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Leaves, berries and herbivorous larvae of bilberry *Vaccinium myrtillus* as sources of metals in food chains at a Cu-Ni smelter site

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Abstract

Ericaceous dwarf shrubs, such as bilberry, *Vaccinium myrtillus*, have an important role in nutrient cycling of boreal forests, but in metal polluted environments they also form a link between heavy metal pool of the soil, primary consumers and upper trophic levels. From the viewpoint of metal transfer in a food chain, we document metallic element (As, Ca, Cd, Co, Cu, Mn, Mo, Ni, Pb, Se, Zn) concentrations in leaves, berries and herbivorous larvae of *V. myrtillus* around a Finnish copper-nickel smelter and compare those with levels in relatively unpolluted reference sites, and with levels documented in soil and feces (a proxy of dietary levels) of an insectivorous bird, the pied flycatcher, *Ficedula hypoleuca*. Herbivorous larvae of the autumnal moth, *Epirrita autumnata* (Lepidoptera: Geometridae), grown experimentally on *V. myrtillus*, showed slower growth rate but not higher mortality in the polluted area. In general, metal levels in leaves, berries and larvae were higher in the polluted area and comparable to those reported at other smelter sites in Europe. The levels of the main toxic metals (As, Cd, Cu, Ni, Pb) followed the general pattern: soil > bird feces > leaves > larvae = berries, and levels in *V. myrtillus*, *E. autumnata* and *F. hypoleuca* reflected soil metal levels. The lowest levels were found in those matrices that are most important sources of food for birds and humans, i.e. leaf-eating larvae and berries, reducing a risk of toxic effects.

Keywords: Autumnal moth, bilberry, dwarf shrubs, food-chain transfer, pied flycatcher, metal pollution

1. Introduction

Ericaceous dwarf shrubs are key species in boreal forests and an important food source for many insects, birds and mammals (Atlegrim 1989; Atlegrim 1992; Nilsson and Wardle 2005; Dahlgren et al. 2007; Honkavaara et al. 2007). They have an important role in nutrient cycling, but in metal polluted environments they also form a link between the soil metal pool, primary consumers and upper trophic levels (Uhlig and Junttila 2001; Beyer and Sample 2017). For example, lepidopteran caterpillars chewing on bilberry, *Vaccinium myrtillus* L., are an ample and important source of food for insectivorous birds but also important agents transferring metals from plants to vertebrates (Eeva et al. 2005). Dwarf shrubs are relatively prone to take up metals from the soil because their roots are generally shallow, and absorption of nutrients mainly takes place in the upper soil layers where airborne metals accumulate (Derome and Lindroos 1998; Uhlig et al. 2001; Salemaa et al. 2001; Ettler 2016). Several dwarf shrub species tolerate long-term metal pollution relatively well, being able to grow, regenerate and spread even at heavily polluted sites (Salemaa et al. 2001; Uhlig and Junttila 2001). High metal concentrations may, however, suppress growth and seedling establishment, decline vegetation cover and affect negatively the performance of herbivorous caterpillars (Ruohomäki et al. 1996; Salemaa et al. 2001; Taulavuori et al. 2013).

Besides leaves, berries of dwarf shrubs make another potential transfer route of soil-accumulated metals to vertebrates, including humans (Barcan et al. 1998; Demczuk and Garbiec 2009). Metal levels in berries are generally considered to be lower than in other parts of the plant, but their significance as a transfer route of metals in heavily polluted environments is not well known (McIlveen and Negusanti 1994). Instead, there has been more interest on measuring metal levels in berries which are used as food stuff for humans (e.g. Kruglikova 1991; Barcan et al. 1998; Demczuk and Garbiec 2009). For example, relatively high Ni concentrations have been measured in berries of *Vaccinium* species at non-ferrous smelter sites (Kruglikova 1991; Barcan et al. 1998). Therefore, metal levels in berries could also have ecotoxicological relevance, e.g. for some frugivorous birds, such as thrushes (*Turdus* sp.).

The aim of our study was to document metallic element (As, Ca, Cd, Co, Cu, Mn, Mo, Ni, Pb, Se, Zn) concentrations in leaves, berries and herbivorous larvae of *V. myrtillus* around a Finnish copper-nickel smelter, which has been emitting metals in the surroundings since 1940's (Kozlov et al. 2009). We also experimentally tested whether feeding on metal-exposed *V. myrtillus* plants would reduce growth rate or increase mortality of the autumnal moth (*Epirrita autumnata* Borkhausen 1794; Lepidoptera: Geometridae) larvae. Information on plant and caterpillar metal levels are further compared to published data on soil metal levels and levels in a diet of an insectivorous passerine bird, the pied flycatcher (*Ficedula hypoleuca* Pallas 1764) in the same study area. As a proxy of dietary metal levels we used metal concentrations in feces of *F. hypoleuca* nestlings (Dauwe et al. 2004). Metal levels of *V. myrtillus* are compared with those found in relatively unpolluted sites farther from the smelter, and other metal polluted and reference sites in Northern Europe. Finally, metal levels in berries are compared with toxicity thresholds for food stuffs and birds.

2. Material and methods

2.1. Study area

The data were collected in summer 2014 around a Cu-Ni smelter (61°20' N, 22°10' E) in Harjavalta, southwestern Finland. Sulphur oxides (SO_x) and heavy metals (e.g. As, Cd, Cu, Ni and Pb) are common pollutants in the area (Tammiranta 2000; Kiikkilä 2003). Elevated heavy metal concentrations occur in the polluted area due to current and historical deposition, and soil metal contents decrease exponentially with increasing distance to the smelter (Derome and Lindroos 1998; Eeva and Penttinen 2009). There is no systematic change in organic layer pH (mean ca. 3.6) along the pollution gradient, but exchangeable Ca level is low in soils near the pollution source (Derome and Lindroos 1998; Salemaa et al. 2001; Eeva and Penttinen 2009). Sandy soils prevail in the area and forests are dominated by Scots pine (*Pinus sylvestris* L.), which forms mixed stands with Norway spruce (*Picea abies* [L.] Karsten) and birch (*Betula* L. spp.). In the understory vegetation, dwarf shrubs *Vaccinium vitis-idaea* L. and *V. myrtillus* dominate. Our focal species, *V. myrtillus*, is a common, long-lived, clonal and deciduous dwarf shrub in the area and persists even at relatively highly contaminated sites (Zvereva and Kozlov 2005; Lyanguzova and Maznaya 2012). However, at sites closest to the factory complex, understory vegetation is patchy and poorly developed, due to the long-term effect of pollution and consequent nutrient imbalance (Salemaa et al. 2001).

Populations of insectivorous hole-breeding passerines, including *F. hypoleuca*, have been studied in this area since 1991 by setting up study sites with nest-boxes to three main directions (southwest, southeast and northwest) from the smelter (Eeva et al. 1997). Eleven of these sites were used in the current study: 6 polluted sites close (<2 km; sites 01, 02, 03, 21, 22, 25; hereafter ‘polluted area’) to the smelter and 5 reference sites farther away (5 – 11 km; sites 06, 07, 09, 16, 12; hereafter ‘reference area’) from the smelter (Fig. 1).

2.2. Larval growth experiment

Autumnal moth, *E. autumnata*, is a common and widely distributed Holarctic species (Tammaru et al. 2001). It occurs naturally in the polluted area of Harjavalta, though the population densities and survival of larvae decrease towards the pollution source (Ruohomäki et al. 1996). Its polyphagous herbivorous larvae feed on a wide variety of trees and shrubs, including *V. myrtillus*, on which it also performs well (Seppänen 1970; Klemola et al. 2003). Among several herbivorous moth species, the carotenoid-rich caterpillars of *E. autumnata* are an important component in the diet for breeding insectivorous passerines (Atlegrim 1992; Eeva et al. 2005; Sillanpää et al. 2009; Eeva et al. 2010).

Overwintering eggs of *E. autumnata* were acquired from an unpolluted area in Northern Finland, and spring-hatched larvae were raised on bilberry twigs in a laboratory of Turku University up to the 2nd instar. Food plant material was collected from an unpolluted area near Turku. Thereafter (7th May), the larvae were taken out to four of our study sites (2 polluted [02, 21] and 2 reference sites [07, 09]; Fig. 1). Short bilberry twigs with three larvae were mounted in 14 bilberry bushes per study site. Bushes were enclosed with mesh-bags (voile curtain fabric, mesh 0.3 mm) to prevent predation (total n = 56 bags). The development of larvae was followed, and when they had reached their 5th instar (26th May) we took them back to the laboratory, together with the bilberry bush still enclosed with the mesh-bag, and measured their fresh mass individually with a precision of 0.01 mg. Samples of bilberry leaves from each bag were further picked up and their fresh mass was recorded. Larvae and leaves were then frozen and later dried in an oven at 40 °C. Finally, the dry mass of larvae and leaves was recorded for calculating their water content (mass %). This enables calculating dry − wet conversion factors for metal levels. Larvae from each bush were thereafter combined as one sample for the metal analysis. Thus we have individual performance parameters but pooled metals levels for the larvae in each mesh bag.

2.3. Heavy metal samples and analyses

After *V. myrtillus* berries ripened, we collected samples of leaves and berries (28th June) at 8 study sites (02, 03, 06, 07, 09, 12, 16, 21; Fig. 1) for measuring their heavy metal content. Five bushes at locations of the larval growth experiment were sampled and fresh leaves and berries were stored in 1.5 ml sealed plastic tubes. In the laboratory, their fresh mass was measured, both materials were oven-dried at 40 °C and dry mass was taken. Three samples from each site were taken for metal analyses (n = 24 for both materials). In addition, six samples of larvae per each of the four study sites were taken to the analyses (n = 24). Samples were ground to powder, weighed accurately (mean sample mass for larvae, leaves and berries were 77, 1635 and 1336 mg, respectively), and 2 ml of Supra-pure HNO_3 and 0.5 ml of H_2O_2 were added to the samples in Teflon bombs for digestion with a microwave system (Milestone High Performance Microwave Digestion Unit mls 1200 mega). The samples were then diluted to 50 ml with de-ionized water. The concentrations of elements (As, Ca, Cd, Co, Cu, Mn, Mo, Ni, Pb, Se, Zn) were determined with ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA). The calibration of the instrument was done with certified solution (Claritas PPT, Multi element solution 2A) from Spex Certiprep. The detection limit for most of the metals was around 1 ng/l. As a reference material we used certified mussel tissue (ERMCE278K-8G; Mo not included). The mean recoveries (±SE) in 8 reference samples were as follows: As 96 ±0.6%, Ca 98 ±5.6%, Cd 91 ±0.6%, Co 101 ±0.5%, Cu 100 ±0.7%, Mn 98 ±1.2%, Ni 120 ±0.9%, Pb 95 ±1.1%, Se 151 ±9.3%, Zn 87 ±0.6%.

Concentrations are expressed as µg/g, dry weight (d.w.). We report our results by using geometric means, but arithmetic means are used in comparisons to corresponding values in literature.

In the same field season, organic top soil samples (n = 20; collected 21st – 22th July) and feces of *F. hypoleuca* nestlings (n = 63; collected 17th – 28th June) were collected for metal analyses in other studies from 10 study sites (site 02 missing). Fecal and soil metal concentrations, together with detailed sampling methods, have been reported earlier by Espín et al. (2016) and Ruiz et al. (2017) and we use here these data sets for comparing associations in metal levels among different parts of a food chain: soil → bilberry leaves → herbivorous larvae → insectivorous bird. Missing fecal (n = 4) and soil (n = 1) samples from site 02 were collected and measured separately for the current study in 2016 and 2017, respectively. Note that the leaves for the metal analyses were collected ca. one month later than the larvae and more metals may have been accumulated in plants meanwhile. According to earlier studies, *F. hypoleuca* parents take ca. 1/3 of the food given to the nestlings from the field layer (Atlegrim 1992; Eeva et al. 1997). Of that, only a proportion is taken from bilberry and therefore the levels should not be interpreted as representing a direct transfer from the bilberry chewing larvae to birds. Furthermore, invertebrate species used by *F. hypoleuca* during their chick feeding are primarily others than our model species, *E. autumnata* (Eeva et al. 2010), whose main larval period is phenologically earlier than the main nestling period of *F. hypoleuca*.

2.4. Statistics

Metal levels in different materials (larvae, leaves and berries) were compared between the polluted and reference areas with linear models (LMs) in the Glimmix procedure of SAS statistical software 9.4 (SAS Institute Inc. 2013). In these models, area (polluted vs. reference) and sampling site nested within area were used as explanatory factors. Metal concentrations were $\log_{10}$ transformed and normality of model residuals was checked visually comparing residual distribution against normal distribution. Model predictions and 95% confidence limits were transformed back to the original scale for Table 1.

Metal levels among top soil, bilberry leaves, bilberries, herbivorous larvae and feces of *F. hypoleuca* nestlings were compared within the polluted area for the five main toxic metals emitted by the smelter (As, Cd, Cu, Ni, Pb; Cu is here considered potentially toxic because of its high emissions). Differences in metal levels among materials were tested with pairwise Tukey's comparisons within each metal (concentrations $\log_{10}$ transformed; p-values adjusted with the number of tests). We also wanted to test for the associations between the metal levels in soil, bilberry leaves, caterpillars and bird feces by using site level data. Because the concentrations of the five metals were generally positively correlated, we first ran principal component analyses for each material to reduce the number of variables for further analyses. In each case only the 1st principal components (PC1) showed an eigenvalue >1 and were used in further analyses. Bartlett's test of sphericity ($p<0.001$ for all materials) indicated that the correlation matrices were suitable for PCA. Associations between PC1s were then tested among site means of PC1s with Pearson correlations.

Differences in larval growth and survival were tested with linear mixed model (LMM; growth) and generalized linear model (GLM; survival). For growth, the larval mass at the end of the experiment was explained by area (polluted vs. reference), site (nested within area) and water content of leaves. In this model, with normal error distribution, the mesh bag number was included as a random factor to control for the non-independence of the three larvae growing on the same bilberry bush. The degrees of freedom were calculated with Kenward-Roger method. Normality of residuals was visually checked. For modeling survival, we used binomial error distribution with logit link function by using events/trials syntax of Glimmix: n alive at the end / n alive at the beginning. Association between growth (mean of larvae in the same bilberry bush) and PC1 of toxic metals in larvae was further tested with Pearson correlation.

3. Results

In general, metal levels in leaves, berries and larvae were significantly higher in the polluted area (Table 1). This was not the case, however, for Ca, Mn and Zn in all materials, Mo in berries and larvae, and Cd in berries (Table 1). The highest enrichment factors (element concentration in polluted area /

element concentration in reference area in each sample type) were shown by As (range among the three sample types: 7.3 – 12.3), Co (3.5 – 4.1) and Pb (2.5 – 4.5). The lowest enrichment factors were shown by Mn (0.47 – 0.68), Zn (0.93 – 1.0) and Ca (0.83 – 1.2), Mn and Ca of larvae showing significantly higher values in the reference area (Table 1). Water content (mean ±SD) was 59.3 ±8.4% (n = 40) for leaves, 83.4 ±3.2% for berries (n = 40) and 69.8 ±4.6% for larvae (n = 84).

The levels of the main toxic metals (As, Cd, Cu, Ni, Pb) in different materials from the polluted area followed the general pattern: soil > feces > leaves > larvae = berries (Figure 2). Among leaves, berries and larvae, leaves generally contained more of the other elements too (especially Ca, Co and Mn; Table 1). The most prominent exception to this was Zn, which showed markedly higher values in larvae (Table 1). Calcium and Mn also showed relatively high bioaccumulation factors from soil to leaves (Table 1). Site level correlations of general toxic metal exposure (PC1s of the five main toxic metals) among different materials were all positive and relatively strong, though the sample sizes were low for some combinations (Figure 3). Respectively, in most cases individual metals showed strong site level correlations among materials, though these were not always significant due to low sample size (Table S1).

At the end of the larval growth experiment, *E. autumnata* larvae were 12.6% heavier in the reference area as compared to the polluted area (Table 2). Larval body mass at the end of the experiment correlated negatively with their metal level (PC1 of As, Cd, Cu, Ni and Pb; $r = -0.62$, $p = 0.0012$, $n = 24$). Overall mortality of the larvae was relatively high (50%) but there was no significant effect of pollution on their survival (Table 2). Relatively cold days during the first week of the experiment may have increased larval mortality (mean daily temperature in the nearest weather station was 7.7 °C). Leaf water content was not statistically different between the areas (polluted 63.3%, reference 66.8%; $F_{1,80} = 2.7$, $p = 0.10$), and it did not affect either growth or survival of larvae (Table 2).

4. Discussion

4.1. Metal accumulation in leaves

Vaccinium myrtillus leaves appeared to be a good monitor for metal pollution, showing increased values in the polluted area for most elements, with the exception of Ca and the essential micronutrients Mn and Zn (see also Mróz and Demczuk 2010). The result is expected since the plant metal levels and enrichment factors in great deal reflect soil concentrations (McIlveen and Negusanti 1994; Derome and Nieminen 1998). While the most typical toxic Cu-Ni smelter pollutants (As, Cd, Cu, Ni, Pb) show increased organic soil concentrations near the smelter (Uhlig et al. 2001; Ruiz et al. 2017), Ca and Mn levels have decreased due to displacement of these elements by excessive amounts of Cu and Ni (Derome and Lindroos 1998; Uhlig and Junttila 2001; Mróz and Demczuk 2010). In general, Mn levels in leaves were relatively high, as this element is known to efficiently accumulate in shoots of *V. myrtillus* (Parzych 2014; Kandziora-Ciupa et al. 2017; Wojtun et al. 2017). On the other hand, soil Zn levels are relatively high near the smelter but this pattern was not reflected in *V. myrtillus* leaves, probably because Zn homeostasis is relatively well regulated within plants (Gjengedal and Steinnes 1994; Derome and Lindroos 1998). The highest enrichment factors in leaves (and in other matrices) were found for As, which is likely due to a strong pollution gradient of this element.

The focus of our study was the food chain transfer of metals and thus we did not try to remove external contamination. Therefore, besides soil pool, aerial fallout is an additional source of metal-rich dust particles on the surface of leaves (Nieminen et al. 2002). For example, a significant part of Ni was accumulated on the surface of birch (*Betula* sp.) leaves in the same study area (Koricheva and Haukioja 1995). Field studies in heavily polluted areas near a Ni-Cu smelter of Monchegorsk indicate that *V. myrtillus* is relatively tolerant to high metal levels (Zvereva and Kozlov 2005). This is likely due to effective detoxification and metal storage on below-ground organs (Taulavuori et al. 2013). The levels found in our study are comparable to those found in *V. myrtillus* leaves around other point sources of metals (Table 3). Copper levels are much lower than those measured in the same study area in the 1980’s (Table 3), reflecting temporal decrease in emissions: between 1984 and 2014, annual Cu emissions from the Harjavalta smelter have decreased from 300 t/a to 0.44 t/a (Kozlov et al. 2009; European Environment Agency 2017).

4.2. Metal levels and growth of larvae

Leaf-chewing caterpillars of *V. myrtillus* showed growth retardation in the polluted area. Slower growth rates and decreased pupal weights have also been documented on *E. autumnata* experimentally fed with birch leaf material collected from the same study area (van Ooik et al. 2007). Slow growth could be due to metal toxicity, sub-optimal ambient conditions (e.g. microclimate) or lowered food quality. In general, metal levels in larvae followed the patterns found in leaves, with the typical smelter pollutants showing higher values, and Ca and Mn lower values near the pollution source, with highest enrichment factor for As and generally high levels of Zn. Arsenic concentration of 18 µg/g d.w. in *Mamestra configurata* larvae was found to increase mortality but exceeds the level in our study 37 times (Andrahennadi and Pickering 2008). Matić et al. (2016) report that the lowest-observed-effect concentration (LOEC) of Cd in food of *Lymantria dispar* larvae was 30 µg/g d.w., which clearly exceeds the dietary level in our study (0.21 µg/g d.w.). Larval performance and population density of birch leaf-chewing *E. autumnata* were found to be decreased when leaf Cu and/or Ni concentrations exceeded 20 µg/g, d.w. (Ruohomäki et al. 1996). Mean levels in our study reached that threshold (Cu 20.6 and Ni 12.9 µg/g, d.w.) and maximum values clearly exceeded it (Cu 82.2 and Ni 30.2 µg/g, d.w.). Considering the levels of these and the other metals it is likely that slower growth is a consequence of pollution, either directly via toxic effect or indirectly via changed food quality.

Several studies have found that nutritional composition of *V. myrtillus* leaves changes in metal-polluted sites (Loponen et al. 1997; Bialonska et al. 2007; Mróz and Demczuk 2010). For example, pollution-related increase in the amount of secondary metabolites (e.g. phenols and alkaloids) in leaves could have negative effects on larval performance (Suomela et al. 1995; Haukioja 2003). Forests near the smelter are sparse, the forest floor more lighted and upper soil water holding capacity reduced as compared to more distant and shady sites, potentially affecting the water balance of plants (Derome and Nieminen 1998; Salemaa et al. 2001; Kozlov et al. 2009). However, we did not find any difference in leaf water content between areas and it did not explain the variation in larval growth. Sillanpää et al.

(2009) found no marked differences between polluted and reference areas of Harjavalta in body mass of *E. autumnata* larvae (including individuals of different instars) collected from birch trees, but the total biomass of caterpillars was lower in the polluted area due to lower density of larvae. This resulted in lower breeding success of an insectivorous bird, the great tit, *Parus major* (Sillanpää et al. 2009).

Comparable data on metal levels in caterpillars included in bird diet is scanty but we can compare the metal levels in the current study with unpublished values on lepidopteran larvae directly collected from adults of *P. major* and *F. hypoleuca* feeding their nestlings in the beginning of 2000's near the pollution source in the same study area (sampling described in: Eeva et al. 2005). For the main toxic metals, five samples of lepidopteran larvae (mainly Noctuidae and Geometridae; 3–5 larvae per sample) showed on average 5.6 times higher metal concentrations (geometric means: As 2.4, Cd 0.22, Cu 49.5, Ni 6.83, Pb 0.74 µg/g, d.w.) as compared to the current levels in larvae in the polluted area (Table 1). This difference may primarily reflect decreased emissions in time, but also different species composition and feeding substrate: on the basis of identified species (*Panolis flammea, Bupalus piniarius, Operophtera brumata, E. autumnata*), they partly originate from tree foliage, and caterpillars collected from trees showed higher metal levels than those collected from ground layer vegetation (Eeva et al. 2005). In all, this comparison suggests that the current levels of toxic metals in caterpillars of *V. myrtillus* are relatively low even in the polluted area.

4.3. Metal levels in berries

Metal levels (per dry mass) in berries were generally much lower than in leaves, again with the highest enrichment factor for As and the lowest for Zn, Mo and Mn. In general, the levels were comparable to those found in *V. myrtillus* berries around other point sources of metals, though Pb levels seem comparatively very low (Table 3). According to European Commission regulations, a maximum level of Pb in fruit used as foodstuff for humans is 0.10 µg/g, w.w. (European Commission 2015). Using a wet → dry conversion factor of 6.04 (water content 83.4%) for berries, the threshold level for Pb would be 0.604 µg/g, d.w. Both mean (0.067 µg/g, d.w.) and maximum (0.235 µg/g, d.w.)

concentrations in the polluted area are below this threshold. Corresponding threshold levels for Cd in fruits are 0.050 µg/g, w.w. and 0.302 µg/g, d.w. (European Commission 2006). The mean (0.052 µg/g, d.w.) of Cd concentration in the polluted area is below the threshold level but two individual samples (0.305 and 0.604 µg/g, d.w.) exceed it. European Commission gives no threshold value for As level in fruits but European Food Safety Authority (EFSA) considers 0.3 – 0.8 µg/kg body mass/day as a range of benchmark levels for safe inorganic As intake in humans (EFSA Panel on Contaminants in the Food Chain 2009). Based on average metal concentrations, a person weighing 70 kg would achieve the lower level of the range when eating 846 g of fresh berries from the polluted area. According to tolerable daily Ni intake (2.8 µg/kg body mass/day) the corresponding daily amount of fresh berries from the polluted area would be 323 g (EFSA Panel on Contaminants in the Food Chain 2015). However, because the bilberry growth coverage in the most polluted area is relatively small (< 5% of the land area), does not yield high crops and is not commonly used for picking berries, we consider the risk for humans very low.

NOAEL (no-observed-adverse-effect-level) values proposed for an adult passerine (American robin, *Turdus migratorius*) are much higher than the benchmark levels for humans: As: 5100, Cd: 1450, Pb: 1130, Cu: 47000 µg/kg body mass/day (Beyer and Sample 2017). For example, estimated daily food consumption of bilberry-eating fieldfare (*Turdus pilaris*; body mass ca. 90 g) would be 110g of fresh berries (equation 20 in: Sample et al. 1996). This is much less than the amount needed to achieve NOAEL levels for the above-mentioned metals (e.g. for Cu: 3.8 kg). Following these benchmark levels, the relatively low levels in berries do not therefore pose a toxic risk for frugivorous birds.

4.4. Associations between metal levels in soil, bilberry, caterpillars and birds

For the potentially most toxic metals in the surroundings of the smelter (As, Cd, Cu, Ni, Pb), the lowest levels were found in those matrices that are most important sources of food for humans and birds, i.e. berries and leaf-eating larvae, reducing the transfer and risk of toxic effects. Although metal concentrations in biota in general, and bird tissues more specifically, reflect those present in soil (e.g.

Fritsch et al. 2012), and insectivorous birds in our study area accumulate metals in their internal organs (e.g. liver) via their invertebrate food, the levels of individual elements are currently below the thresholds considered toxic (Berglund et al. 2011). For two highly toxic metals, Pb and As, this has also been shown by experimental studies on *P. major* (Eeva et al. 2014; Sánchez-Virosta et al. 2017). The combined effect by mixture of all metals is more difficult to evaluate due to the lack of experimental studies, but despite elevated metal levels, *F. hypoleuca* nestlings currently show very limited signs of direct toxic effects in our study area (Berglund et al. 2011; Espín et al. 2017).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure captions:

Fig. 1. Map of the study area, showing 11 study sites where samples were collected for this study around a copper-nickel smelter (in the middle).

Fig. 2. Geometric means (±95% CL) of the levels of five main polluting metals in different materials (soil, bilberry leaves, bilberries, bilberry chewing larvae of *E. autumnata* and feces of *F. hypoleuca* nestlings; all dried) from the polluted area of Harjavalta. Numbers above the bars show the actual values and the letters below bars indicate significant differences among materials within each metal (Tukey's test: means with the same letter within each metal are not statistically different).

Fig. 3. Scatter plot matrix between 1st principal components of metal concentration (As, Cd, Cu, Ni, Pb) in different materials (bilberry leaf, herbivorous larvae, top soil and feces of *F. hypoleuca* nestlings). The numbers at symbols denote the study sites within two areas (polluted and reference; see Figure 1). Corresponding Pearson correlations showed above the diagonal.

Table 1.

Element concentrations (geometric means and 95% confidence limits; µg/g, d.w.) in leaves, berries and leaf-eating larvae (geometrid *Epirrita autumnata*) of bilberry *Vaccinium myrtillus* in a polluted area at non-ferrous smelter and in a reference area. Linear model (LM)[1] results for differences between areas. Bioaccumulation factors are shown for soil → leaf and leaf → larva transfer routes for the polluted area.

	Leaves			Berries			Larvae			Bioaccumulation factors	
Element	Polluted n = 12	Reference n = 12	F_{df} p	Polluted n = 12	Reference n = 12	F_{df} p	Polluted n = 12	Reference n = 12	F_{df} p	Soil-leaf	Leaf-larva
As	1.02 (0.79-1.29)	0.14 (0.11-0.18)	**591.2$_{1,16}$** **<0.0001**	0.15 (0.095-0.23)	0.020 (0.013-0.031)	**185.8$_{1,16}$** **<0.0001**	0.48 (0.36-0.64)	0.039 (0.029-0.052)	**322.2$_{1,20}$** **<0.0001**	0.12	0.47
Ca	7222 (5054-10320)	6872 (4809-9819)	0.17$_{11,6}$ 0.68	939 (632-1396)	765 (514-1137)	2.41$_{1,16}$ 0.14	758 (664-866)	917 (803-1047)	**8.96$_{1,20}$** **0.0072**	12.2	0.10
Cd	0.12 (0.050-0.29)	0.056 (0.023-0.14)	**6.67$_{1,16}$** **0.020**	0.052 (0.018-0.15)	0.025 (0.0086-0.072)	4.28$_{1,16}$ 0.055	0.026 (0.018-0.039)	0.0081 (0.0055-0.012)	**38.8$_{1,20}$** **<0.0001**	0.22	0.22
Co	0.30 (0.21-0.45)	0.074 (0.050-0.11)	**123.5$_{1,16}$** **<0.0001**	0.044 (0.027-0.069)	0.012 (0.0074-0.019)	**69.9$_{1,16}$** **<0.0001**	0.039 (0.028-0.054)	0.011 (0.0080-0.015)	**66.2$_{1,20}$** **<0.0001**	0.05	0.13
Cu	20.6 (13.0-32.9)	6.12 (3.84-9.76)	**61.1$_{1,16}$** **<0.0001**	6.64 (5.31-8.29)	5.38 (4.31-6.72)	**7.99$_{1,16}$** **0.012**	12.1 (9.65-15.1)	8.81 (7.05-11.0)	**8.58$_{1,20}$** **0.0083**	0.04	0.59
Mn	414 (169-1017)	654 (266-1605)	2.32$_{1,16}$ 0.15	69.4 (38.1-126)	102 (55.8-185)	3.64$_{1,16}$ 0.075	63.8 (47.6-85.6)	135 (100-180)	**28.1$_{1,20}$** **<0.0001**	12.0	0.15
Mo	0.26 (0.16-0.45)	0.076 (0.045-0.13)	**51.7$_{1,16}$** **<0.0001**	0.095 (0.041-0.22)	0.13 (0.056-0.29)	1.18$_{1,16}$ 0.29	0.15 (0.10-0.23)	0.24 (0.15-0.36)	**4.61$_{1,20}$** **0.044**	0.22	0.58
Ni	12.9 (8.67-19.3)	3.40 (2.28-5.06)	**100.6$_{1,16}$** **<0.0001**	3.66 (2.60-5.16)	1.67 (1.19-2.35)	**47.3$_{1,16}$** **<0.0001**	0.99 (0.66-1.49)	0.37 (0.25-0.56)	**25.3$_{1,20}$** **<0.0001**	0.13	0.08
Pb	0.85 (0.48-1.52)	0.19 (0.11-0.35)	**58.4$_{1,16}$** **<0.0001**	0.067 (0.044-0.10)	0.027 (0.018-0042)	**41.4$_{1,16}$** **<0.0001**	0.20 (0.15-0.27)	0.079 (0.059-0.11)	**40.9$_{1,20}$** **<0.0001**	0.03	0.23
Se	0.19 (0.13-0.30)	0.084 (0.055-0.13)	**34.9$_{1,16}$** **<0.0001**	0.052 (0.034-0.078)	0.024 (0.016-0.037)	**29.3$_{1,16}$** **<0.0001**	0.23 (0.17-0.30)	0.074 (0.056-0.098)	**69.1$_{1,20}$** **<0.0001**	0.25	1.21
Zn	15.6 (10.0-24.2)	16.5 (10.7-25.6)	0.17$_{1,16}$ 0.69	9.46 (6.71-13.3)	10.2 (7.24-14.4)	0.44$_{1,16}$ 0.52	61.7 (56.2-67.8)	59.1 (53.8-64.9)	0.92$_{1,20}$ 0.35	0.25	3.96

[1] LMs with area and sampling site (nested within area) as explanatory factors.

Table 2

Linear model estimates (least squares means) for final body mass (g) and survival probability of *Epirrita autumnata* larvae in polluted and reference areas.

	Body mass[1]		Survival[2]	
	n	mean (95% CIs)	n	mean (95% CIs)
Polluted area	46	0.104 (0.098−0.110)	28	0.54 (0.43−0.65)
Reference area	38	0.117 (0.110−0.124)	28	0.46 (0.35−0.57)
Source of variation	F_{df}	p	F_{df}	p
area	$8.40_{1,37.9}$	0.0062	$0.98_{1,51}$	0.33
site(area)	$0.15_{2,36.9}$	0.86	$0.47_{2,51}$	0.63
leaf water (%)	$0.00_{1,31.3}$	0.99	$1.19_{1,51}$	0.28

[1] Linear mixed model with mesh bag number as a random factor (n = 84 larvae).

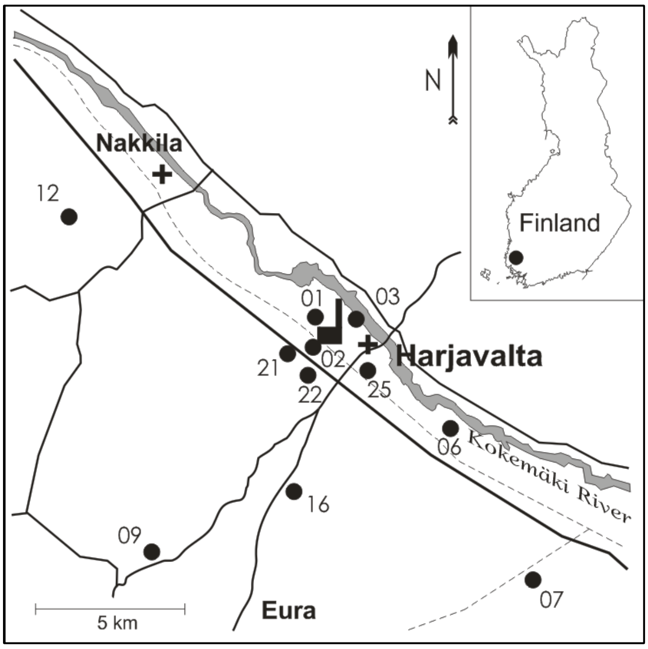
[2] Generalized linear model with binary error distribution and logit link function (n = 56 mesh bags).

Table 3

Examples of published arithmetic mean/median heavy metal concentrations (µg/g, d.w.) in leaves and berries of bilberry *Vaccinium myrtillus* from metal polluted and reference sites. Values marked with tilde (~) were read from figures.

Location	Country	Year	Pollution source	Concentration					Reference
Leaves				As	Cd	Cu	Ni	Pb	
Harjavalta	Finland	2014	Cu-Ni smelter	1.61	0.21	31.3	17.2	1.64	Current study
Głogów	Poland	2007	Cu smelter	−	0.10	24.1	3.4	8.7	(Mróz and Demczuk 2010)
Monchegorsk	Russia	1999	Cu-Ni smelter	−	0.039	15.8	48.9	0.81	(Reimann et al. 2001)[1]
Sør-Varanger	Norway	1990	Ni smelter	−	−	8.1	7.2	−	(Uhlig and Junttila 2001)
Harjavalta	Finland	1984	Cu-Ni smelter	−	−	66	−	−	(Aulio 1987)
Harjavalta control	Finland	2014	No	0.18	0.18	6.66	4.19	0.25	Current study
Słowiński National Park	Poland	2011	No	−	−	~1.7	−	−	(Parzych 2014)
Głogów control	Poland	2007	No	−	0.04	7.6	3.2	3.5	(Mróz and Demczuk 2010)
Kuopio	Finland	2007	No	−	−	−	~0.8	~0.08	(Roivainen et al. 2011)[1]
Northern Europe	Russia, Finland, Norway	1999	No	0.03	0.009	6.5	1.0	0.13	(Reimann et al. 2001)[1,3]
Berries				As	Cd	Cu	Ni	Pb	
Harjavalta	Finland	2014	Cu-Ni smelter	0.22	0.11	6.83	4.53	0.094	Current study
Głogów	Poland	2009	Cu smelter	−	0.05	6.87	0.52	1.34	(Demczuk and Garbiec 2009)
Skellefte	Sweden	1998	Sulphide ore mine	0.91	0.048	7.00	0.55	0.91	(Rodushkin et al. 1999)[4]
Kola Peninsula	Russia	1987-1992	Cu-Ni smelter	0.076	0.036	8.5	6.4	1.14	(Barcan et al. 1998)[2]
Harjavalta	Finland	1984	Cu-Ni smelter	−	−	10	−	−	(Aulio 1987)
Mazovia	Poland	2016	No	−	0.015	1.84	1.63	2.40	(Dróżdz et al. 2018)[4]
Harjavalta control	Finland	2014	No	0.028	0.050	5.52	1.83	0.029	Current study
Milicz	Poland	2009	No	−	0.02	4.31	0.39	0.86	(Demczuk and Garbiec 2009)
Luleå	Sweden	1998	No	0.056	0.006	4.73	0.31	0.031	(Rodushkin et al. 1999)[5]
Lapland	Finland	1990	No	−	0.024	6.96	0.90	−	(Laine et al. 1993)

[1] Median values. [2] Means of samples in Table V. [3] Some samples from a polluted area included. [4] Means for *V. myrtillus* from Table 1 were transformed with wet-dry conversion factor 6.04. [5] Transformed with wet-dry conversion factor 9.09.


N
Nakkila
12
01
03
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21
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Harjavalta
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Eura
5 km
Finland

Figure 1.

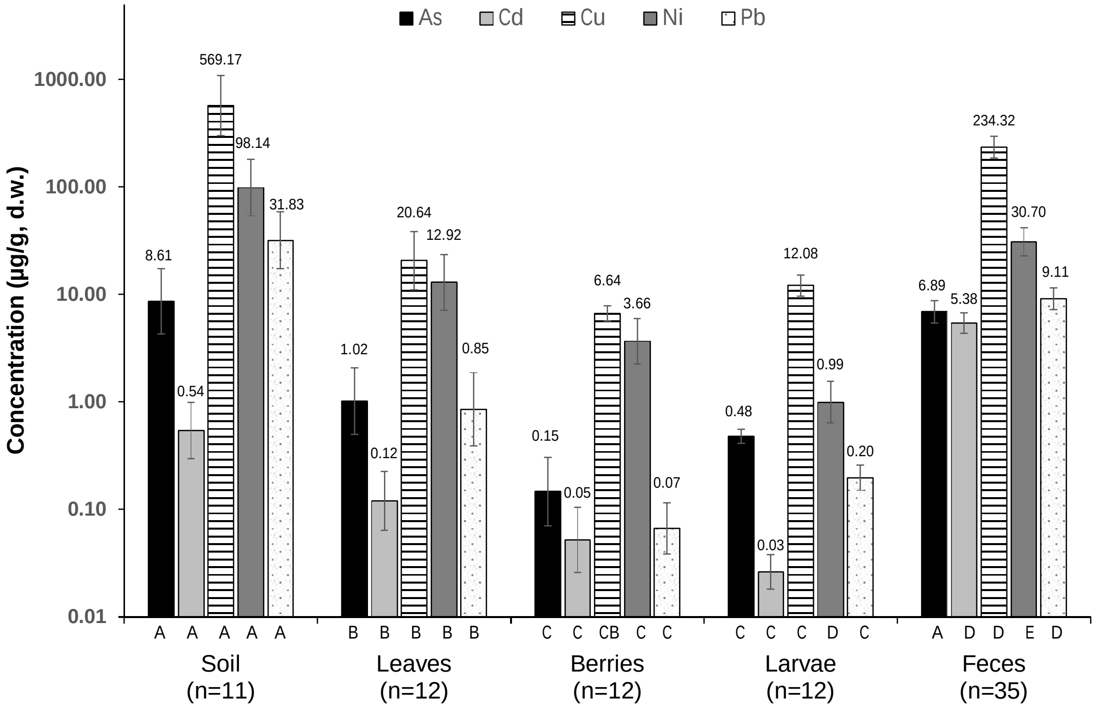
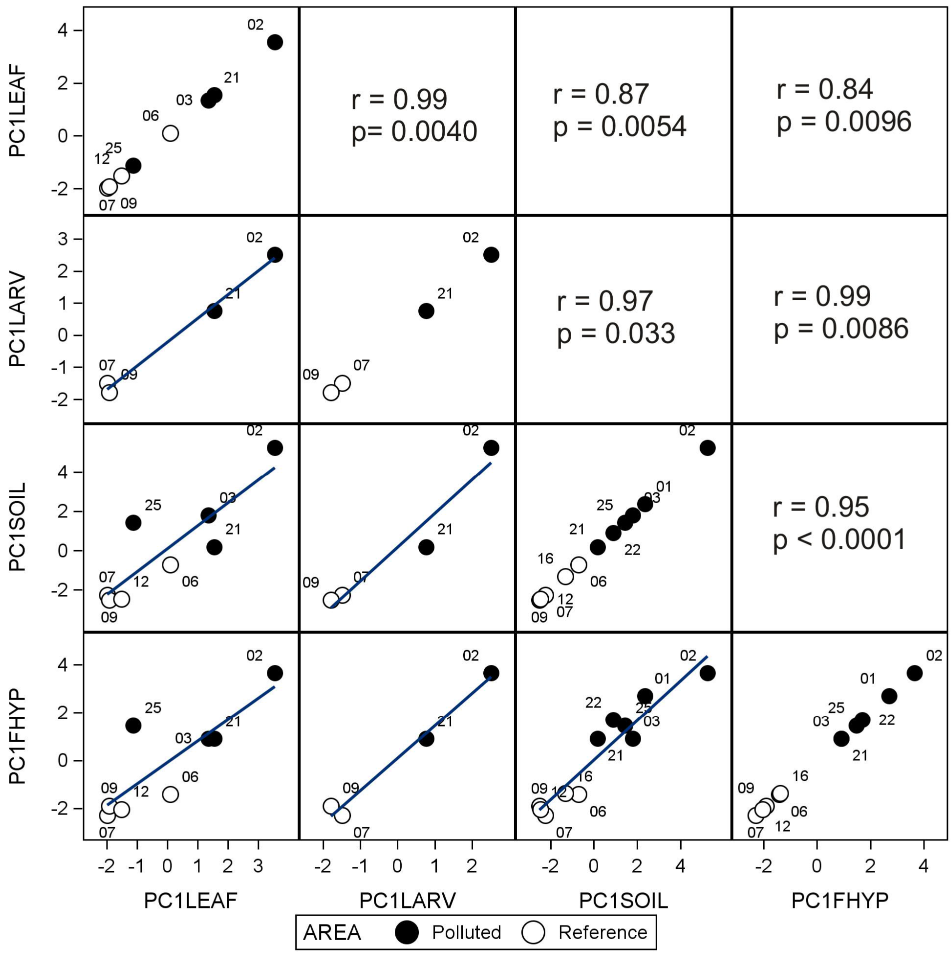

■ As
▨ Cd
▤ Cu
■ Ni
□ Pb
Concentration (µg/g, d.w.)
1000.00
100.00
10.00
1.00
0.10
0.01
8.61
0.54
569.17
98.14
31.83
1.02
0.12
20.64
12.92
0.85
0.15
0.05
6.64
3.66
0.07
0.48
0.03
12.08
0.99
0.20
6.89
5.38
234.32
30.70
9.11
A A A A A
B B B B B
C C CB C C
C C C D C
A D D E D
Soil
(n=11)
Leaves
(n=12)
Berries
(n=12)
Larvae
(n=12)
Feces
(n=35)

Figure 2.


PC1LEAF
PC1LARV
PC1SOIL
PC1FHYP
r = 0.99
p= 0.0040
r = 0.87
p = 0.0054
r = 0.84
p = 0.0096
r = 0.97
p = 0.033
r = 0.99
p = 0.0086
r = 0.95
p < 0.0001
-2
-1
0
1
2
3
4
AREA
● Polluted
○ Reference
01
02
03
06
07
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12
16
21
22
25

Figure 3.