silva *et*

345   *al.* (2015), assessing dry wildfire ash in a predominantly eucalypt ecosystem in Portugal ($\sum$16

346   EPA PAHs: 1100 ng g^{-1} ash). The full range of PAH concentrations found here are within the

347   range of 1000-50,000 ng n^{-1} ($\sum$16 EPA PAHs) found by Santín *et al.* (2017) analysing PAHs

348   in pine forest floor and wood under wildfire charring and slow-pyrolysis.

349   The UK ash shows a much higher PAHs concentration than the other types (Table 4). It is

350   unclear why this is the case as no other research has been conducted on the PAHs composition

351   of wildfire ash originating from comparable grassland ecosystems. The type of fuel and

352   variations in combustion temperatures and oxygen availability are thought to strongly affect

353   the concentration and type of PAHs in ash (Enell *et al.* 2008; Rey-Salgueiro *et al.* 2018). Chen

354   *et al.* (2018) found that PAHs concentrations were significantly higher in black wildfire ash

355   (moderate burn severity) in comparison to white wildfire ash (severe burn severity). This was

356   also true of the ash types tested here with the darker (dark grey-black) ash samples (UK, URIA,

357   CAN) containing a much higher concentration of PAHs than the lighter (light grey-white)

358   samples (AUS, USA, SPA) (Table 4). Although, variations in combustion completeness could

359   be related to PAHs content here, the proportion of methylated PAHs in the UK ash is similar

360   to that of the other samples tested (Table 4). The proportion of methylated/total PAHs is usually

361   considered an indicator of combustion completeness as during combustion the methylated

362   component of the compound is lost first (Keiluweit *et al.* 2012) (Table 4).

363   The high presence of low molecular weight and therefore, greater volatility PAHs (i.e. Nap and

364   Phe) in the ash tested here may seem contradictory as it can be expected that these compounds

365   would be lost during a fire. It is, however, likely that these PAHs preferentially re-condense in

366   the ash layer and are retained in microporous structures of pyrogenic material (Santín *et al.*

367   2017). Other studies support this idea, reporting high concentrations of Naph and Phe (Kim *et*

368   *al.* 2011) or Naph, Chry, BaA and Acy (Campos *et al.* 2012) from wood burning. Ash studies

369   of beech and similar species (Bundt *et al.* 2001) were dominated by Nap and, to a slight extent,

370   by BghiPer, BbF, BkF, Chry, Triph and Phe.

371   Caution is required when making comparisons between the PAHs values across studies as there

372   are important variations in the methodologies employed. Some studies examine PAHs in ash

373   (Enell *et al.* 2008; Silva *et al.* 2015) or sediment (Olivella *et al.* 2006; Kim *et al.* 2011) and

374   others in stream water (Olivella *et al.* 2006), pond water (Vila-Escalé *et al.* 2007), runoff water

375   (Campos *et al.* 2012) or aqueous extracts (Enell *et al.* 2008; Silva *et al.* 2015) meaning

376   concentration and compositional differences are to be expected. It is likely the high to very

377   high PAHs concentrations recorded in the ash studied here would be dramatically reduced if

378   the leachable fraction of the samples was tested, as opposed to total concentrations, therefore,

379   making the portion more accessible to interact with aquatic fauna lower (Frišták *et al.* 2019).

380

381   *Implications for toxicology*

382   The wildfire ash analysis conducted here not only demonstrates the high variability in the

383   concentration of chemical components of ash produced in contrasting ecosystems (Table 2),

384   but also the differences in its potential toxic effects in aquatic systems (Table 5 & 6).

385   Significant toxicity was observed on *D. magna* over the acute exposures for three of the six ash

386   types tested: AUS, USA and CAN (Figure 2; Table 5 & 6). Ash type and composition,

387   therefore, seems crucial to the level of toxicity on cladoceran species, as also demonstrated

388   previously (Campos *et al.* 2012; Silva *et al.* 2015; Brito *et al.* 2017).

389   The combination of the chemical data with the *D. magna* immobilisation results highlights a

390   number of possible relationships (Figure 1). The PCA identified pH and EC as two of the

391   parameters strongly characteristic of the three ash types causing significant *D. magna*

392   immobilisation (AUS, USA, CAN) (Table 3, Figure 1). It is well established that extreme

393   values of pH and EC have a detrimental impact on zooplankton species (Mount *et al.* 1997;

394   Franklin *et al.* 2000; Silva *et al.* 2015). The pH values in the bioassays themselves, however,

395   were notably lower and less variable than in the leachate results used during the PCA analysis

396   and within a range thought acceptable for the survival of *D. magna* and similar cladoceran

397   species (OECD 2004) (Table 5 & 6). Crucially however, the relationship between pH and

398   immobilisation is very similar between the leachates and bioassays pH results with higher pH

399   values, characteristic of the ash types, producing immobilisation in *D. magna*. This perhaps

400   suggests that pH has an indirect effect on *D. magna* immobilisation as pH can also influence

401   the dissolution of elements from ash into water and therefore the relative toxic potential of

402   other ash components (Fedje *et al.* 2010). Low pH values, for example, encourage the leaching

403   of oxyanion-forming (As, B, Cr, Sb and V) and cation-forming elements (Ca), and neutral pH

404   greatly reduces the leaching of amphoteric elements (e.g. Al, Cd, Cu, Pb and Zn) (Fedje *et al.*

405   2010). The more neutral pH of the UK sample, however, does not seem to have reduced the

406   leaching of Al, Cd, Cu and Pb. pH has an inconsistent relationship with toxicity, and, often,

407   results are difficult to interpret (Wilde *et al.* 2006; Silva *et al.* 2015).

408   The influence of key nutrients on *D. magna* immobilisation is perhaps less well established

409   (Smith *et al.* 2011) (Figure 1), as ions such as, Cl^- and NO_3- are required at minimum levels to

410   support aquatic life. However, the PCA also identified high concentrations of Cl^- and NO_3- as

411   being key characteristic components of the three toxic ash types, particularly the more toxic

412   AUS and USA ash (Table 3 and Figure 1). Many anthropogenic (e.g. oil/gas production,

14

413   irrigation methods and industrial/agricultural processes) and natural (e.g. sediment pore waters

414   and burning) circumstances have been shown to increase nutrient concentrations to toxic levels

415   (e.g. Hoke *et al.* 1993; Ferreira *et al.* 2005; Mast and Clow 2008). Scott and Crunkilton (2005)

416   demonstrated NO_3^- produces immobilisation of *D. magna* at 462 mg L^{-1} with no observable

417   effect concentration at 358 mg L^{-1}. Similarly, Mount *et al.* (1997) estimated a concentration of

418   1000 – 2000 mg L^{-1} as the concentration of Cl^- required to produce EC_{50} in *Ceriodaphnia*

419   *dubia*. This suggests despite the correlations between NO_3^- and Cl^- with the toxic ash types

420   found here, the relatively low quantities of these components alone are not likely capable of

421   causing the observed toxicity (Table 2). The limited number of studies focusing on Na^+, Cl^-,

422   SO^- and NO^- exports after fire have found maximum levels sampled in ash fall well below

423   recommended limits (Smith *et al.* 2011).

424   The relatively high PAHs concentrations found in the ash tested here appear to produce no

425   observable toxicity on *D. magna* and furthermore, higher PAHs concentration seem to be

426   associated with reduced toxicity. It has to be noted that PAHs concentrations were only

427   determined in bulk ash samples. PAHs have limited solubility in water, particularly of the

428   larger ring size PAHs (> 3 rings) (Chen *et al.* 2018). The lack of relationship between high

429   levels of PAHs and toxicity found here and in other studies (Campos *et al.* 2012; Silva *et al.*

430   2015) raise questions about the bioavailability of PAHs in this context. In an assessment of the

431   methylated PAHs composition of sludge-derived pyrogenic material, Frišták *et al.* (2019)

432   found during pyrolysis methylated aromates mainly bind to insoluble carbon fractions or get

433   trapped in microporous structures of pyrogenic material and, therefore, are unlikely to be

434   bioavailable and hazardous to freshwater systems. This may be one reason why the PAHs

435   concentrations are not associated with toxicity in *D. magna* here. The potential more subtle and

436   longer term impacts of PAHs on aquatic biota such as, reductions in the rate of growth,

437   metabolic activity, reproduction or increased mutation and cancer risk (Hellou *et al.* 2006;

438   Campos *et al.* 2012) were beyond the scope of this study. Potential synergistic, antagonistic

439   and additive effects of the complex and variable PAHs composition of the ash types tested

440   could also not be ruled out as a source of toxicity. Further research should be conducted

441   investigating if these levels of total PAHs pose a greater water contamination risk from a wider

442   ecological or drinking water perspective.

443   Despite the variations in ash composition and the subsequent significant differences in *D.*

444   *magna* immobilisation, it is difficult to isolate the primary causes of toxicity. In addition to the

445   most likely, if indirect, influential parameters, pH and electrical conductivity, there are also

446   likely components that are not necessarily toxic by themselves, but could be variables
447   influencing toxicity in certain concentrations (e.g. DOC, Na, Ca and Mg) (Freitas and Rocha
448   2011; Simplício *et al.* 2016). Physical characteristics of the ash types may also be a possible
449   cause of immobilisation as variations in particle size and distribution of the suspended
450   particulate matter in the unfiltered samples used could have compromised the food intake and
451   locomotive ability of *D. magna* leading to immobilisation or death (Brito *et al.* 2017). Even
452   when using a standardised laboratory approach, as employed here, it remains difficult to
453   untangle the effects of such components from those caused by other variables in such complex
454   samples (Wilde *et al.* 2006; Silva *et al.* 2015; Brito *et al.* 2017).

455   Earlier work has suggested macroinvertebrate species such as *D. magna* are less sensitive to
456   contamination than lower trophic species (Campos *et al.* 2012) and thus, the effects of ash
457   contamination on these higher trophic organisms are expected to be primarily indirect through
458   the propagation of toxicity across the food chain via bottom-up, bioaccumulation processes
459   (Abrantes *et al.* 2008). A few notable studies have demonstrated this premise with no
460   observable effect of ash toxicity on daphnid survival or reproduction rates over both acute (48
461   h) and chronic (21 day) exposures, but significant impacts have been observed on lower trophic
462   species (bacteria, algae and macrophytes) (Campos *et al.* 2012; Silva *et al.* 2015).
463   Understanding the mechanisms influencing the bioaccumulation/availability of ash
464   contaminants in freshwater systems should thus be a focus of future research. The results
465   presented here, along with other studies, appear to justify the concerns around the impacts of
466   wildfire ash on aquatic biota and water quality even without the assessment of bioaccumulation
467   processes (Campos *et al.* 2012; Brito *et al.* 2017).

468

469   **Conclusion**

470   The chemical characterisation of the six wildfire ash types shows an overall similar
471   composition of elements, but significant variations in the concentration, reactivity and
472   solubility of these elements. Solubility of all elements was low for all ash types comparing the
473   total and leachate characterisation data.

474   Our results demonstrate significant immobilisation of *D. magna* over acute exposure (48 h) to
475   three of the six ash types (AUS, USA and CAN). The principle characteristics of these ash
476   types producing immobilisation, derived from PCA, were high values of pH, EC, NO_3^-, Cl^-, Hg
477   and SO_4^{2-}. None of these components, however, appear likely to have directly caused the

478   immobilisation response (ecotoxicity) observed. It is perhaps more likely that these

479   components, and possible others (e.g. Ca), have contributed indirectly to the observed toxicity.

480   Elevated water-soluble concentrations of metal and metalloid contaminants (Mn, Fe, Zn, Pb,

481   Cu and As) did not produce any significant inhibition and tended to be characteristic of the

482   non-toxic ash types. The total PAHs concentrations were also not linked to significant

483   inhibition. It continues to prove difficult to identify specific causes of toxicity in aquatic biota

484   using test substances as complex and variable as wildfire ash.

485   Combining the detailed chemical characterisation of the ash types with the ecotoxicology

486   results helps to provide further insight into the composition and variations in ash produced in

487   contracting ecosystems and potential implications of wildfire ash contamination on the

488   environment. A detailed understanding of the interactions and impacts of metals, nutrients and

489   PAHs in different ecosystem types is essential for evaluating the pollution risk of fires and for

490   informing management. The results presented here justify the concerns around the down-

491   stream contamination potential of ash in certain ecosystems on aquatic biota and highlight the

492   need for a greater understanding of possible direct/indirect chemical causalities. Further

493   research is therefore, required in order to (i) identify and predict conditions creating certain

494   chemical signatures in ash and (ii) to investigate the specific direct (or indirect) causality of

495   toxicity in key groups of aquatic species.

505

506   **Conflicts of interest**

507   The authors declare no conflicts of interest.

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717 **Table 1. Fire and vegetation characteristics of the six ash types used in this study.**

Sample name	Location	Vegetation cover	Burn description
Australian eucalypt (**AUS**)	West of Sydney (33°52'14" S; 150°36'01" E)	Open, dry sclerophyll forest with a dense shrubby undergrowth. Key species; ironbark (*Eucalyptus. fibrosa*), stringybarks (*Eucalyptus eugenioides, Eucalyptus oblonga*), *Banksia* sp., *Leptospernum* sp., *Acacia* sp. and *Petrophile* sp..	Moderate to high severity prescribed fire in 2014. Fire did not affect tree canopy but complete combustion of understory fuels and mostly fine ash, light in colour was generated (Santín *et al.* 2018).
USA chaparral (**USA**)	South western California (34°25'14" N; 119°30'39" W)	Mixed chaparral with the dominant species including coast live oak (*Quercus agrifolia*), Toyon (*Heteromeles arbutifolia*), coyote brush (*Baccharis pilularis*), holly-leaf cherry (*Prunus ilicifolia*)	Large-scale, extreme high-severity wildfire (Thomas fire) in 2018, achieving almost complete combustion of above surface fuel.
Canadian spruce (**CAN**)	Northwest Territories (61°34'55" N; 117°11'55" W)	Very dense tree canopy comprised of mostly black spruce (*Picea mariana*) and jack pines (*Pinus banksiana*) with very little understory vegetation with the exception of young spruce and moss species.	Very high intensity experimental crown fire in 2015, all fine fuels aboveground were consumed. The forest floor was only slight affected (<1cm depth of burn).
Spanish heathland (**URIA**)	North western Spain (43°6'17'' N 6°50'52'' W)	Dominant species included, heather (*Calluna vulgaris*), western gorse (*Ulex gallii*) and a variety of Erica sp. (e.g. *Erica tetralix*).	Extreme hot and dry conditions producing a severe experimental wildfire in 2017. Combustion completeness very high (most fine fuel consumed).
Spanish pine forest (**SPA**)	Eastern Spain (40°18'36" N; 1°01'59" W)	Forested area dominated by Aleppo pine (*Pinus halepensis*).	High-severity wildfire producing a very high level of combustion completeness. All surface fuel consumed.
UK grassland (**UK**)	South Wales (51°50'11" N; 3°25'44" W) and (51°41'02" N; 3°38'37" W)	Upland graminoid dominant areas. Dominant vegetation in these species-poor areas consisted of purple moor-grass (*Molinia caerulea*), soft rush (*Juncus effuses*), mat-grass (*Nardus stricta*) and heath plait-moss (*Hypnum jutlandicum*).	Moderate severity wildfires in 2018. Consumed most above surface fuel and litter, but did not penetrate soil surface. Composite from two fires created in weight ratio 2:1 (g) of ash from site one and site two, respectively.

718

26

719 **Table 2**. **Water-soluble chemical composition of the six ash types obtained by leaching**

720 **tests.**

721 Solubility of elements provided in brackets as a percentage (%) of the total ash composition.

722 Electrical conductivity (E.C.) given in µS cm^{-1}. N.D (not detected) represents components

723 with quantities <0.01 mg kg^{-1}. The symbol (-) is used to denote values not able to be

724 calculated due to the dry weight of the component not being tested for or the value being 0.

		Ash types										
		AUS		USA		CAN		URIA		SPA		UK	
	pH	11.1	-	11.2	-	10.3	-	10.3	-	9.1	-	7.9	-
	E.C	3880	-	2570	-	2500	-	1505	-	233	-	293	-
	Al	0	-	4	(0.02)	0	-	20	(0.2)	0	-	0	-
	Si	45	(2.17)	182	(8.81)	27	(1.54)	133	(5.59)	25	(1.13)	27	(1.86)
	Ca	55	(0.03)	136	(0.06)	5864	(3.60)	580	(1.97)	1101	(0.83)	114	(0.97)
	PO_4^{3-}	10	(0.65)	10	(0.06)	1	(0.01)	27	(0.35)	10	(0.17)	620	(7.50)
mg kg^{-1}	NH_4^+	8	-	9	-	0	-	33	-	4	-	20	-
	DOC	496	-	130	-	1331	-	1272	-	93	-	198	-
	Cl^-	1509	-	1494	-	1139	-	955	-	230	-	228	-
	NO_3^-	207	-	232	-	206	-	104	-	24	-	26	-
	SO_4^{2-}	4065	-	10180	-	32289	-	5600	-	3370	-	1203	-
	B	85	-	17	-	6	-	12	-	4	-	1	-
	Na	3893	(77.2)	831	(18.1)	860	(27.6)	1766	(49.6)	17	(1.49)	148	(22.3)
	Mg	377	(3.81)	26	(0.12)	3067	(25.6)	328	(5.13)	232	(4.23)	172	(6.36)
	F^-	340	-	3260	-	460	-	5080	-	9300	-	800	-
	Mn	0	-	0	-	68	(0.01)	656	(0.07)	136	(0.04)	9292	(0.65)
	Fe	205	-	643	-	553	(0.06)	2172	(0.03)	406	-	4378	(0.06)
	Ni	0	-	0	-	0	-	844	(3.84)	0	-	59	(0.37)
µg kg^{-1}	Cu	423	(2.01)	280	(0.54)	198	(0.68)	5158	(12.9)	147	(0.49)	340	(0.68)
	Zn	0	-	0	-	0	-	0	-	0	-	140	(0.08)
	As	18	(1.17)	26	(1.12)	6	(1.21)	329	(7.40)	102	(1.05)	259	(5.96)
	Cd	1	(0.42)	0	-	1	(0.59)	0	-	7	(2.83)	2	(0.18)
	Hg	1	(44.9)	2	(57.4)	2	(10)	1	(5.96)	1	(17.9)	1	(4.91)
	Pb	16	(0.05)	7	(0.02)	3	(0.01)	5	(0.01)	7	(0.01)	64	(0.06)

725

27

726 **Table 3**. **Relative contribution of the 24 water-soluble ash constituents and parameters to**

727 **four of the significant principle components of the six ash types derived from principle**

728 **components analysis.**

729 Cumulative proportion (%) of the variance explained by each principle component also

730 provided.

731

	PC1	PC2
pH	-0.29	-0.11
E.C	-0.26	-0.02
Al	0.00	-0.40
Si	-0.10	-0.26
Ca	-0.13	0.18
PO_4^{3-}	0.27	0.08
NH_4^+	0.15	-0.36
DOC	-0.12	-0.16
Cl^-	-0.27	-0.08
NO_3-	-0.27	0.01
SO_4^{2-}	-0.18	0.15
B	-0.14	-0.03
Na	-0.16	-0.14
Mg	-0.14	0.17
F^-	0.05	-0.10
Mn	0.28	0.07
Fe	0.27	-0.07
Ni	0.04	-0.39
Cu	0.02	-0.40
Zn	0.27	0.10
As	0.23	-0.27
Cd	0.11	0.18
Hg	-0.19	0.14
Pb	0.26	0.10
Cumulative prop. (%)	0.41	0.64

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738 **Table 4. Concentration and composition of PAHs found in each ash type (ng g⁻¹).**

739 PAHs displayed followed with the notation * are U.S. Environmental Protection Agency

740 priority PAHs (Keith 2015).

PAH (ng/g)	Ash type					
	AUS	USA	CAN	URIA	SPA	UK
Naphthalene*	744.9	1148.6	4540.3	2861.4	1147.4	8010.9
Biphenyl	293.5	654.3	1851.1	1953.3	1019.1	1677.6
Acenaphthylene*	75.2	9.7	377.3	323.7	28.1	3337.2
Acenaphthene*	13.2	1.9	84.5	44.3	9.6	198.2
Fluorene*	18.1	3.4	99.7	104.0	26.7	380.4
Dibenzothiophene	4.7	3.5	45.6	11.9	7.7	29.2
Phenanthrene*	140.5	121.2	1049.5	487.4	170.5	1131.8
Anthracene*	19.1	10.4	126.3	76.1	15.6	193.2
Fluoranthene*	36.1	27.5	285.6	128.1	26.7	262.2
Pyrene*	37.3	16.9	215.8	112.9	20.7	257.8
Benzo(c)phenanthrene	2.9	3.0	12.7	9.7	2.9	12.7
Benz(a)anthracene*	11.9	8.4	32.3	23.9	4.4	35.3
Triphenylene	7.7	53.7	44.6	16.0	7.1	14.7
Chrysene*	14.3	26.5	38.9	27.4	7.3	36.3
Benzo(b)fluoranthene*	16.3	29.2	335.0	83.1	7.9	95.4
Benzo(k)fluoranthene*	4.2	8.9	92.8	25.0	3.0	32.3
Benzo(e)pyrene	11.3	44.1	291.8	90.9	8.8	57.3
Benzo(a)pyrene*	7.2	6.1	74.9	31.7	2.4	34.2
Perylene	3.4	1.3	20.1	14.8	1.9	13.2
Indeno (1,2,3-c,d) pyrene*	5.2	5.5	30.2	15.9	1.5	27.0
Dibenzo(a,h)anthracene*	1.9	2.6	10.5	14.8	0.9	5.2
Benzo (g,h,i) perylen*	9.6	18.2	92.5	33.0	2.8	40.7
2-Methylnaphtalene	225.7	66.9	1702.3	751.4	274.3	2118.2
1-Methylnaphthalene	168.2	34.3	1204.2	683.7	318.7	1663.5
2,3-Dimethylnaphthalene	43.2	7.1	388.0	138.0	166.8	235.0
2,3,6-Trimethylnaphtalene	19.4	3.4	140.5	50.0	20.9	80.0
4-Methyldibenzothiophene	7.9	1.0	32.5	49.5	32.8	35.0
2-Methylphenanthrene	21.2	6.3	154.3	87.7	44.7	153.6

29

2,8-Dimethyldibenzothiophene	3.0	0.7	14.1	12.0	5.2	29.4
1,6-Dimethylphenanthrene	31.9	5.5	147.2	109.0	51.5	120.0
2,4,7-Trimethyldibenzothiophene	0.3	0.1	0.8	1.0	0.5	3.3
1,2,8-Trimethylphenanthrene	8.4	2.1	67.2	47.8	43.4	35.5
1-Methylpyrene	9.8	1.4	24.5	31.3	11.3	32.9
2-Methylchrysene	3.8	1.7	4.5	5.9	1.9	6.4
7,12-DimethylB(a)A	2.9	0.5	1728.4	64.2	90.9	12.5
Σ16 PAHS	1155	1445	7486	4393	1476	14078
Σ35 PAHS	2024	2336	15360	8521	3586	20408
Σ Methylated	546	131	5608	2031	1063	4525
Σ Non-methylated	1479	2205	9752	6489	2523	15883
% Methylated	27	6	37	24	30	22

741

Table 5. Immobilisation percentage of *Daphnia magna* at 24 h.

Estimates of Lowest Observed Effect Concentration (LOEC) (Dunnetts test; $p < 0.05$), EC_{10}*
and EC_{50} (g L^{-1}). One-way analysis of variance p-values also provided, testing if observed
immobilisation of each ash type was significantly different to the control. *EC_x = the
concentration of substance required to produce x% (10 or 50) of the test individuals to
become immobilised. The symbol (-) is used to denote values not able to be calculated.

| | Concentration (g L^{-1}) | | | | | | | pH | LOEC (g L^{-1}) | EC$_{10}$ (g L^{-1}) | EC$_{50}$ (g L^{-1}) | p value |
	Control	3.12	6.25	12.5	25	50	75					
AUS	0	5	10	75	100	100	100	8.81	6.25	6.25	11	<0.001
USA	0	0	0	5	35	100	100	8.78	25	14	30	<0.001
CAN	0	0	0	5	10	100	100	8.23	50	25	37	<0.001
URIA	0	0	0	0	0	0	5	8.17	-	-	-	0.451
SPA	0	0	0	0	0	5	0	7.88	-	-	-	0.451
UK	0	0	0	0	0	0	0	7.56	-	-	-	-

Table 6. **Immobilisation percentage of *Daphnia magna* at 48 h.**

Estimates of Lowest Observed Effect Concentration (LOEC) (Dunnetts test; $p < 0.05$), EC_{10}*
and EC_{50} (g L^{-1}). One-way analysis of variance p-values also provided, testing if observed
immobilisation of each ash type was significantly different to the control. *EC_x = the
concentration of substance required to produce x% (10 or 50) of the test individuals to
become immobilised. The symbol (-) is used to denote values not able to be calculated.

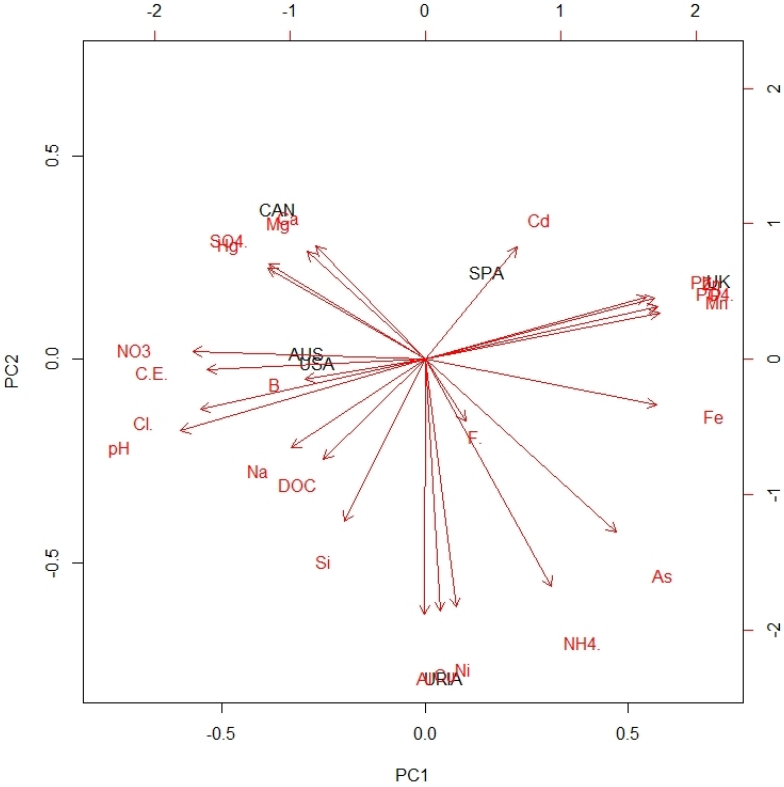| | Concentration (g L^{-1}) | | | | | | | pH | LOEC (g L^{-1}) | EC$_{10}$ (g L^{-1}) | EC$_{50}$ (g L^{-1}) | p value |
	Control	3.12	6.25	12.5	25	50	75					
AUS	0	10	15	85	100	100	100	8.93	6.25	5.5	9.5	<0.001
USA	0	0	5	5	65	100	100	9.08	6.25	14	20	<0.001
CAN	0	0	0	5	40	100	100	7.81	25	14	26	<0.001
URIA	0	0	0	0	0	0	10	8.03	-	-	-	0.451
SPA	0	0	0	0	0	5	0	7.58	-	-	-	0.451
UK	0	0	0	0	0	0	0	7.31	-	-	-	-

31

Fig.1: Representation of the ordination in the first two axes (PC1 and PC2) produced by principle component analysis (PCA) of the water-soluble chemical composition of the six ash types studies.
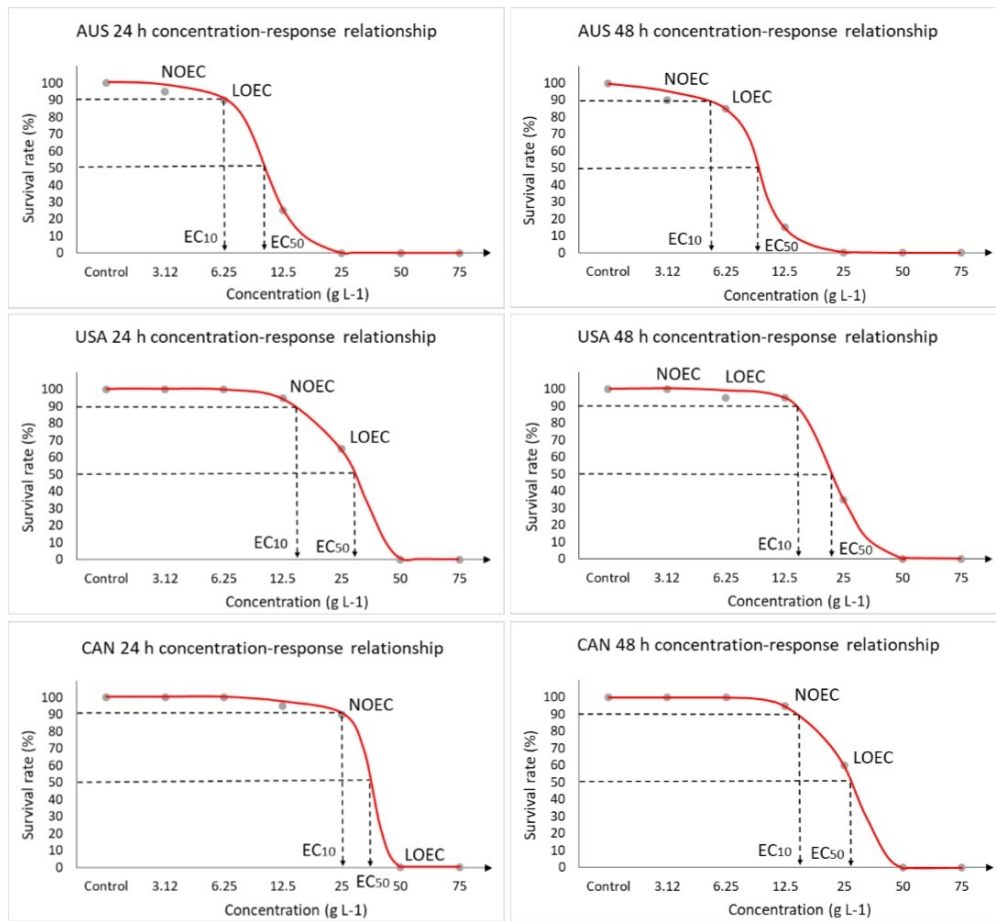
248x210mm (96 x 96 DPI)

Fig. 2: Concentration response relationship after 24 and 48 hours of exposure. NOEC = no observed effect concentration, LOEC = lowest observed effect concentration, EC10 = concentration at which 10% of daphnids are immobilised, EC50 = concentration at which 50% of daphnids are immobilised.

184x169mm (150 x 150 DPI)

1 **Supplementary material**

2

3 1. Methodology

4 *Chemical analysis of ashes*

5 pH and EC measurements were conducted on a 1:20 sample:water ratio solution (unground

6 sample) after shaking for 5 minutes and allowing to settle for another 10 minutes (same

7 procedure than for the leaching tests below but without filtering). pH was measured with a

8 Crison micropH 2000 pH meter, with buffer solutions of pH 4, 7 and 9 and EC was measured

9 with a Crison GLP 31 apparatus, previously calibrated with a 0.1 M KCl solution.

10

11 *Total and soluble concentration of major and trace elements*

12 Total concentrations of major (Si, Al, Fe, Ca, Mg, Na, K) and trace elements (B, As, Cu, Ni,

13 Pb, Cu, Hg) were determined in acid extracts of the samples (9 ml of HNO_3 65% and 3 ml of

14 HCl 37% added to 0.5 g of ground sample) after microwave digestion (Ethos Plus Milestone

15 microwave) at 200 ºC during 50 minutes. After digestion, the extracts were completed to 50 ml

16 with ultrapure water (1:100 sample:solution). Certified soil standards were used to validate the

17 method of trace metal extraction (SRM 2709a, SMR2710a, from NIST, U.S.A.), with a mean

18 recovery rate of >93%) (Santín *et al.* 2015).

19 Leaching tests were carried out according to the methodology proposed by (Hageman 2007).

20 3 g of unground ash samples were weighed into 125 ml bottles. Then, 60 ml ultrapure water

21 (sample:water ratio 1:20) was added and the bottles were capped and shaken for 5 minutes.

22 After shaking, the contents were allowed to settle for 10 minutes. The leachate was then filtered

23 using a 0.45 μm pore-size nitrocellulose capsule filter. A sub-sample of the filtrate was

24 collected in plastic bottles and refrigerated for ion chromatography analysis. Another sub-

25 sample of the filtrate was acidified to pH < 1.5 with suprapur grade HNO_3 for atomic absorption

26 spectroscopy and inductively coupled plasma mass spectrometry analysis.

27 Dissolved organic carbon (DOC) was analysed in a loop flow analysis system (Systea).

28 Phosphate (PO_4^{3-}) (given as Total Phosphorous - TP), Nitrate (NO_3^-) and ammonium (NH_4^+)

29 concentrations in leachate extracts were determined by colorimetry (Kempers 1974) using a

30 Jasco V-630 spectrophotometer. Fluoride (F^-) concentrations were measured using an ion-

1

31 selective fluoride electrode (Metrohm 692 pH/Ion Meter). Sulfate and Cl$^-$ were determined by

32 ion chromatography (Dionex 4500i system).

33 Major and trace elements in acid and leachates extracts were determined using Inductively

34 Coupled Plasma Mass Spectrometry (ICP-MS) using a VARIAN 820-MS ICP-MS

35 spectrometer, except; Fe, Si and Al that were determined by atomic absorption (PerkinElmer

36 Spectrometer 1100B).

37

PAHs Analysis

39 Ash samples (~ 3 g) were extracted in Soxhlet with a 1:3 acetone:hexane mixture spiked with

40 a mixture of six deuterated PAHs. The extracts were then cleaned-up using aluminium oxide

41 and copper was added to remove sulphur interferences. PAHs were identified with a GC/MS

42 Thermo mod DSQ II (Thermo Electron Corporation, Austin, TX USA). The GC (TRACE GC

43 Ultra) was equipped with a DB-5 MS column (J&W Scientific Folsom, CA; 0.25 mm i.d., 0.25

44 μm film, 60 m, 5% phenylsubtituted methylpolysiloxane phase) and helium was used as carrier

45 gas. The operating conditions were: held at 50°C for 3 min, ramped to 200°C at 6°C min^{-1} and

46 finally to 315°C at 4°C min^{-1} holding that temperature for 15 min. The sample was injected

47 using an on-column injection system with the purge valve activated 30 s after the injection.

48 Transfer line and ion source temperatures were held at 280°C and 220°C, respectively. The MS

49 was operated in the electron impact (EI) mode at 70 eV and the spectrum divided into 8 SIM

50 windows, each scanned for up to 7 molecular masses, previously optimized, for the

51 corresponding analyses and the deuterated internal standards eluting in this windows.

52 Quantitation of parent and alkylated PAHs was performed using Thermo Scientific^{TM}

53 Xcalibur^{TM} software package.

54 Chemical and reagents used during analysis (Suprasolv grade acetone, hexane, neutral alumina

55 (70–230 mesh) and anhydrous sodium sulphate for analysis) were obtained from Merck

56 (Darmstadt, Germany). A standard mixture of deuterated PAHs containing naphthalene-d8,

57 biphenyl-d10, anthracene-d10, dibenzpthiophene-d8, pyrene-d10 and benz[a]pyrene-d12 was

58 obtained from CIL (Massachusetts, USA). A PAH mixture containing Naphthalene, Biphenyl,

59 2-Methylnaphthalene,   1-Methylnaphthalene,   2,3-Dimethylnaphthalene,   Acenaphthylene,

60 Acenaphthene, 2,3,6-Trimethylnaphthalene, Fluorene, Dibenzothiophene, Phenanthrene,

61 Anthracene,   4-Methyldibenzothiophene,   2-Methylphenanthrene,   2,8-

62 Dimethyldibenzothiophene,   1,6-Dimethylphenanthrene,   Fluoranthene,   2,4,7-

2

63   Trimethyldibenzothiophene,   Pyrene,   1,2,8-Trimethylphenanthrene,1-Methylpyrene,

64   Benzo(c)phenanthrene, Benz(a)anthracene, Triphenylene, Chrysene, 2-Methylchrysene,7,12-

65   DimethylB(a)anthracene,   Benzo(b)fluoranthene,   Benzo(k)fluoranthene,   Benzo(e)pyrene,

66   Benzo(a)pyrene, Perylene, Indeno(1,2,3-c,d)pyrene, Dibenzo(a,h)anthracene, Benzo (g,h,i)

67   perylene was prepared from mixtures from CPA Chem (Bulgaria) and Chiron (Trondheim,

68   Norway).

69

70   For the quality assurance and quality control of analysis (QA/QC), reagents blanks were

71   analyzed and recovery procedures checked to assure that no contamination or losses occurred

72   during extraction or other analytical procedures. Blanks result in no detectable PAHs

73   concentrations and average recovery of PAHs ranged from 55 to 114 % for Acenaphthylene

74   and Acenaphthene, respectively. The method detection limits (LOD) for individual PAHs

75   calculated using the signal-noise ratio standard deviation were in the range of 0.25 to 2.62 ng

76   g^{-1} d.w. A minimum of five levels of a PAHs mixture standards were run with every batch of

77   samples to build the linear regression curves by plotting the peak area ratios versus each PAH

78   concentration. Four different sediments used in the lab in various intercalibration exercises

79   organised by Quality Assurance of Information for Marine Environmental Monitoring

80   (QUASIMEME) were used as internal reference materials (Viñas *et al.* 2009; Pérez-Fernández

81   *et al.* 2015).

3

82 2. Tables

83

84 **Table 1. Total dry chemical composition of the six ash types tested (mg kg⁻¹).**

85

	AUS	USA	CAN	URIA	SPA	UK
			Ash type			
	AUS	**USA**	**CAN**	**URIA**	**SPA**	**UK**
Al	7000	22600	1320	10000	32800	2805
Si	2079	2068	1782	2376	2255	1595
Ca	177000	215000	163000	29400	133000	11800
TP	477	5342	5826	2418	1866	2645
Na	5043	4603	3113	3563	1123	663
Mg	9900	22000	12000	6400	5500	2700
Mn	510	710	830	1000	320	1430
Fe	4300	19100	979	8600	30600	7100
Ni	16	99	15	22	32	16
Cu	21	52	29	40	30	50
Zn	144	112	144	101	172	181
As	1.57	2.37	0.46	4.45	9.67	4.35
Cd	0.17	0.21	0.22	0.18	0.26	1.13
Hg	N.D	0.05	N.D	0.01	0.01	0.02
Pb	35	38	24	35	59	112

102 N.D (not detected) represents elements in concentrations <0.01 mg kg⁻¹.

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