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# Heavy metal resistance of two boreal dwarf shrubs in a polluted environment

Satu Monni

VANTAAN TUTKIMUSKESKUS - VANTAA RESEARCH CENTRE

METLA

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Finnish Forest Research Institute
Vantaa Research Centre

Academic dissertation in
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Faculty of Science
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Vantaa, October, 2000

Satu Mauri

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## LIST OF ORIGINAL PUBLICATIONS

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which in the text will be referred to by their Roman numerals.

I Monni, S., Salemaa, M., Millar, N., 2000. The tolerance of *Empetrum nigrum* to copper and nickel. Environmental Pollution 109, 221-229.

II Monni, S., Salemaa, M., White, C., Tuittila, E., Huopalainen, M., 2000. Copper resistance of *Calluna vulgaris* originating from the pollution gradient of a Cu-Ni smelter, in southwest Finland. Environmental Pollution 109, 211-219.

III Monni, S., Uhlig, C., Junttila, O., Hansen, E., Hynynen, J, 2000. Chemical composition and ecophysiological responses of *Empetrum nigrum* to aboveground element application. Environmental Pollution (in press).

IV Monni, S., Uhlig, C., Hansen, E., Magel, E., 2000. Ecophysiological responses of *Empetrum nigrum* to heavy metal pollution. Environmental Pollution 112 (in press).

V Monni, S., Bücking, H., Kottke, I., 2000. Ultrastructural element localization by EDXS in *Empetrum nigrum*. Manuscript.

# 1. INTRODUCTION

## 1.1. Mechanisms of plants to resist elevated heavy metal concentrations in the substrate

Heavy metal resistance is species- and metal-specific (Baker and Walker, 1990), and it can be achieved by two strategies: tolerance and avoidance (Baker, 1981, 1987). Tolerance is the ability of a plant to cope with metals that have excessively accumulated in the plant parts. Avoidance is the ability to prevent excessive metal uptake into plant parts. Tolerance mechanisms of higher plants include the production of intracellular metal-binding compounds (e.g. organic acids or proteins), alteration of the metal compartmentation patterns (e.g. translocation of metals to older plant parts or different cell organelles), changes in cellular metabolism (increased enzyme synthesis) or alterations to the membrane structure. Avoidance can be achieved through changes in the metal-binding capacity of the cell wall or increased exudation of metal-chelating substances (Figure 1) (Verkleij and Schat, 1990; Kochian and Garvin, 1999). The ericoid mycorrhizas of dwarf shrub roots have been suggested to accumulate heavy metals, leading to a reduction in heavy metal concentrations in the shoot (Bradley et al., 1981, 1982; Burt, 1984). Especially long-lived plants can avoid metals by proliferating roots in uncontaminated zones of the soils (Turner and Dickinson, 1993). However, even if the rate of heavy metal uptake can be controlled by plants, total avoidance of metal uptake is not possible (e.g. Baker, 1981).

Tolerance to heavy metals can either be based on the evolution of tolerant genotypes, it can occur without evolution or it may be environmentally induced in the adaptation of plant populations to toxic soils (Antonovics et al., 1971; Baker, 1987; Wu, 1990). Bradshaw and Hardwick (1989) have defined constitutive adaptation (classical evolutionary change) as an inevitable evolutionary change in the population in a situation where particular stress is occurring consistently, providing that an appropriate genetic variability exists in the populations affected. Facultative adaptation (phenotypic plasticity), in contrast, is produced within single genotype. The ways to achieve the adaptation in constitutive and facultative systems are fundamentally different, but there are not necessarily any differences in the physiological mechanisms (Bradshaw and Hardwick, 1989).

Different plant species have different degrees of metal tolerance, and they may possess varying levels of genetic variability and innate plasticity for tolerance. These mechanisms may interact with different types of polluted environments determining the success or failure of plant colonization (Wu, 1990). Multiple resistance to several metals is usually associated with the co-occurrence of high levels of these metals in the soil (Gregory and Bradshaw, 1965).

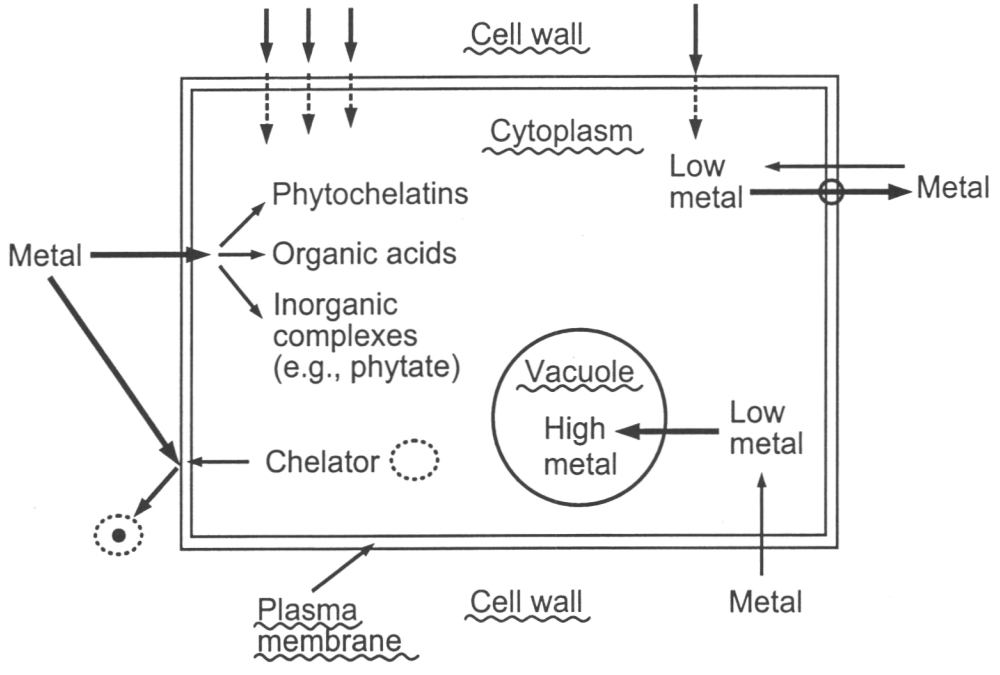



Figure 1. Resistance mechanisms of plants (modified after Tomsett and Thurman, 1988; Marschner, 1995).

## 1.2. Toxicity of Cu and Ni and parameters used to evaluate the responses of plants to heavy metals

### *1.2.1. Toxic Cu and Ni concentrations for plants*

Although copper is an essential element for plants and participates in many metabolic processes of the cell (Marschner, 1995), elevated concentrations are toxic. Allaway (1968) reported that a Cu concentration in leaf tissue of above 20 μg $g^{-1}$ dw is generally toxic to terrestrial plants. For most crop species a concentration of 20 μg $g^{-1}$ dw is toxic (Beckett and Davis, 1977; Davis and Beckett, 1978), whereas the yield was zero in the concentrations of

40-100 μg $g^{-1}$ Cu dw (Beckett and Davis, 1977). Balsberg Påhlsson (1989) defined a Cu concentration in the leaves of 15-20 μg $g^{-1}$ dw as being toxic for plants. However, the tolerance varies widely between species, for example sitka spruce (*Picea sitchensis*) is comparatively insensitive, tolerating 88 μg $g^{-1}$ Cu dw in the needles (Burton and Morgan, 1983)

Ni is also a mineral nutrient for higher plants, and so far it is known to be a component of one enzyme (urease) (Marschner, 1995). Leaf concentrations of 55 μg $g^{-1}$ dw have been reported to be potentially toxic for plants (Allaway, 1968). The critical concentration varies between species, for example, the upper critical Ni concentration for sitka spruce has been determined to be 6 μg $g^{-1}$ dw (Burton and Morgan, 1983), and for crops around 11-16 μg $g^{-1}$ Ni dw (Beckett and Davis, 1977; Davis and Beckett, 1978). The lethal concentration is much higher; the zero yield of barley was obtained in the tissue concentration of 250-850 μg $g^{-1}$ Ni dw (Beckett and Davis, 1977). The tissue of hyperaccumulators growing mainly in serpentine soils may contain 10 000 - 30 000 μg $g^{-1}$ dw (Lee et al., 1978).

### *1.2.2. Elongation and biomass*

According to Baker and Walker (1989), the tolerant individuals can be separated from the non-tolerant individuals in their ability to establish, survive and reproduce in metal-contaminated substrates. Growth in terms of biomass or length growth provides information about intraspecific differences in the effect of metal treatments. Root growth is usually rather sensitive to the presence of metal toxins (Baker and Walker, 1989), and the root elongation test has been used to determine the metal tolerance index of higher plants (Wilkins, 1978). The root elongation test is based on the assumption that tolerance will be manifested as a genotype/environment interaction. When the concentration of the metal in the solution increases, the root growth of tolerant plants will be less affected than that of the less tolerant plants. The commonly used technique is the Tolerance Index (TI), which is root growth in a toxic solution / root growth in a control solution (Macnair, 1990).

### *1.2.3. Physiological measurements*

In addition to morphological measurements, several physiological parameters such as the chlorophyll, abscisic acid (a plant hormone), organic acid contents, water potential, photosynthesis of plants etc. have been used to indicate the responses of plants to elevated heavy

metal levels. Photosynthetic rate and chlorophyll concentrations of plants has been found to be decreased by Cu, Ni, Cd and Pb (Lamoreaux and Chaney, 1978; Becerril et al., 1989; Angelov et al., 1993; Bishnoi et al., 1993; Pandolfini et al., 1996), and connections between drought stress and Ni, Zn and Cd have been reported (Rauser and Dumbroff, 1981; Bishnoi et al., 1993). In experimental studies, the stomatal conductance and water potential of leaves have decreased and the ABA content, which regulates the water status of plants, increased when plants were exposed to Ni (Rauser and Dumbroff, 1981; Bishnoi et al., 1993). Organic acids play a central role in detoxifying metals in Ni- and Zn-accumulating plants (Ernst, 1975; Mathys, 1977; Lee et al., 1978; Yang et al., 1997), and the amount of organic acids in plant parts usually increases with increasing Zn or Ni concentrations in the soil (e.g. Lee et al., 1978).

### *1.2.4. Electron microscopical investigations*

The ultrastructural localization of a large number of nutrients and heavy metals in plants is nowadays possible by electron dispersive X-ray spectroscopy (EDXS) and electron energy loss spectroscopy (EELS) (e.g. Mullins et al., 1985; Turnau et al., 1993 a, b; Neumann et al., 1995, 1997; Lichtenberger and Neumann, 1997), although both methods have their advantages and limitations (Stelzer and Lehmann, 1993; Kottke, 1994; Bücking et al., 1998). Very accurate information is obtained about the heavy metal resistance of plants when the metals are localized in specific cell compartments. Connections between the location of metals and nutrients in cell organelles or tissues provide information about detoxifying substances. However, the plant under study usually has to contain relatively high amounts of metals for them to be detected.

## 1.3. Ecology and special characteristics of dwarf shrubs colonising heavy metal contaminated areas

### *1.3.1. Ecology of* Empetrum nigrum

Crowberry (*Empetrum nigrum* (L.) ssp. *nigrum* and ssp. *hermaphroditum*) is a xeromorphic (Miller, 1975; Carlquist, 1989; Wollenweber et al, 1992), evergreen dwarf shrub with an ericoid mycorrhiza (Read, 1983). It is characterised by clonal growth. It is wind pollinated

and black berries are long distance dispersed by animals and birds passing through their gut (Bell and Tallis, 1973).

*E. nigrum* occurs on a variety of substrates. It can tolerate soil pH values ranging from 2.5 to at least 7.7 (Bell and Tallis, 1973). In the northern hemisphere *E. nigrum* is limited to the cooler regions, but it is also common in mountains at lower latitudes (Good, 1927; Bell and Tallis, 1973). In the subarctic plant communities it is often dominant species (Ojala, 1991). In boreal forests it grows on nutrient-poor, light dry heaths (Sarvas, 1937), and can form single-species plant communities in clearcut areas (Nilsson, 1992). It also grows on ombrogenous peat and peaty podsols (Good, 1927; Bell and Tallis, 1973; Eurola et al., 1990; Laine and Vasander, 1990) and serpentine soils (Proctor and Woodell, 1971).

The ecology of *E. nigrum* has been widely studied in both boreal forests and arctic ecosystems (Good, 1927; Bell and Tallis, 1973; Malmer and Nihlgård, 1980; Elvebakk and Spjelkavik, 1995; Michelsen et al., 1996a, b; Tybirk et al., 2000). One reason for this is its special ecology (dominance) in boreal forest ecosystems (Nilsson et al., 1998). One interesting feature of *E. nigrum* is its ability to influence the establishment of other species (Zackrisson et al., 1997); its leaf extracts inhibit, for example, the establishment of Scots pine (*Pinus sylvestris* L.) seedlings (e.g. Nilsson and Zackrisson, 1992; Zackrisson and Nilsson, 1992; Nilsson, 1994). However, this feature is not typical for *Empetrum* species alone, because allelopathic interactions between other plant species and trees have been also found in coniferous forests (e.g. Pellissier and Souto, 1999).

Tybirk et al., (2000) summarizes profoundly the function and susceptibility of *E. nigrum* dominated ecosystems to environmental changes. *E. nigrum* is relatively tolerant to simulated acid rain (Shevtsova, 1998), tropospheric ozone (Johnsen et al., 1991) and it responds relatively slowly to enhanced UV-B radiation (Johanson et al., 1995). It is a weak competitor for light (Tybirk et al., 2000) and sensitive to waterlogging (Bell, 1969), mechanical disturbances and fire (Tybirk et al., 2000). Gerdol et al., (2000a) found that length growth of the current-year shoots does not vary considerably between years though interannual fluctuations occur. Shevtsova (1998) reported also, that the number of interactions between the effects of warming and watering have an effect on the growth and reproduction of *E. nigrum*.

### *1.3.2. Ecology of* Calluna vulgaris

The heather (*Calluna vulgaris* (L.) Hull.) dominated oceanic heathland ecosystems are perhaps the most widely studied among dwarf shrub dominated ecosystems (e.g. Gimingham, 1972). *C. vulgaris* is a widespread and common species in Europe that usually grows on acidic and nutrient-poor soils (Gimingham, 1960, 1972). It is found on heathlands (e.g. Mohamed and Gimingham, 1970; Haapasaari, 1988), moors, bogs, fixed sand dunes and in forests (Gimingham, 1960). In Finland, *C. vulgaris* is one of the 10 most common vascular plant species in forest and peatland vegetation (National Forest Inventory 1995, unpublished results).

*C. vulgaris* reproduces both vegetatively and from seeds (e.g. Gimingham, 1960, Mallik et al., 1988). Although *C. vulgaris* produces a vast number of seeds each year and there exists a huge seed reserve in the soil, the germination and subsequent establishment of these plants do not occur very readily (Mallik et al., 1984). Environmental factors and the seedbed substrates affect seed germination (Mallik et al., 1988).

*C. vulgaris* contains high amounts of carbon-based secondary metabolites such as phenolics and tannins (Iason et al., 1993), and therefore the litter of *C. vulgaris* is rich in phenolic compounds (Jalal and Read, 1983), which decompose slowly and modify the soil causing acidification and organic matter accumulation. This leads to soil conditions that are optimal for the growth of *C. vulgaris* and impairs the growing conditions of other species (Grubb and Suter, 1971; Robinson, 1971; Haslam, 1977; Leake, 1988).

The dominance of *C. vulgaris* in nutrient-poor environments has been explained on the basis of the effective use of nutrients; the ericoid mycorrhizal endophyte of *C. vulgaris* roots is also able to utilise organic sources of nitrogen (Bajwa et al., 1985; Leake and Read, 1989). However, contrary to the most of the studies, no significant differences between mycorrhizal and non-mycorrhizal plants in N concentration of *C. vulgaris* shoots were observed in experimental studies (Strandberg and Johansson, 1999).

Long-term studies and greenhouse experiments have shown that an increased nitrogen load first strongly increases the shoot biomass of *C. vulgaris* (Carroll et al., 1999; Hartley and

Amos, 1999), but might be harmful in the long run by increasing the susceptibility of *C. vulgaris* to other environmental stresses such as frost and drought (Carroll et al., 1999).

### *1.3.3. Dwarf shrubs in heavy metal polluted areas*

Species of the family *Ericaceae* ja *Empetraceae* colonise soils with naturally elevated metal concentrations such as serpentine soils (Proctor and Woodell, 1971; Marrs and Bannister, 1978) and soils contaminated with metals (Laaksovirta and Silvola, 1975; Marrs and Bannister, 1978; Bagatto and Shorthouse, 1991; Bagatto et al., 1993; Chertov et al., 1993; Lukina et al., 1993; Helmisaari et al., 1995; Shevtsova, 1998; Mälkönen et al., 1999; Reimann et al., 1999; Salemaa et al., 2000a, b; Uhlig et al., 2000) as a result of mining and smelting activities. However, also very resistant ecotypes and metal hyperaccumulators are found among herbs and grasses (e.g. Amiro and Courtin, 1981; Boyd et al., 1999), and for example near the Sudbury Cu-Ni smelter in Canada, the grasses, herbs and decidious trees are the dominant vegetation in the polluted soils (Freedman and Hutchinson, 1980; Winterhalder 1995; Bagatto and Shorthouse, 1999). The situation for trees and other long-lived perennial and clonal plants is different from many annual or colonizing plants, because the long generation time prevents rapid selection for tolerance (Dickinson et al., 1991).

Among dwarf shrubs, *Vaccinium angustifolium* is one of the plants surviving near the Cu-Ni smelter at Sudbury, Canada (Bagatto and Shorthouse, 1991; Bagatto et al., 1993; Shorthouse and Bagatto, 1995), and *Vaccinium uliginosum* and *Arctostaphylos uva-ursi* near the Cu-Ni smelter at Harjavalta, SW Finland (Salemaa et al., 1999). *E. nigrum* occurs in the immediate vicinity of the Cu-Ni smelter at Harjavalta (Helmisaari et al., 1995; Uhlig et al., 2000; Mälkönen et al., 1999; Salemaa et al., 2000a, b) and those in the Kola Peninsula, Russia (Chertov et al., 1993; Lukina et al., 1993; Shevtsova, 1998; Reimann et al., 1999). In most of the publications it is described as a species resistant to metals (Väisänen, 1986; Helmisaari et al., 1995; Shevtsova, 1998; Salemaa et al., 2000a, b). *C. vulgaris*, however, is reported to be resistant or sensitive depending on the geographical region (Gilbert, 1975; Marrs and Bannister, 1978; Eltrop et al., 1991; Salemaa et al., 2000a, b). In Great Britain and Germany, *C. vulgaris* has been found to be a resistant species occurring on a variety of substrates, e.g. serpentine and polluted soils (Marrs and Bannister, 1978; Eltrop et al., 1991) and colonising waste heaps in metal-polluted areas (Burt, 1984). In the northern parts of its

distribution *C. vulgaris* is absent from severely heavy metal polluted areas in Fennoscandia (Salemaa et al., 2000a, b) and the Kola Peninsula (Mikhail Kozlov, unpublished results), and it is considered to be a species sensitive to metals (Laaksovirta and Silvola, 1975, Salemaa et al., 2000a, b).

The soils that are colonised by the dwarf shrub species are generally acidic and nutrient deficient (Bell and Tallis, 1973; Marrs and Bannister, 1978). It is thought that dwarf shrubs are generally adapted to grow in soils where metal availability is high, and they therefore must have an intrinsic ability to tolerate high levels of metals (Meharg and Cairney, 2000). The resistance of *C. vulgaris* and *Vaccinium* species to metals has been suggested to be based on the ericoid mycorrhiza of the roots (Bradley et al., 1981, 1982; Burt, 1984).

## 1.4. Aims

The overall aim of this thesis is to provide more information about the heavy metal resistance of two important, common dwarf shrubs, *E. nigrum* and *C. vulgaris*, in boreal forests. These species were chosen because the former grows near smelters, whereas the latter is absent from the polluted areas mentioned in the above. The sensitivity and tolerance to metals were studied using greenhouse experiments and field studies. The greenhouse studies were carried out in order to eliminate factors other than specific heavy metals (I, II, III), and the field studies to determine the actual situation in nature (IV, V). The specific aims of the thesis were;

- to study the chemical composition, growth, biomass and discoloration of leaves of dwarf shrubs in response to elevated concentrations of Cu and/or Ni applied to the roots of *E. nigrum* (I) and *C. vulgaris* (II) in greenhouse conditions.

- to study the chemical composition and aboveground element uptake of *E. nigrum* after nutrient and heavy metal applications to the aboveground parts. Ecophysiological responses (chlorophyll concentration, water potential, ABA content, dark respiration and maximum photosynthesis) to the applications were also studied (III).

- to determine the responses of *E. nigrum* to heavy metal pollution by measuring physiological parameters (chlorophyll contents, organic acids, stem water potential and ABA contents of *E. nigrum* leaves and stems) in the high and low contaminated areas near the Cu-Ni smelter at Harjavalta (IV).

- to study the localization of heavy metals and nutrients in *E. nigrum* growing in the high and low contaminated areas near the Cu-Ni smelter at Harjavalta (V).

## 2. MATERIALS AND METHODS

The materials, measured parameters and implementation of studies in papers I-V are shown in Table 1.

Table 1. The materials, measured parameters and implementation of studies in papers I-V.

| Paper | Species | Parameters | Elements applied | Implementation |
|---|---|---|---|---|
| I | *E. nigrum* | Chemical composition, elongation, biomass | *Cu, Ni, Cu+Ni + nutrients | Greenhouse |
| II | *C. vulgaris* | Chemical composition, elongation, biomass | *Cu + nutrients | Greenhouse |
| III | *E. nigrum* | Chemical composition, chlorophyll, $CO_2$-exchange rate, stem water potential, ABA | Cu, Ni, Pb, Zn, Fe, Cd, Cr, Mn, Mg, Ca, P, K, C, N | Greenhouse |
| IV | *E. nigrum* | Chlorophyll, organic acids, stem water potential, ABA, soil moisture | | Field; 0.5 and 8 km distances from the smelter |
| V | *E. nigrum* | Electron microscopical element localization | | Field; 0.5 and 8 km distances from the smelter |

*Only Cu and Ni concentrations were increased in the treatment solution series, the nutrient concentrations being kept constant.

### 2.1. Greenhouse experiments

The greenhouse experiments were carried out in the greenhouse of the Finnish Forest Research Institute at Ruotsinkylä (60°21' N, 25°00' E). The light conditions were natural, and the mean temperature was regulated at +20-22 °C during the day and +15 °C at night. The mean relative humidity in the greenhouse was approximately 60 to 70% (I, II, III).

The three- to five-year-old (I) and four- to five-year-old (III) *E. nigrum* cuttings originated from an unpolluted area in SW Finland. The cuttings were rooted in a mixture of sand and peat and transferred to quartz sand for the Cu and Ni experiment (I) or grown in quartz sand

(on the bottom) and peat substrate for the aerial heavy metal and nutrient application (Cu, Ni, Fe, Pb, Zn, Cd, Cr, Mn, Mg, Ca, P, K, N) experiment (III).

In Cu and Ni experiment (I) the cuttings were watered twice a week (50 ml/pot) with a nutrient solution modified by Stribley and Read (1976) containing P, K, Ca, Mg, Fe, Mn, B, Zn, Mo and $NH_4$- and $NO_3$-N. Six levels of Cu (0.1, 1, 10, 22, 46 and 100 mg $l^{-1}$), five levels of Ni (0, 10, 22, 46 and 100 mg $l^{-1}$) and nine combinations of Cu and Ni [Cu (mg $l^{-1}$)/Ni (mg $l^{-1}$): 10/10, 22/10, 46/10, 100/10, 22/22, 46/22, 100/22, 46/46, 100/46] were used. Cu was given as $CuSO_4$ and Ni as $NiCl_2$. The experimental design was completely random, and the total number of seedlings treated was 152 (8 plants/treatment) (I).

In the aerial heavy metal and nutrient application experiment (III) solutions with six different compositions of 13 elements were sprayed twice a week on the seedlings. The aerial parts of the seedlings were enclosed in a plastic container, the spraying treatment being applied via openings in the container. The seedlings were sprayed from two opposite directions with 15 ml of solution from each direction (altogether 15 times). The composition of wet deposition near the Cu-Ni smelter at Harjavalta, SW Finland, was simulated. Treatment I represented control, the nutrient concentrations being the same in treatments I and II. Treatment II contained about the same concentration of nutrients and heavy metals as rainwater at a distance of 0.5 km from the smelter reported by Helmisaari et al. (1994) and Helmisaari (personal communication). In treatments III to VI, the concentrations of all the components increased exponentially, the relative concentrations thus being constant in the different treatments (see table 1 in paper III). The experimental setup was completely random and there were nine plants per treatment (III).

The seed bank samples of *C. vulgaris* (II) were collected from three peatland sites located 1.2 km to the NW (Lammaistensuo) and 2.5 km (Kotosuo) and 5.5 km to the NE (Pyhäsuo) of the Harjavalta Cu-Ni smelter (61°19'N, 22°9'E). The seeds were germinated in a mixture of sand and peat and the nine-month-old seedlings planted in quartz sand for the Cu experiment. The cuttings were watered twice weekly (50 ml/pot) and the same nutrients as above (I) were given (see plant culture system in paper II). Five concentrations of Cu (1, 10, 22, 46 and 100 mg $l^{-1}$), applied as $CuSO_4$, were used with ten replicates per treatment. The

total number of seedlings was 150 (3 origins × 5 treatments) and the experimental setup was completely random (II).

## 2.2. The study sites near Harjavalta Cu-Ni smelter

The Harjavalta Cu-Ni smelter, situating SW Finland (61°19' N, 22°9' E), started operating 55 years ago. Cu smelter was established in 1945 and the Ni smelter in 1960. Sulphur dioxide and heavy metals have been emitted into the environment for the past 40-60 years. The deposition of metals near the smelter was considerably reduced in the 1990's after a new taller stack and electrostatic filters were built (Rantalahti, 1995). The prevailing wind direction has been from the south, south-west and south-east (Derome, 2000). A detailed description of the emissions and smelter activity has been presented by Derome (2000).

Samples of *E. nigrum* for the ecophysiological (IV) and electronmicroscopical (V) studies were collected at two distances (0.5 and 8 km) from the Harjavalta Cu-Ni smelter (Figure 2). For ecophysiological studies ten separate *E. nigrum* patches were marked situating more than four meters from each other, and these plants were used for all physiological measurements at both locations (0.5 and 8 km). The material collection was done on July 21$^{st}$, 26$^{th}$, August 18$^{th}$-19$^{th}$, 26$^{th}$ and October 9$^{th}$, 1997 (IV). The sampling for electronmicroscopical studies was done on August 6$^{th}$, 1998 (V).

The sites were chosen SE from the smelter, because it was the only direction where the two sites represented the same forest site type (*Calluna* site type) and soil type (orthic podzol) (Figure 2). The pH of the organic layer was 3.5 at 0.5 km distance and 3.6 at 8 km distance from the smelter (Derome and Lindroos, 1998a). The sulphur deposition has not had an effect on soil acidity in the organic layer or upper mineral soil layers, although an increase in exchangeable acidity and Al in deeper mineral soil at 0.5 km was found (Derome, 2000). At 0.5 km distance the site is located in a heavily polluted area where the total Cu and Ni concentrations in the organic layer are over 5 800 and 460 mg $kg^{-1}$ dw respectively, which has resulted a displacement of Ca, Mg, K from exchange sites and a decrease in plant available concentrations of these cations. This is also caused by a partial inhibition of the mineralisation of these nutrients from the litterfall (Derome, 2000). The concentrations of other heavy metals (Fe, Zn, Cd, Pb, Cr) in the organic layer are also elevated near the smelter (Derome

Figure 2. Sampling sites at a) 8 and b) 0.5 km distance from the Cu-Ni smelter at Harjavalta, SW Finland.

and Lindroos, 1998a; see also metal concentrations in article V). The site at 8 km is only slightly polluted, the total Cu and Ni concentrations in the organic layer being 150 and 40 mg $kg^{-1}$ dw, respectively. The concentrations of other heavy metals are also much lower at 8 km than at 0.5 km, but are higher than background values (Derome and Lindroos, 1998a).

There are several examples about the influence of heavy metal and sulphuric acid deposition as well as nutrient deficiency on the ecosystem (e.g. Helander, 1995; Fritze et al., 1996; Pennanen et al., 1996; Nieminen et al., 1999; Mälkönen et al, 1999; Salemaa et al., 1999, 2000b; Uhlig et al., 2000). For example Scots pine (*Pinus sylvestris*) suffers from Mg deficiency (Nieminen et al., 1999) near the smelter and the number of pine needles infected by specific endophyte, *Cenangium ferrucinosum* Fr.:Fr. is decreased (Helander, 1995). Also fine root mass (Mälkönen et al., 1999) and soil microbial and fungal biomasses of the soil are decreased (Fritze et al., 1996; Pennanen et al., 1996). At the site 0.5 km the vegetation is almost totally absent apart from the few patches of mosses (*Pohlia nutans*, *Ceratodon purpureus*) and the dwarf shrub *E. nigrum* ssp. *nigrum*. *Carex globularis* and *Vaccinium uliginosum* have also survived in partially paludified depressions. Abundances of many species at 8 km are rather typical for dry heath forests (Salemaa et al., 2000b).

### 2.3. Chemical analyses

The total concentrations of Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, P, Pb and Zn in the peat (III) and different plant parts [stems, leaves by year growth (I, II, III) and roots (II), detailed descripition see papers I, II, III] were determined by dry digestion (+550 °C), followed by extraction of the ash with 2-3 ml of 6 M HCl (pro analysi). The solutions were analysed by induction coupled plasma atomic emission spectrometry (ICP-AES) (I, II, III). The C and N concentrations were determined on the dry material using a Leco CHN analyser (Nelson and Sommers, 1982) (III). The results are given as concentrations (mg $kg^{-1}$) (Timmer, 1991).

### 2.4. Physiological measurements

For the analysis of chlorophyll *a* and *b*, freeze-dried plant material (Hetosicc freeze dryer, type CD 52) was extracted with 80% acetone and the absorbances (A) at 647 and 664 nm recorded on a spectrophotometer (Shimadzu UV-1201, UV-VIS) (Graan and Ort, 1984) (III,

IV). The results are calculated as chlorophyll concentration (μmol $l^{-1}$) (III) or content in the tissue (μmol chlorophyll $g^{-1}$ dw) (IV).

Citric and malic acids in freeze-dried material were determined enzymatically by a method modified from Boehringer (1989) and Hampp et al. (1984). The organic acids were extracted by 0.1 N HCl, and measured by a spectrophotometer at 340 nm (Kontron). The results are shown as contents (nmol $mg^{-1}$ dw) (IV).

Pressure chamber determinations were carried out to estimate the total water potential of the xylem sap of *E. nigrum*. The stems were cut with a razor blade, and the stem water potential measured using a Scholander pressure bomb (Scholander et al., 1965; Ritchie and Hinckley, 1975; Richter, 1997) (III, IV).

The soil moisture measurements were made at the same time as the stem water potential measurements. The soil moisture was measured using a ThetaProbe soil moisture sensor (type ML1). The ground vegetation was removed and the measurements were made horizontally in three soil horizons (organic layer = O, mineral soil = A and B horizons) (IV).

For the analysis of abscisic acid, freeze-dried (Hetosicc freeze dryer, type CD 52), homogenised plant material was suspended in 0.05 M phosphate buffer (pH 8.0), and 50 ng internal standard ($[^2H_4]$ABA) was added. The residue was dissolved in 80% methanol and injected into a Radial Pak™ (Waters, Milford, USA) $C_{18}$ HPLC column. The fraction containing ABA was collected (15.5-17.5 min), and reduced to dryness *in vacuo*. The residue was dissolved in methanol and methylated. The dried sample was dissolved in 12 μl of heptane, and 1 μl of this solution was injected into the GC-MS. Ions of m/z 162, 190, 193 and 194 were monitored. The results are shown as contents ng $g^{-1}$ (III, IV). Note that here the term content is used for (ng $g^{-1}$) unlike in chemical analyses the term concentration (mg $kg^{-1}$) is used.

The $CO_2$ exchange rate of *E. nigrum* was measured using a battery-operated Li-Cor LI-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Measurements were carried out with a quarter litre chamber. Six different irradiance levels (0, 50, 110, 330, 600

and 820 μmol $m^{-2}$ $s^{-1}$) of photosynthetically active radiation (PAR) were used. Dark respiration was measured by covering the chamber with a black plastic sheet (III).

## 2.5. Electron microscopy

Previous-year stems of *E. nigrum* were collected in the field, cryofixed and stored in liquid nitrogen (-192 °C). The stem pieces were freeze-dried (CFD, Leica, Germany) and pressure infiltrated directly in 100 % Spurr's epoxy resin (Spurr, 1969), using a method described by Fritz (1980). For energy dispersive X-ray spectrometry (EDXS) the embedded samples were dry sectioned (0.5 μm), placed on filmed Ni or Cu grids and carbon coated (V).

The EDXS studies were carried out under standardised conditions using a Philips EM 420 provided with the EDAX DX-4 system. EDXS spectra were collected between 0 and 20 keV with a Si(Li) X-ray detector. EDXS point measurements were made in the xylem, phloem and parenchyma of the primary ray of the stems in both high and low contaminated samples (V).

## 2.6. Statistical analysis

Two-factor ANOVA (analysis of variance) was used in analysing the effects of Cu and/or Ni or origin on the response variables of *E. nigrum* and *C. vulgaris* (GLM procedure, SAS Institute Inc., 1994; Sokal and Rohlf, 1995). Pairwise comparisons between the treatments and between the Cu and Ni series were performed by the t-test (I, II). Relationships between Cu and Ni concentrations in plant parts and the applied concentrations were studied by regression models (REG and NLIN procedures, SAS Inst.). Regression equations are also given for Cu and Ni uptake as a function of the amounts of Cu and Ni applied (SAS Institute Inc., 1994; Sokal and Rohlf, 1995) (I). Pearson correlations were calculated between the concentrations of Cu and other elements in the different plant parts (II).

Non-parametric Kruskal-Wallis ANOVA (analysis of variance) was used in analysing the effects of spraying treatments on the stem water potential of *E. nigrum*. Pairwise comparisons between the treatments were performed by the Kruskal-Wallis, comparison of mean ranks test. The Spearman rank correlations were calculated between Cu and Fe concentra-

tions in different plant parts and stem water potential, chlorophyll and ABA contents of *E. nigrum* leaves. In order to determine the element uptake via the roots from the peat and possible contamination of the peat by the spraying solutions, the Spearman rank correlations between the element concentrations in peat and different parts of *E. nigrum* were calculated (Sokal and Rohlf, 1995; Statistix, 1996). The effect of treatment on the $CO_2$ exchange rate of *E. nigrum* was evaluated by comparing the dark respiration at the irradiance level of 0 μmol $m^{-2}$ $s^{-1}$ and the maximum photosynthesis at the irradiance levels of 600 or 820 μmol $m^{-2}$ $s^{-1}$ in the different treatments. One-way analysis of variance was performed and the differences between the treatment means were compared by the t-test (SAS Institute Inc., 1994) (III).

In order to evaluate the effects of heavy metal deposition on *E. nigrum* derived from the two sites, the means of the measured parameters (soil moisture, chlorophyll, organic acid, ABA contents, stem water potential) (IV) and the means of different elements in cell compartments (V) were compared by the t-test after logarithmic transformations had been performed to normalise the data. Otherwise the Kruskal-Wallis, comparison of mean ranks test, was used (Sokal and Rohlf, 1995; Statistix, 1996) (IV, V).

## 3. RESULTS

### 3.1. Chemical composition of *E. nigrum* and *C. vulgaris* in response to element applications (I, II, III)

Table 2 summarizes the maximum concentrations of heavy metals in different parts of living *E. nigrum* and *C. vulgaris* when exposed to heavy metals in greenhouse (I, II, III). The maximum metal concentrations in plants were usually the result of the highest applied metal concentrations in the nutrient solution with some exceptions. The experimental setup and thus the maximum concentrations in nutrient solutions applied to the plant roots (I, II) or aboveground parts (III) see papers I-III.

Table 2. The maximum element concentrations in different parts of living *E. nigrum* and *C. vulgaris* after element applications to the roots (I, II) or aboveground parts (III).

| Species | Metal applied | Plant part | Maximum concentrations in the living tissue mg $kg^{-1}$ |
|---|---|---|---|
| *C. vulgaris* | Cu to roots (II) | old roots | 2240 |
| | | stems of the leading shoots | 1370 |
| | | stems of the side branches | 710 |
| | | discoloured leaves | 710 |
| | | new roots | 540 |
| | | green leaves | 60 |
| *E. nigrum* | Cu to roots (I) | older stems | 2030 |
| | | older discoloured leaves | 470 |
| | | younger stems | 180 |
| | | younger discoloured leaves | 140 |
| | | younger green leaves | 80 |
| | Ni to roots (I) | older stems | 2130 |
| | | younger stems | 580 |
| | | younger discoloured leaves | 340 |
| | | older discoloured leaves | 310 |
| | | younger green leaves | 100 |
| *E. nigrum* | Cu to aboveground | bark (older) | 2150 |
| | parts (III) | leaves (older) | 260 |
| | | stems (older) | 80 |
| | Ni to aboveground | bark (older) | 160 |
| | parts (III) | leaves (older) | 20 |
| | | stems (previous-year) | 30 |
| | Fe to aboveground | bark (older) | 660 |
| | parts (III) | leaves (previous-year) | 250 |
| | | stems (previous-year) | 200 |
| | Pb to aboveground | bark (older) | 50 |
| | parts (III) | leaves (older) | 10 |
| | | stems (current-year) | 6 |
| | Zn to aboveground | bark (older) | 330 |
| | parts (III) | leaves (older) | 80 |
| | | stems (current-year) | 30 |
| | Cd to aboveground | bark (older) | 6 |
| | parts (III) | leaves (older) | 0.6 |
| | | stems (older) | 0.4 |

### *3.1.1. Accumulation of Cu and/or Ni in* E. nigrum *(I)*

During the six-week course of the experiment, *E. nigrum* accumulated increasing amounts of Cu and/or Ni with increasing Cu and/or Ni levels in the nutrient solution. Metal accumulation increased with age, the younger parts containing less Cu and Ni than the older ones. The accumulation pattern was similar independant whether the Cu and Ni were applied separately or in combination, and it was similar over the whole concentration range. The highest Cu and Ni concentrations were measured in the oldest stems (Table 2). The roots were not analysed due to technical problems.

### *3.1.2. Accumulation of Cu, Ni, Pb, Zn, Fe, Cd, Mn, Mg, Ca, P, K, N in* E. nigrum *(III)*

The concentrations of Cu, Ni, Pb, Zn, Fe and Cd in the leaves and bark of *E. nigrum* generally increased due to increasing concentrations of those elements in the spraying solution. In contrast, the Mn, Mg, Ca, P, K and N concentrations did not increase in any plant parts in response to increasing nutrient applications. Cu, Ni, Pb, Zn, Fe and Cd accumulation was the highest in the older bark (Table 2) and increased with increasing age in the leaves and bark. In the stems, the age-dependant accumulation was not found (III).

### *3.1.3. Accumulation of Cu in* C. vulgaris *(II)*

During the six-week course of the experiment, Cu concentrations increased in all parts of *C. vulgaris* with increasing Cu levels in the nutrient solution. The highest Cu concentrations were measured in the old roots and stems of the leading shoots (Table 2). In the living plants the concentrations were considerably lower than in the dead plants (II).

## 3.2. Ecophysiological responses of E. nigrum to metals in the greenhouse and field (III, IV)

### *3.2.1. Photosynthesis of* E. nigrum *(III, IV)*

The sum of chlorophyll ($a+b$) did not change, while the chl $a/b$ ratio increased slightly with increasing element (Cu, Ni, Pb, Zn, Fe, Cd, Cr, Mn, Mg, Ca, P, K, N) applications in the

greenhouse (III). Unlike in the greenhouse, the plant chlorophyll (*a+b*) contents were lower at 0.5 than at 8 km from the Cu-Ni smelter, and the differences between the means were statistically significant throughout the whole season ($p < 0.05$) (IV). The means of the chlorophyll (*a+b*) contents varied between 1.9 to 3.2 μmol chlorophyll $g^{-1}$ dw, and were the lowest in October compared to the other sampling dates. The chlorophyll *a/b* ratio was generally lower at a distance of 8 km than at 0.5 km, and the difference between the means at the two distances was statistically significant ($p < 0.05$) in mid August (IV).

In the greenhouse the highest $CO_2$ exchange rate of *E. nigrum* was measured at the irradiance levels of 600 and 820 μmol $m^{-2}$ $s^{-1}$. Increasing element concentrations (Cu, Ni, Pb, Zn, Fe, Cd, Cr, Mn, Mg, Ca, P, K, N) in the spraying solution decreased dark respiration and maximum photosynthesis of the *E. nigrum* current-year shoots (III).

### *3.2.2. Stem water potential and ABA of* E. nigrum *(III, IV)*

The increasing treatment levels in the greenhouse had no consistent effects on the water potential of *E. nigrum*, which varied between -11 to -13 bars (III). In the field the stem water potential of *E. nigrum* varied more and was more negative during the day (-15 to -21 bars) than at night (-4 to -12 bars) (IV). During the day, the water potential of plants at 8 km was more negative than those at 0.5 km. However, during the night the opposite occurred (more negative at 0.5 km). The means of the stem water potential measured during the day and at night in July and during the day in August differed significantly between the plants growing at the two sites ($p < 0.05$) (IV).

In the greenhouse the abscisic acid contents varied between 13 to 66 ng $g^{-1}$ dw. The spraying treatment appeared to affect the ABA contents, the highest contents being in treatments IV and VI, and the lowest values in treatments II and III (III). In the field, the abscisic acid content varied more (IV) than in the greenhouse studies (III). The mean ABA contents in the leaves varied between 66-232 ng $g^{-1}$ dw and 56-196 ng $g^{-1}$ dw, and in the stems between 45-113 ng $g^{-1}$ dw and 33-71 ng $g^{-1}$ dw at 0.5 and 8 km, respectively. *Empetrum* plants growing at 0.5 km had higher abscisic acid contents in their stems compared to those growing at 8 km. With the exception of the July sampling, a similar pattern was also ob-

served in the leaves. No statistical differences were found between the means ($p > 0.05$). In late autumn the ABA contents decreased in the stems, but not in the leaves (IV).

### *3.2.3. Organic acid contents of* E. nigrum *in the field (IV)*

The citric acid contents in the leaves and stems of *E. nigrum* were higher at 8 km than at 0.5 km. In the stems, the difference between the means was statistically significant ($p < 0.05$). In the leaves, the mean pools of citric acid were 16 nmol $mg^{-1}$ dw at 8 km and 13 nmol $mg^{-1}$ dw at 0.5 km, and thus exceeded those in the stems of 14 nmol $mg^{-1}$ dw and 11 nmol $mg^{-1}$ dw, respectively (IV).

The pools of malic acid in the leaves and stems of *E. nigrum* were higher at 8 km than at 0.5 km. The difference between the means of the malic acid content of the stems was statistically significant ($p < 0.05$). In the leaves, the mean malic acid values exceeded those in the stems, and were 17 nmol $mg^{-1}$ dw at 8 km and 14 nmol $mg^{-1}$ dw at 0.5 km, compared to 13 nmol $mg^{-1}$ dw and 8 nmol $mg^{-1}$ dw, respectively, in the stems (IV).

## 3.3. Ultrastructural element localization of *E. nigrum* in the field (V)

The heavy metal localization in stems of *E. nigrum* analysed by EDXS varied according to the metal and tissue, and the amounts (peak to background ratio) of Cu, As and Fe were higher near to the smelter than in the stems from the control site. The highest Cu amounts were measured in the vessel lumens and primary wall of the ray cells, and Cu amounts were elevated both in living (ray cells, phloem, parenchyma of the primary ray) and in dead cells (xylem, sclereids). Cu was rather evenly distributed among the tissue. The highest As amounts were measured in the outer regions of the stem cross-section and lower As amounts were generally detected in vessels and tracheids of the xylem. In general, ray cells, phloem and sclereids had higher Fe amounts than other tissues from the contaminated stem samples. Unlike in the Fe amounts, the Al amount in many cell compartments was higher in the highly contaminated site, but the difference between the two distances was very small, and the variation could not be explained by the effect of the site. The highest Al amounts were detected in the electron-dense material of the ray cells. In contrast to the elements described above, the Mn amounts in the different cell compartments of the stems were lower near to the smelter. High Mn amounts were detected in low contaminated samples, especially in

living tissue (ray cells, phloem, parenchyma of the primary ray) and the cell walls of sclereids of the stem. In contrast, the amounts were very low in the xylem of the vascular tissue (V).

The amounts of macronutrients varied in the different cell compartments. The Ca amounts were generally higher at 8 km than at 0.5 km distance from the smelter, the highest amounts occurring in the electron-dense material of vessel lumens. Ca was mainly located in the living parts of the tissue, in ray cells, phloem and parenchyma of the primary ray, and especially in the primary cell walls. The amounts in the primary cell walls of dead tissue (vessels, tracheids, sclereids) were lower. The K amounts were higher at 0.5 km than at 8 km. The highest K amounts were measured mainly in the living parts of the tissue, i.e. in the phloem and parenchyma of the primary ray. The P and S amounts did not vary according to the site. There were relatively high P amounts in the ray cells, phloem and parenchyma of the primary ray, whereas only low amounts were detected in the vessels and tracheids of the xylem and sclereids. In the living tissue of the stem cross-section, P and S were mainly located in the cytoplasm or electron-dense material. In contrast, only low amounts were found in the vacuoles and lumens (V).

## 3.4. Growth of *E. nigrum* and *C. vulgaris* in response to Cu and/or Ni applications (I, II)

### *3.4.1. The elongation and biomass of* E. nigrum *in response to Cu and/or Ni (I)*

Elevated Cu or Ni concentrations in the nutrient solution decreased the elongation of the shoots and the dry weights of the current-year shoots (leaves and stems combined) and roots, and increased the biomass of discoloured leaves of *E. nigrum*. The maximum growth reduction of the elongation of the leading shoot and side branches was 72-79% when treated with 100 mg $l^{-1}$ Cu. The corresponding reductions for 100 mg $l^{-1}$ Ni were 96% and 85%. Ni therefore reduced elongation more than Cu. When Ni was added to the highest Cu level (100 mg $l^{-1}$) there were no differences in length growth compared to the plants treated only with Cu (100 mg $l^{-1}$ ). The overall survival was not affected (I).

Both Cu and Ni suppressed biomass production. Compared to the highest dry weight, the dry weight of the current-year shoot was limited by 78 and 79 % in plants treated with 100 mg $l^{-1}$ Cu or Ni, respectively. At low Cu levels (Cu <46 mg $l^{-1}$) Ni increased the growth suppression, but at higher Cu concentrations (46, 100 mg $l^{-1}$) there was no effect (I).

Root growth was affected already at relatively low levels of Cu and Ni. The root growth of *E. nigrum* was only about 1 to 4% of the total belowground biomass when the Cu or Ni concentrations in the nutrient solution were more than 22 mg $l^{-1}$. When a combination of the two metals was given, the maximum proportion of new root dry weight out of the total below ground biomass was 6% (46 mg $l^{-1}$ Cu and 10 mg $l^{-1}$ Ni applied) (I).

### *3.4.2. The elongation and biomass of* C. vulgaris *in response to Cu (II)*

In greenhouse experiment, the growth and survival of *C. vulgaris* were strongly decreased due to Cu application. The maximum growth reduction of the leading shoots of *C. vulgaris* was 86-99%, and that of the side branches 84-92%, compared to that for the control treatment (1 mg $l^{-1}$ Cu) (II).

The total shoot biomass of *C. vulgaris* decreased less than the length growth, the maximal reduction in biomass varying from 52 to 67% in the different seedling origins. The biomass of discoloured leaves of *C. vulgaris* increased with increasing metal applications (II).

Root growth was affected already at relatively low levels of Cu. Root growth of *C. vulgaris* was almost totally inhibited by 100 mg $l^{-1}$ Cu, the proportion of new roots being only 2-3% at the highest Cu concentration (II).

# 4. DISCUSSION

## 4.1. Heavy metal accumulation in *C. vulgaris* and *E. nigrum* in controlled conditions

### *4.1.1. Measured concentrations*

The greenhouse studies showed that maximum concentrations in the living roots (over 2000 mg $kg^{-1}$ Cu dw) and stems (over 1000 mg $kg^{-1}$ Cu dw) of *C. vulgaris* were at the same level as those reported in the experimental study of Burt (1984) on non-mycorrhizal plants. Bradley et al. (1981, 1982) reported even higher shoot and root concentrations in mycorrhizal and non-mycorrhizal plants. The Cu concentrations in plants growing in Cu-polluted soil have been considerably lower. In Great Britain, the Cu concentration in *C. vulgaris* fine roots was only about 140 mg $kg^{-1}$ and in brown shoots 90 mg $kg^{-1}$, even though the total Cu concentration in the spoil heap was 2500 mg $kg^{-1}$ (Burt, 1984). 115 mg $kg^{-1}$ Cu has been reported in green shoots (Marrs and Bannister, 1978) (II).

The Cu and Ni concentrations (over 2000 mg $kg^{-1}$) in the different plant parts of *E. nigrum*, when applied to the roots, were higher than those measured earlier in the living tissue of *E. nigrum*. The highest reported Cu concentration in older living parts has been 1450 mg $kg^{-1}$ (Helmisaari et al., 1995) and the Ni concentration in leaves 1510 mg $kg^{-1}$ in the field (Chertov et al., 1993) (I). When Cu was applied to aboveground parts, the bark contained a maximum of over 2000 mg $kg^{-1}$ Cu even though the tissue was washed with distilled water before analysis (III). These measured concentrations are high compared to e.g. barley of which the zero yield was obtained in the tissue concentration of 250-850 μg $g^{-1}$ Ni dw or 40-100 μg $g^{-1}$ Cu dw (Beckett and Davis, 1977). However, the tissue of hyperaccumulators growing mainly in serpentine soils may contain 10 000 - 30 000 μg $g^{-1}$ Ni dw (Lee et al., 1978).

Because the metal concentrations (Cu, Fe, Pb, Cd, Ni and/or Zn) increased with increasing treatment levels when metals were applied to the roots (I, II) or to the aboveground parts (III) of the dwarf shrubs, these results suggest that not only root uptake of Cu and Ni but

also surface contamination significantly contribute to the levels of these metals in the aboveground parts of plants exposed to aerial pollution.

### *4.1.2. Accumulation pattern*

The Cu concentrations in *C.vulgaris* generally decreased in the order (also dead plants included): old roots > new roots > stems > discoloured leaves > green leaves (II). When Cu and Ni were applied to the roots of *E. nigrum*, the accumulation pattern of Cu and Ni was stems > discoloured leaves > green leaves. Roots of *E. nigrum* were not studied but in the field relatively lower concentrations of Cu have been found in the roots compared to the older tissues (Salemaa and Vanha-Majamaa, 1998; Uhlig et al., 2000). The accumulation pattern of these metals within *E.nigrum* was in good agreement with the values reported for *E. nigrum* growing in the field (Uhlig et al., 2000) (I). When metals (Cu, Fe, Pb, Cd, Ni, Zn) were applied to the aboveground parts the concentrations decreased generally in the following order: bark > leaves > stems (III). The accumulation pattern in all the studies (I, II, III) was similar over the whole concentration range. Because this pattern was not restricted to the highest metal applications, it seems that root-to-shoot transport as well as restricted transport to the green leaves occur (I, II).

Increasing heavy metal concentrations with age is a pattern generally found in the aboveground parts of *E. nigrum* growing in the field (Helmisaari et al., 1995; Uhlig et al., 2000). Both greenhouse studies confirmed the results obtained in the field, suggesting that *E. nigrum* accumulates metals in its older tissues especially (I, III). The accumulation pattern seems to be the same irrespective of whether Cu and Ni are applied to the roots (I) or to the aboveground parts (III). The stems of other dwarf shrub species, *Vaccinium uliginosum* and *Arctostaphylos uva-ursi*, have been found to have higher concentrations of Cu in the older than in the younger parts. However, the difference in concentrations between the age classes for *A. uva-ursi* is not as high (Salemaa and Vanha-Majamaa, 1998) as for *E. nigrum*. The strong surface binding of heavy metals in the older tissue of *E. nigrum* (III) is also in good agreement of the results of Uhlig et al. (2000), who found that the litter of *E. nigrum* contains considerable amounts of heavy metals.

## 4.2. Ecophysiological responses of *E. nigrum* to elements in greenhouse and field conditions

### *4.2.1 Photosynthesis of* E. nigrum

The chlorophyll contents of *E. nigrum* were not affected by the aerial application of a mixture of heavy metals (Cu, Fe, Pb, Cd, Cr, Ni, Zn) in the greenhouse (III). At 0.5 km from the smelter, where high heavy metal concentrations enter the plant from the toxic soil via the roots, the chlorophyll concentrations of *E. nigrum* were 8 to 30% lower than the values for plants growing at 8 km (IV). Similar decreases in chlorophyll concentrations have been reported for several broadleaved species exposed to metals (Angelov et al., 1993; Bishnoi et al., 1993). The chlorophyll *a/b* ratio increased slightly with increasing metal treatments in the greenhouse (III), and this was also seen in the *E. nigrum* leaves near to the Cu-Ni smelter in the field (IV). The chlorophyll *a/b* ratio is used as a stress indicator and has been reported to increase as a result of environmental stress (Delfine et al., 1999).

The light saturation for *E. nigrum* occurred between 600 and 800 μmol $m^{-2}$ $s^{-1}$ and was about the same as found for *Vaccinium angustifolium* (500 - 600 μmol $m^{-2}$ $s^{-1}$) (Hicklenton et al., 2000). Although the chlorophyll concentrations were not affected in a study where a mixture of metals was applied to the aboveground parts (III), there were signs of a decrease in dark respiration and maximum photosynthesis due to the aerial application. No positive correlation was found between the applied N concentrations and $CO_2$ exchange, although an increased N content generally increases the photosynthetic rate (Hoogesteger and Karlsson, 1992). The positive correlation between leaf-N concentration and $CO_2$-rate of *Vaccinium vitis-idaea* and *Vaccinium myrtillus* has also been found (Gerdol et al., 2000b). However, despite the high N concentration in the spraying solution, the leaf N concentration of *E. nigrum* did not increase (III). Also the form of nitrogen applied affects; $CO_2$-exchage rate of *Vaccinium corymbosum* L. was higher when the roots were supplied to ammonium nitrogen with or without calciumcarbonate than in a case when nitrate nitrogen was applied (Claussen and Lenz, 1999).

### *4.2.2. Water stress of* E. nigrum

The mixture of heavy metals had no clear effect on the water potential of *E. nigrum* in the greenhouse (III). The fact that heavy metal pollution affected the water potential in the field (IV) indicates that the metals applied to the aboveground parts remained on the surface of *E. nigrum* (III). In contrast, the ABA contents of *E. nigrum* leaves and stems increased slightly with increasing metal treatments (III) or deposition in the field (IV), the results being partly in agreement with earlier studies showing heavy-metal induced ABA accumulation (Rauser and Dumroff, 1981; Poschenrieder et al., 1989). In manipulation studies the ABA content increased 2-fold as a result of excess Ni in bean leaves (Rauser and Dumbroff, 1981). This increase is similar to that in the *E. nigrum* leaves exposed to the highest heavy metal treatment (III). The overall ABA contents of the *E. nigrum* leaves were lower in the greenhouse experiment (III) than in the field (IV). The time of the year also affected the ABA contents when measured in the field. The ABA contents were high in the leaves in October, which may be related to environmental conditions, e.g. to decreasing temperature and ageing of the leaves (IV).

The ABA content in the leaves usually rises as the leaf water potential falls (Beardsell and Cohen, 1975). The leaf ABA content was higher and stem water potential lower at 0.5 than at 8 km distance from the Harjavalta smelter in July, but not in August. However, in the early stages of soil desiccation, ABA is transported as a chemical signal from the roots to the leaves where, by enhancing stomatal closure, it prevents a decline in the water potential (Davies and Zhang, 1991). Furthermore, the production of ABA in nutrient-deficient plants seems to occur at less negative water potentials (Radin, 1984). Near to the smelter at Harjavalta, *E. nigrum* is suffering from low concentrations of Mg and Mn, although the concentrations of other nutrients (N, P, Ca, K) in the plant tissue are not significantly lower (Uhlig et al., 2000). In addition to nutrient deficiency, the plants may require less water for growth and photosynthesis as these metabolic processes have declined. The roots, where ABA synthesis occurs (Marschner, 1995), are damaged near to the smelter and this could, in turn, affect ABA synthesis and subsequently the measured ABA contents (IV).

### *4.2.3. Organic acid contents of* E. nigrum

The organic acid contents were measured in the field in order to determine whether there is any connection between organic acid and Ni and Zn concentrations in the plant. However, the organic acid contents in *E. nigrum* stems and leaves were lower in the plants growing at 0.5 km than at 8 km distance from the smelter, and therefore the results of our study do not follow the generally accepted pattern. Elevated pools of organic acids are normally associated with elevated heavy-metal levels in the substrate (especially Ni and Zn) (Lee et al., 1978; Godbold et al., 1984; Yang et al., 1997). Organic acids are known to take part in the uptake and transport of metals, and to accumulate in the cytosol or vacuoles of plants (Ernst, 1975; Ernst et al., 1992). For this reason, their concentrations increase in plant parts as a results of Ni or Zn stress (Lee et al., 1978; Godbold et al., 1984; Yang et al., 1997). At Harjavalta, however, the lower metabolic efficiency measured by the decreased chlorophyll contents might explain the decreased production of organic acids near the smelter (IV).

## 4.3. Ultrastructural localization of elements in *E. nigrum*

The higher Cu, As and Fe amounts in the cellular compartments of stems of *E. nigrum* collected from the highly contaminated area compared to the low contaminated plots were in good agreement with greenhouse study (I) and earlier results obtained near to the Harjavalta Cu-Ni smelter (Helmisaari et al., 1995; Uhlig et al., 2000). Although arsenic concentrations in the soil and plants have not been earlier reported, the emissions from the smelter do in fact also contain As (Rantalahti, 1995). The studies of Derome and Lindroos (1998a) and Uhlig et al. (2000) support our results about higher Mn amounts in different cell compartments at 8 km than at 0.5 km.

The cellular K, P and S amounts by EDXS reflected the macronutrient concentrations in the plant parts at the two sites, the nutrient concentrations (Ca, K, P and S) in *E. nigrum* being approximately the same or higher near the smelter than further by chemical analyses (Uhlig et al., 2000). Although the accumulation of metals was not clearly associated with the elevated amounts of macronutrients, the localization of nutrients in different cell compartments

was in agreement with the function of these elements in the cells (e.g. Marschner, 1995; Keltjens and van Beusichem, 1998).

No connection was found between the location of heavy metals and nutrients. The highest element (Ca, P, S, Al, As) peaks were found in the electron-dense material but these were not localized in the same tissue or distance. According to light microscopical examinations this electron-dense material consisted partly of phenolic material. Due to the higher frequency of this phenolic, electron-dense material and the high heavy metal amounts measured by EDXS in the stems from the highly polluted site as well as the importance of phenolics in the ecology of *E. nigrum* (e.g. Nilsson, 1992; Wallstedt, 1998), it can be assumed that this phenolic electron-dense material might have a function in the heavy metal tolerance of *E. nigrum*. Kukkola (1999) reported increased tannin production in pine needles, which was seen as dark staining of the central vacuole in the Cu and Ni treatments, and suggested that the dark staining may be caused by metal accumulation in the vacuoles (Kukkola, 1999). The increase in tannin amount in mesophyll cell vacuoles of pine needles, however, could be also due to the nutrient deficiencies (Kukkola et al., 1997) as shown by Holopainen and Nygren (1989).

In *Minuartia verna* leaves Cu and Fe were found in the cell walls of parenchyma and Cu, Fe and Al in leaf surface, whereas the cell organelles did not contain any metals (Neumann et al., 1997). In this study, especially As was localized more frequently in the primary cell walls of living cells (ray cells, phloem) than dead cells, although it also accumulated in the cytoplasm and vacuoles. Fe was more frequent in the living (ray cells, phloem) than dead tissue (xylem) and Al did not show very clear pattern between the distances. Cu was not clearly distributed in certain cell organelles of stems. Also Neumann et al. (1995) reported that Cu was located in several cell organelles in leaves and roots of tolerant *Armeria maritima* by electron microscope. Kukkola (1999) found that needles of CuNi irrigated pines in the arctic (North Finland) contained dark accumulation in cytoplasm and concluded that it might be partly due to metal accumulation as Cu is present in different deposits of the cells (Kukkola, 1999).

Although the spectras showed differences in metal amounts in different cell compartments, the elemental mappings made in this study showed that Cu, Fe, As and Al were relatively

evenly distributed among the tissue. Because the heavy metals were not only localized in dead tissue, the heavy metal complexing agents could be involved in heavy metal tolerance of *E. nigrum*. However, heavy metal tolerance is metal-specific (Baker, 1981, 1987), and the specification of complexing agents of certain metals could not be done. Because of the homogeneous distribution of metals in the tissue, it seems that not only one mechanism is involved in heavy metal tolerance of *E. nigrum*. Most of the heavy metals are present in the xylem as free cations or complexed with organic acids, while Cu is mainly transported in the xylem in complexed form (Graham, 1979; White et al., 1981; Kochian, 1991). Cu tolerance has been found to be achieved by chelating the Cu with proteins and vacuolar phenolic compounds in leaves and roots (e.g. Rauser, 1984; Neumann et al., 1995). Cu and Fe accumulation in *C. vulgaris* ericoid mycorrhizal roots has been suggested to be responsible for the reduction of Cu and Fe transport to the shoots when exposed to high Cu and Fe concentrations (e.g. Bradley et al., 1981, 1982; Shaw et al., 1990). In *E. nigrum* the considerable amounts of metals are transported also to the shoot. Several studies have shown the connection between Al tolerance and organic acids (e.g. Foy et al., 1990; Larsen et al., 1998). Especially in crops the Al-induced organic acid release from roots is an important Al tolerance mechanism (Kochian and Garvin, 1999). Ahonen-Jonnarth (2000) also found that the oxalic acid production of *Pinus sylvestris* mycorrhizal and non-mycorrhizal roots increased when exposed to Al or Cu.

### 4.4. The morphological responses of *C. vulgaris* and *E. nigrum* to Cu and/or Ni in controlled conditions

In this study, as well as in previous studies (e.g. Roth et al., 1971; Taylor and Crowder, 1983; Krämer et al., 1996; L'Huillier et al., 1996), the growth parameters of plants were found to clearly respond to Cu and Ni. A clear decrease in the elongation and biomass of shoots and roots indicate the toxicity of elevated concentrations of Cu and/or Ni to *C. vulgaris* and *E. nigrum* (I, II) shown also by ecophysiological parameters of *E. nigrum* near the Cu-Ni smelter at Harjavalta (IV). The growth decrease in greenhouse was higher than that measured in the field at Harjavalta (Salemaa et al., 1995) and in the Kola Peninsula (Shevtsova, 1998). Shevtsova (1998) reported 30% decrease in length growth and 65% decrease in biomass of *E. nigrum* in the Kola Peninsula compared to the unpolluted areas, where both increased heavy metal concentrations and $SO_2$ affects on the growth of the

plants. In greenhouse, the maximum applied concentrations were high, because other factors (e.g. organic matter) that could affect the availability of metals in the soil-plant system were not present. In addition, at Harjavalta, the sulphuric acid and heavy metal load have strongly decreased since 1980's (Rantalahti, 1995) and the pollutants enter the plant as dry or wet deposition. The most severe stress for plants, however, are the toxic metal concentrations in the soil. At Harjavalta, the total metal pool is not available for plants, because the Cu and Ni are in the soil partly in the complexed form (Derome and Lindroos, 1998b). At 0,5 km distance Cu is accumulated in the organic layer whereas Ni is less readily bound and more uniformly ditributed down the soil profile. This indicates that Cu is strongly retained in the organic layer and forms rather stable complexes with organic matter while Ni is more mobile (Goryainova and Nikonov, 1993) and therefore more plant available than Cu (Derome and Lindroos, 1998b).

The relatively high decrease in growth already at the concentration of 10 mg $l^{-1}$ was surprising, because according to the pilot study, the growth of *E. nigrum* increased two times in the presence of 5 mg $l^{-1}$ Cu compared to that of 0 mg $l^{-1}$ Cu, indicating that *E. nigrum* would need some amounts of Cu for maximal growth (Monni and Salemaa, 1998). However, the pilot study was done earlier in the growing season, which might affect on the measured length growth of *E. nigrum*.

## 4.5. The heavy metal resistance of *C. vulgaris* and *E. nigrum*

### *4.5.1. Mechanisms*

As mentioned above, *E. nigrum* tolerated elevated concentrations of Cu and Ni in the stems, while transport to the green leaves was restricted (I). In the cellular level elevated As, Cu and Fe amounts were localized in cell walls, cytoplasm and vacuoles and phenolic electron-dense material might also contribute to the heavy metal tolerance of *E. nigrum* (V). *C. vulgaris* had higher Cu concentrations in discoloured leaves, stems and roots (II). According to the growth responses and survival, *E. nigrum* was more tolerant to Cu than *C. vulgaris* (I, II) as reported also by Laaksovirta and Silvola (1975). Also Gilbert (1975) reported that dying *C. vulgaris* was replaced by *E. nigrum* near aluminium smelters.

The greenhouse experiment partly helps to explain occurrence of *E. nigrum* in highly polluted areas near the smelters (I). As mentioned above, according to Baker and Walker (1989), tolerant individuals can be separated from non-tolerant ones on the basis of their ability to establish, survive and reproduce in metal-contaminated substrates. *E. nigrum* is one of the surviving species near the smelters (e.g. Salemaa et al., 2000b). It is capable of producing berries near the Cu-Ni smelter at Harjavalta and viable seeds occur at Harjavalta (personal observation) and in seedbank soil at Kola Peninsula (Komulainen et al., 1994) but, because of the toxic metal concentrations in the soil (Derome and Lindroos, 1998a; Uhlig et al., 2000), no new seedlings or vegetative reproduction have been observed. In natural habitats where the heavy metal concentrations are low, *E. nigrum* usually reproduces vegetatively (Ojala, 1991).

Not only the innate tolerance of *E. nigrum*, but also the heterogeneity of the contaminated soil may partly explain the successful survival of *E. nigrum* in Harjavalta and Kola Peninsula (Uhlig et al., 2000). Uhlig et al., (2000) reported that beneath *E. nigrum* patches (microsites) the concentrations of Cu, Ni, Fe, Pd, Zn, Al in the organic soil were considerably higher, but those in the mineral soil considerably lower than the concentrations of the surrounding soil, where *E. nigrum* was not growing. They suggested that phenolics might have some role in immobilizing metals beneath *E. nigrum*. In polluted areas the taproot of *E. nigrum* penetrates in the mineral soil, and the living fine roots occur in the less polluted mineral soil. In the reference areas, in contrast, the fine roots lie in the organic soil. Watmough and Dickinson (1995) discussed that the spatial heterogeneity of metal concentrations and availability in surface soils might provide partial explanation for the survival of long-lived plants. Turner and Dickinson (1993) showed that the *Acer pseudoplatanus* L. (sycamore) did not tolerate metals although growing on the metal contaminated soils. They found that roots could grow in uncontaminated zones of the soils, and therefore seedlings survived in the contaminated soil. They concluded that the phenotypic plasticity might have an importance in long-lived plants (Turner and Dickinson, 1993).

The environmental conditions may regulate the occurrence of *C. vulgaris* and explain why it grows in heavy metal polluted areas on oceanic heathlands but not in polluted boreal forests. In addition to high metal concentrations (Derome and Lindroos, 1998a), drainage and a deficiency of base cations near the Cu-Ni smelter at Harjavalta (Derome and Nieminen, 1998)

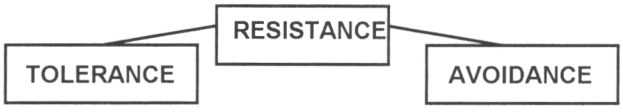
may explain the absence of *C. vulgaris*. The water supply is known to be critical for seedling establishment of *C. vulgaris* (e.g. Gimingham, 1972) (II).

It has been suggested in earlier studies that the Cu resistance of *C. vulgaris* is based on an ericoid mycorrhiza, which accumulates the metals and thus prevents Cu transportation to the shoots of *C. vulgaris* (Bradley et al., 1981, 1982; Burt, 1984). Indications of true Cu tolerance in *C. vulgaris* have also been reported (Burt, 1984). The higher Cu concentrations in the roots than in the shoots found in this study support the hypothesis of Bradley et al. (1981, 1982) about the avoidance mechanism. Hashem (1987) compared the copper tolerance between the ectomycorrhizas and ericoid mycorrhiza *Hymenoscyphus ericae* colonizing the roots of *C. vulgaris* and found that *Hymenoscyphus ericae* was more tolerant than other species (Hashem, 1987). Shaw (1987), in contrast, researched the Al and Fe concentrations in the shoots and roots in mycorrhizal and non-mycorrhizal *C. vulgaris* seedlings, and found that the accumulation was metal-specific. Shoot Fe concentrations were significantly higher in mycorrhizal than non-mycorrhizal plants in low and high Fe concentrations. In contrast, Al concentrations in shoots and roots of mycorrhizal *C. vulgaris* seedlings were significantly lower than those in non-mycorrhizal seedlings (Shaw, 1987).

Based on the high concentrations in the stems, Cu was also transported to the stems of *C. vulgaris*. The discoloured leaves had higher Cu concentrations than the green leaves, indicating that in higher concentrations *C. vulgaris* could not restrict the Cu transport to the leaves. Marrs and Bannister (1978) and Burt (1984) concluded that *C. vulgaris* could detoxify and remove metals by accumulating them in older shedding leaves and stems (II).

The results of this thesis, combined with the findings of earlier studies, provide a lot of new information about the heavy metal resistance of dwarf shrubs in the polluted area. However, although this study has answered many questions, many questions still remain open. According to the earlier field studies and this thesis, the specific tolerance and avoidance mechanisms of *E. nigrum* and *C. vulgaris* can be assumed to follow the pattern shown in Figure 3, especially concerning the heavy metal resistance of *E. nigrum*.

In addition to tolerance and avoidance mechanims the natural capability to adapt in changing environment and conditions where metal availability is high, is probably partly the reason for the successful survival of *E. nigrum* in the polluted areas.




| TOLERANCE | AVOIDANCE |
|---|---|
| • Accumulation of Cu, Ni, Fe, Pb, Zn, Cd in older tissues (*E. nigrum*) -field (Helmisaari et al., 1995; Uhlig et al., 2000), greenhouse (I)<br>• Accumulation of Cu, Zn and Pb in roots (*C. vulgaris*). Connection to mycorrhizas not shown. -greenhouse (II), field (Marrs and Bannister, 1978; Burt, 1984)<br>• Accumulation of elevated Cu, Zn and Pb in discoloured leaves and stems (*C. vulgaris*) -field (Marrs and Bannister, 1978; Burt, 1984), greenhouse (II).<br>• Restriction of the metal transport to the green leaves (*E. nigrum*) (Cu, Ni, Fe, Pb, Zn, Cd) -field (Uhlig et al., 2000), greenhouse (I)<br>• Localization of metals (Cu, As, Fe) in cell walls, cytoplasm and vacuoles and possible detoxification of metals by phenolics (*E. nigrum*) -field (V) | • The role of ericoid mycorrhiza in accumulating metals (Cu, Zn, Fe) and preventing the transport to the shoots (*C. vulgaris*) -greenhouse (Bradley et al., 1981, 1982; Burt, 1984; Shaw et al., 1990)<br>• Accumulation and immobilization of metals in the litter beneath *E. nigrum* patches, which could explain the low concentrations of metals in the mineral soil, where the roots of *E. nigrum* occur (near Cu-Ni smelter) -field (Uhlig et al., 2000)<br>• Heterogeneity of the contaminated soil allowing the growth of roots to the uncontaminated zones shown by different root distribution of *E. nigrum* near the Cu-Ni smelters and control soils -field (Uhlig et al., 2000) |

Figure 3. The possible tolerance and avoidance mechanisms of *E. nigrum* and *C. vulgaris* based on this thesis and earlier studies.

### *4.5.2. The estimation of heavy metal resistance in the greenhouse and field*

The positive feature about greenhouse studies is that factors other than heavy metals can be eliminated, whereas in the field the observed responses may be also due to other environmental factors. First of all, the greenhouse conditions are different from those in the field,

where the presence of organic matter, interactions with other species etc. affect metal availability for plants. Secondly, greenhouse experiments are of relatively short duration and therefore only give an approximation of what happens in the field. This is especially the case for long-lived plants although very short experimental periods to estimate the tolerance index by root growth have been used. Punshon and Dickinson (1997) showed that the acclimation of willows to toxic metals was achieved by the gradual increase of the toxic metals in the substrate rather than by a short-term greenhouse experiment. Therefore, the results in the greenhouse should always be compared with the situation in the field where the actual processes take place.

## 5. SUMMARY AND CONCLUSIONS

Dwarf shrub species, besides growing naturally in nutrient-poor and dry conditions, seem to be resistant to heavy metals. The greenhouse studies confirmed the observations made in the field that *C. vulgaris* is more sensitive to Cu than *E. nigrum*. Toxicity symptoms also occurred earlier in *C. vulgaris* than in *E. nigrum*. The exposure of *C. vulgaris* seedlings to Cu caused clear toxicity symptoms in the roots of the plants, and strongly affected survival. Toxicity symptoms also appeared on *E. nigrum* and growth was suppressed, but the metals did not affect survival during the six-week experiment (I, II).

Both species were concluded to be resistant to elevated concentrations of Cu and/or Ni, because the concentrations in the plant parts were well above the level generally assumed to be toxic for plants (e.g. Allaway, 1968; Marschner, 1995). However, the difference in the concentrations between different plant parts was not as clear for *C. vulgaris* (II) as for *E. nigrum* (I). *C. vulgaris* accumulated high concentrations of Cu in its living parts, especially in the roots, stems and discoloured, shedding leaves during the short exposure period, and the accumulation pattern was similar throughout the concentration range (II). In *E. nigrum*, the Cu and Ni concentrations were the highest in the stems, and transport to the green leaves appeared to have been restricted, as reflected by the relatively constant Cu and Ni concentrations in the green leaves. The tolerance mechanism of *E. nigrum* was concluded to be partly achieved through the accumulation of metals in the older stems (I), and partly through the metal accumulation in cell walls, vacuoles and cytoplasm of stems in the cellular level. Heavy metal tolerance might also be achieved through the detoxification of metals in the electron-dense material of the phloem and ray cells. At the cellular level, it seems that not only one specific mechanism contribute to the heavy metal tolerance of *E. nigrum* (V).

Alhough surviving near Cu-Ni smelters in the northern hemisphere (Helmisaari et al., 1995; Uhlig et al., 2000) and accumulating high concentrations of Cu and Ni (I), the vitality of *E. nigrum* near the smelters has clearly been decreased by the heavy metal load. This was apparent from the lower organic acid and chlorophyll contents and higher ABA contents near the smelter (IV). However, the uptake of metals by the leaves seemed to be effectively restricted, because the heavy metals applied to the aerial parts of *E. nigrum* in the greenhouse

were accumulated strongly on the bark and leaves of *E. nigrum*. Surface contamination had negative effects on photosynthesis, although it did not have any effect on the water potential and chlorophyll pigments. It decreased dark respiration and maximum photosynthesis and increased leaf ABA content (III).

An ability to grow in heavy-metal polluted areas makes *E. nigrum* a suitable species for the remediation of contaminated soil. It is one of the plant species, which has been used in a revegetation experiment in the vicinity of the Harjavalta smelter (Kiikkilä et al., 1996). *C. vulgaris*, on the other hand, was more sensitive to metals and is therefore not the most suitable species for the remediation of heavy metal polluted soil in the northern hemisphere.

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# The tolerance of *Empetrum nigrum* to copper and nickel

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**"Capsule":** *Increasing copper and nickel concentration increased adverse effects on growth of* Empetrum nigrum.

**Abstract**

The Cu and Ni tolerance of 3- to 5-year-old cuttings of crowberry (*Empetrum nigrum*) were tested in controlled conditions. Six levels of Cu (0.1–100 mg $l^{-1}$), five levels of Ni (0–100 mg $l^{-1}$) and nine levels of Cu + Ni were applied. The elongation of the shoots, new shoot and root dry weights indicated an adverse effect of increasing Cu and Ni concentrations. At low Cu levels the addition of Ni decreased the dry weights more than at high Cu levels. The results show that *E. nigrum* accumulated high concentrations of Cu and Ni mainly in old stem tissue, which contained a maximum of over 3000 mg $kg^{-1}$ Cu and 1000 mg $kg^{-1}$ Ni. The concentrations of Cu and Ni in *E. nigrum* were higher than those measured in plants growing in areas near to Cu–Ni smelters, but the accumulation pattern was similar. The survival of the cuttings was not affected suggesting that *E. nigrum* possesses an internal heavy metal tolerance. 

*Keywords:* *Empetrum nigrum* (crowberry); Copper; Nickel; Heavy metal tolerance; Cu and Ni accumulation

## 1. Introduction

Recent plant ecological studies have concentrated on crowberry (*Empetrum nigrum* L.) because of its wide ecological amplitude (Elvebakk and Spjelkavik, 1995) and its special ecology in boreal forest ecosystems (Zackrisson et al., 1997; Nilsson et al., 1998). *E. nigrum* is an evergreen dwarf shrub that occurs on a variety of substrates. It can tolerate soil pH values ranging from 2.5 to at least 7.7 (Bell and Tallis, 1973). In the northern hemisphere *E. nigrum* is limited to the cooler regions, but it is also common in mountains at lower latitudes (Good, 1927; Bell and Tallis, 1973). In boreal forests it grows on nutrient-poor, light dry heaths (Sarvas, 1937) and can form single-species plant communities in clear-cut areas (Nilsson, 1992). It also grows on ombrogenous peat and peaty podsols (Good, 1927; Bell and Tallis, 1973). One interesting feature of *E. nigrum* is its ability to influence the establishment of other species; its leaf extracts inhibit, for example, the establishment of Scots pine (*Pinus sylvestris* L.) seedlings (e.g. Nilsson and Zackrisson, 1992; Zackrisson and Nilsson, 1992; Nilsson, 1994).

*E. nigrum* is one of the few understorey species which grows on severely heavy-metal contaminated sites in the vicinity of copper–nickel (Cu–Ni) smelters in the northern hemisphere (Laaksovirta and Silvola, 1975; Chertov et al., 1993; Helmisaari et al., 1995; Uhlig et al., 1996; Shevtsova, 1998). For instance, it survives at a distance of 0.5 km from the Cu–Ni smelter at Harjavalta, southwest (SW) Finland, where almost all the other plant species have disappeared. There have been severe changes in the environment at this site (Helmisaari et al., 1995); the total Cu concentration in the organic layer is over 5800 mg $kg^{-1}$ d.m., Ni concentration 460 mg $kg^{-1}$, while base cations (e.g. $Ca^{2+}$, $Mg^{2+}$) have been displaced from the topsoil. The concentrations of other heavy metals (Fe, Zn, Cd, Pb, Cr) in the organic layer are also high near the smelter (Derome and Lindroos, 1998). An ability to grow in heavy-metal polluted areas makes *E. nigrum* a suitable species for the remediation of contaminated soil. It is one of the plant species which has been used in a revegetation experiment in the vicinity of the Harjavalta smelter (Kiikkilä et al., 1996).

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In general, plants possess several mechanisms enabling them to tolerate high heavy metal concentrations (e.g. Antonovics et al., 1971; Baker, 1981, 1987; Woolhouse, 1983). The metals may accumulate in older tissue or in senescing leaves that are shed (Reilly, 1969). The ericoid mycorrhiza of dwarf shrubs has been suggested to accumulate heavy metals in the roots leading to a reduction in heavy metal concentrations in the shoot (Bradley et al., 1981, 1982; Burt, 1984). Even if the rate of heavy metal uptake can be controlled by plants, total avoidance of metal uptake is not possible (e.g. Baker and Walker, 1990). Heavy metals are mainly taken up via the roots, but uptake via leaves and stems has also been discussed (Chamberlain, 1983; Koricheva et al., 1997).

The heavy metal tolerance mechanism of *E. nigrum* is not well known. Besides the accumulation of metals in older plant parts and the restriction of their transport to the younger parts (Helmisaari et al., 1995; Uhlig et al., 1996), the xeromorphic structure of both the leaves and stems (Miller, 1975; Carlquist, 1989; Wollenweber et al., 1992) might also help survival in heavy-metal polluted areas (Pandolfini et al., 1996), where the water-holding capacity of the soil is usually decreased (Derome and Nieminen, 1998). Brooks et al. (1977, 1980) defined a plant to be a hyperaccumulator of Cu and Ni if the tissue concentration exceeds 1000 mg $kg^{-1}$. Based on field studies (e.g. Chertov et al., 1993; Uhlig et al., 1996), it can be suggested that *E. nigrum* is a hyperaccumulator of Cu and Ni. However, the elevated Cu and Ni levels measured in field samples may be partly due to surface contamination.

It is impossible to differentiate between the toxic effects of different heavy metals on a plant in field conditions. It is also difficult to assess aerial deposition of the metals on the surfaces of leaves and stems compared to the inner accumulation of metals taken up by the roots. In the present study the Cu and Ni tolerance of *E. nigrum* is studied for the first time under controlled conditions. The aim of the experiment was: (1) to investigate the growth and leaf discolouration of *E. nigrum* cuttings exposed to elevated levels of Cu, Ni or to both metals; (2) to determine the interactive effects of these metals; (3) to determine the accumulation pattern of Cu and Ni in different plant parts; and (4) to compare the responses in the experiment to those observed in the field.

## 2. Materials and methods

### 2.1. Experimental setup

The 3- to 5-year-old cuttings of *E. nigrum* used in the experiment originated from an unpolluted area in SW Finland. They were rooted in a mixture of sand and peat. When the experiment was being set up the roots of the cuttings were washed, and 15 root samples were taken to check mycorrhizal infection by a modification of the method of Koske and Gemma (1989). All the fine roots examined were moderately colonized by ericoid mycorrhiza. The roots were cut back to 12 cm to standardize the experiment material and to give the roots sufficient growing space. The roots were dipped in activated carbon to be able to separate, at the end of experiment, stained old roots from white new roots formed during the study. For the experiment the plants were randomised and transferred to plastic pots containing quartz sand (0.5 l).

The cuttings were watered twice a week (50 ml/pot) with a nutrient solution modified by Stribley and Read (1976) containing the following nutrients (mg $l^{-1}$); P, 11.3 ($KH_2PO_3$); K, 28.5 (KCl); Ca, 29.2 ($CaCl_2 \times 2H_2O$); Mg, 8.8 ($MgCl_2 \times 6H_2O$); Fe, 3 ($FeCl_3 \times 6H_2O$); Mn, 0.5 ($MnCl_2$); B, 0.5 ($H_3BO_3$); Zn, 0.1 ($ZnCl_2$); Mo, 0.1 ($Na_2MoO_4$); N, 65 (ammonium and nitrate as $NH_4NO_3$ and ammonium as $NH_4Cl$ in the ratio $NH_4$:$NO_3$ 70:30). Six levels of Cu (0.1, 1, 10, 22, 46 and 100 mg $l^{-1}$), five levels of Ni (0, 10, 22, 46 and 100 mg $l^{-1}$) and nine combinations of Cu and Ni [Cu (mg $l^{-1}$)/Ni (mg $l^{-1}$): 10/10, 22/10, 46/10, 100/10, 22/22, 46/22, 100/22, 46/46, 100/46] were used. Cu was given as $CuSO_4$ and Ni as $NiCl_2$. The control solution in the Ni series contained 0.1 mg $l^{-1}$ Cu, because Cu is a micronutrient. In the Cu series the control solution contained 1 mg $l^{-1}$ Cu. The Cu and Ni concentrations used were selected according to preliminary studies. The concentrations measured in the organic layer near the Harjavalta Cu–Ni smelter represent the real ecological conditions for *E. nigrum* in this polluted environment. Because the Cu concentrations in the soil at Harjavalta are higher than the Ni concentrations (Derome and Lindroos, 1998), we studied how the Cu response changed when a less or equal concentration of Ni was combined with the Cu treatment. This explains why all the combinations were not applied and experiment was not fully factorial. The experimental design was completely random, and the total number of seedlings treated was 152 (eight plants/treatment).

The experiment was carried out in the greenhouse of the Finnish Forest Research Institute at Ruotsinkylä (60°21′ N, 25°00′ E) over the period 1 July to 13 August 1996. The light conditions were natural, and the mean temperature was regulated at +20°C during the day and +15°C at night. The relative humidity in the greenhouse was approximately 60–70%.

### 2.2. Estimating the influence of heavy metals on E. nigrum

The variables used to assess metal toxicity were elongation of the main stem and two side branches

and the leaf, stem, and root dry weights. The accumulation and translocation of Cu and Ni by the roots to aboveground parts of the plant were determined by chemical analysis from different plant parts. The total aboveground uptake of Cu and Ni was determined by the following equation: total Cu or Ni uptake (mg) = total aboveground biomass (kg) × total Cu or Ni concentration (mg $kg^{-1}$ dry wt.) in the aboveground parts.

The total initial length of the cuttings was measured before planting, and the new growth during the experiment calculated as the difference between the initial and final lengths of the leading shoot and side branches. For biomass analyses the material was divided into the following 11 parts: (1) green leaves of current-year growth; (2) discoloured leaves of current-year growth; (3) green leaves of previous-year growth; (4) discoloured leaves of previous-year growth; (5) older green leaves; (6) older discoloured leaves; (7) stems of current-year growth; (8) stems of previous-year growth; (9) older stems; (10) new roots; and (11) old roots. The samples were homogenized, dried at +60°C, and weighed, and the total Cu and Ni concentrations of plant parts 1 to 9 were determined by dry ashing (+550°C), extraction of the ash with 0.2 M HCl, and analysis on an inductively coupled plasma atomic emission spectrometer (ICP–AES) (Dahlquist and Knoll, 1978). Eight replicate samples were combined to obtain enough material for the chemical analyses. The current and previous-year leaves and current and previous-year stems were also combined. In addition, Cu and Ni concentrations were analysed similarly from the green leaves and stems of the cuttings not treated with metals, to know the initial Cu and Ni concentrations of these cuttings.

### 2.3. Statistical analysis

Two-factor analysis of variance (ANOVA) was used in analysing the effects of Cu and Ni and their interactions on the length growth and biomass variables of *E. nigrum* (GLM procedure, SAS Institute Inc., 1994; Sokal and Rohlf, 1995). A few missing values were replaced by the group mean. Pairwise comparisons between the treatments and between the Cu and Ni series were performed by the *t*-test. The initial length of the cuttings was used as the covariate in the variance model. Logarithmic transformations lg $(x+1)$ were made to normalise the data. Relationships between Cu and Ni concentrations in plant parts and applied concentrations were studied by regression models (REG and NLIN procedures, SAS Inst.). Regression equations are also given for Cu and Ni uptake as a function of applied Cu and Ni amounts (SAS Institute Inc., 1994; Sokal and Rohlf, 1995).

## 3. Results

### 3.1. The effect of Cu and Ni on shoot elongation

Elevated Cu or Ni concentrations in the nutrient solution decreased the elongation of the leading shoot and side branches (Fig. 1a, b), and the effects were significant ($p < 0.01$) (Table 2). The overall survival was not affected and only four plants died during the following treatments: Cu 10 mg $l^{-1}$ (one plant), Ni 100 mg $l^{-1}$ (two plants), Cu 22 mg $l^{-1}$, Ni 10 mg $l^{-1}$ (one plant). The length growth was the highest at a concentration of 1 mg $l^{-1}$ Cu for the leading shoot and at 0.1 mg $l^{-1}$ Cu for the side branches. The leading shoot and the two side branches behaved differently, with elongation of the leading shoot generally being better than elongation of the side branches when treated with Cu (Fig. 1a). Ni inhibited the elongation of the leading shoot more than Cu (*t*-test

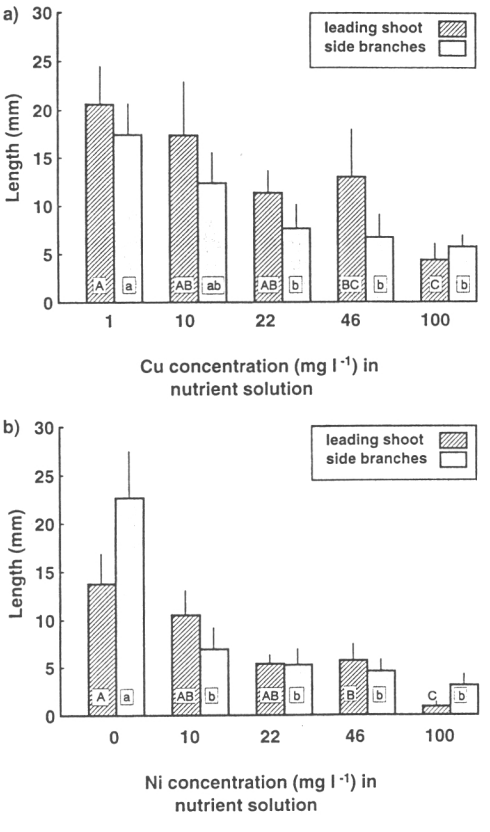


Fig. 1. The mean length growth (mm) of the leading shoot and side branches of *Empetrum nigrum* treated with a nutrient solution containing different concentrations (mg $l^{-1}$) of (a) copper (Cu) or (b) nickel (Ni). The bar indicates the standard error ($n = 8$). Means which do not differ significantly between treatments ($p > 0.05$) are marked with similar capital letters for the leading shoot and small letters for the side branches.

between Cu and Ni series, $p < 0.01$). Growth retardation started at lower Ni concentrations than at comparable Cu concentrations (Fig. 1a, b). Compared to the highest growth (1 mg $l^{-1}$ Cu, 0 mg $l^{-1}$ Ni for leading shoot and 0.1 mg $l^{-1}$ Cu, 0 mg $l^{-1}$ Ni for side branches), the growth reduction of the leading shoot was 79% and of the side branches 72% when treated with 100 mg $l^{-1}$ Cu. The corresponding reductions for Ni were 96 and 85%.

In general, the addition of Ni limited growth to a similar extent at all Cu levels, because the interaction term was not significant (Tables 1 and 2). However, when Ni was added to the highest Cu level (100 mg $l^{-1}$) there were no differences in length growth compared to the plants treated with only Cu (100 mg $l^{-1}$) (Fig. 1a, Table 1).

### 3.2. The effect of Cu and Ni on biomass

The dry weight of the current-year shoots (leaves and stems combined) decreased significantly ($p < 0.001$) with increasing Cu or Ni concentrations in the nutrient solution (Table 2). The dry weight was the highest with the 0.1 mg $l^{-1}$ Cu and 0 mg $l^{-1}$ Ni treatment (Fig. 2a). Cu suppressed biomass production more than Ni (*t*-test between Cu and Ni series, $p < 0.05$). Compared to the highest dry weight (treatment 0.1 mg $l^{-1}$ Cu, 0 mg $l^{-1}$

Table 1
Means for length growth (mm) and biomass (mg) of *Empetrum nigrum* treated with combinations of Cu and Ni[a]

| Cu and Ni applied (mg $l^{-1}$) | Elongation | | Dry weight | | |
|---|---|---|---|---|---|
| Cu/Ni | Leading shoots (mm) | Side branches (mm) | New shoots (mg) | New roots (mg) | Discoloured leaves (mg) |
| 10/10 | $13^{a}$ | 7 | 42 | 2 | $14^{a}$ |
| 22/10 | $8^{ab}$ | 6 | 36 | 3 | $27^{ab}$ |
| 46/10 | $4^{b}$ | 4 | 33 | 3 | $28^{ab}$ |
| 100/10 | $5^{ab}$ | 3 | 29 | 2 | $34^{ab}$ |
| 22/22 | $6^{ab}$ | 3 | 23 | 1 | $18^{ab}$ |
| 46/22 | $5^{b}$ | 7 | 36 | 1 | $52^{b}$ |
| 100/22 | $3^{b}$ | 5 | 31 | 1 | $33^{ab}$ |
| 46/46 | $4^{b}$ | 3 | 23 | 1 | $33^{ab}$ |
| 100/46 | $4^{b}$ | 3 | 27 | 3 | $27^{ab}$ |

[a] $n = 8$, except for side branches $n = 6$–8. Significant differences between the treatment means (elongation of leading shoots and dry weight or discoloured leaves) are marked with different letters. Otherwise there were no significant differences between the treatment means by *t*-test ($p < 0.05$).

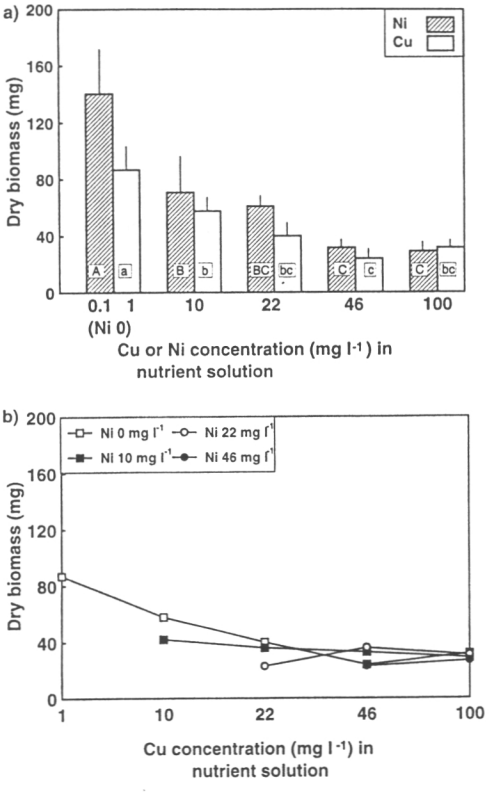


Fig 2. The mean dry biomass (mg) of the current-year growth of *Empetrum nigrum* treated with a nutrient solution containing different concentrations of (a) nickel (Ni) or copper (Cu) (mg $l^{-1}$) or (b) combinations of both Ni and Cu. The bar indicates the standard error ($n = 8$). Means which do not differ significantly between treatments ($p > 0.05$) are marked with similar capital letters for the Ni treatment and small letters for the Cu treatment.

Table 2
*F* and *p* values of two-factor analysis of variance (ANOVA) for log-transformed response variables[a]

| Source of variation | df | Elongation | | | | Dry weight | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Leading shoot | | Side branches | | New shoots | | New roots | | Discoloured leaves | |
| | | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| Cu | 5 | 4.73 | 0.001 | 3.24 | 0.009 | 10.25 | 0.000 | 5.32 | 0.000 | 1.76 | ns |
| Ni | 4 | 7.14 | 0.000 | 7.05 | 0.000 | 6.12 | 0.000 | 3.41 | 0.011 | 5.41 | 0.001 |
| Cu•Ni | 9 | 0.61 | ns | 1.20 | ns | 4.11 | 0.000 | 2.67 | 0.007 | 1.44 | ns |
| Length | 1 | 0.50 | ns | 0.00 | ns | 11.88 | 0.001 | 0.00 | ns | 43.57 | 0.000 |

[a] Initial length of leading shoot has been used as a covariate. ns, not significant.

Ni), the dry weight of the current-year shoot was limited by 78% for plants treated with 100 mg $l^{-1}$ Cu and 79% when treated with 100 mg $l^{-1}$ Ni.

The statistical interaction between Cu and Ni in the dry weights was significant ($p < 0.001$) (Table 2). The effect of adding Ni varied at different Cu levels. At low Cu levels (Cu < 46 mg $l^{-1}$), Ni increased the growth suppression, but at higher Cu concentrations (46, 100 mg $l^{-1}$) there was no effect (Fig. 2b). Compared to the highest dry weight, Cu and Ni in combination suppressed the dry weight by a maximum of 86% when 22 mg $l^{-1}$ Cu and 22 mg $l^{-1}$ Ni or 46 mg $l^{-1}$ Cu and 46 mg $l^{-1}$ Ni were applied.

The dry weight of the discoloured leaves increased as the Ni concentration increased and the effect was significant ($p = 0.001$) (Table 2). A similar trend was also observed with increasing Cu concentrations (Fig. 3a), but the effect was not significant (Table 2). At the lowest Cu and Ni concentrations, 1–4% of the total leaf biomass was discoloured in the current and previous-year growths. The maximum proportion of discoloured leaves out of the total leaf biomass was 43% with the highest Cu concentration and 56% with the highest Ni concentration.

Apart from the highest Cu concentration (100 mg $l^{-1}$), adding Ni to the Cu solution increased discolouration (Fig. 3b). If 10 or 22 mg $l^{-1}$ Ni were added to the 22 mg $l^{-1}$ Cu solution, or 22 mg $l^{-1}$ Ni were added to the 46 mg $l^{-1}$ Cu solution, the discolouration increased significantly ($p < 0.05$) compared to the treatments in which corresponding amounts of Cu only were applied. The highest maximum proportion of discoloured leaves was 66% when 46 mg $l^{-1}$ Cu and 46 mg $l^{-1}$ Ni were given in combination.

The amount of new root biomass produced was less if Cu or Ni was increased ($p < 0.05$) (Fig. 4, Table 2). The maximum proportion of new roots out of the total root biomass was 25% when treated with 1 mg $l^{-1}$ Cu and 0 mg $l^{-1}$ Ni. The growth of new roots was inhibited more at lower Cu and Ni levels compared to the growth decrease of the aboveground biomass. The root growth was only about 1–4% of the total belowground biomass when the Cu or Ni concentrations in the nutrient solution were more than 22 mg $l^{-1}$.

The statistical interaction term between Cu and Ni was significant ($p < 0.01$) on root growth (Table 2). Ni decreased root growth at lower Cu levels, but the response varied at the highest concentrations (Fig. 4, Table 1). The proportion of new root dry weight out of the total below ground biomass was at a maximum of 6% when 46 mg $l^{-1}$ Cu and 10 mg $l^{-1}$ Ni were given.

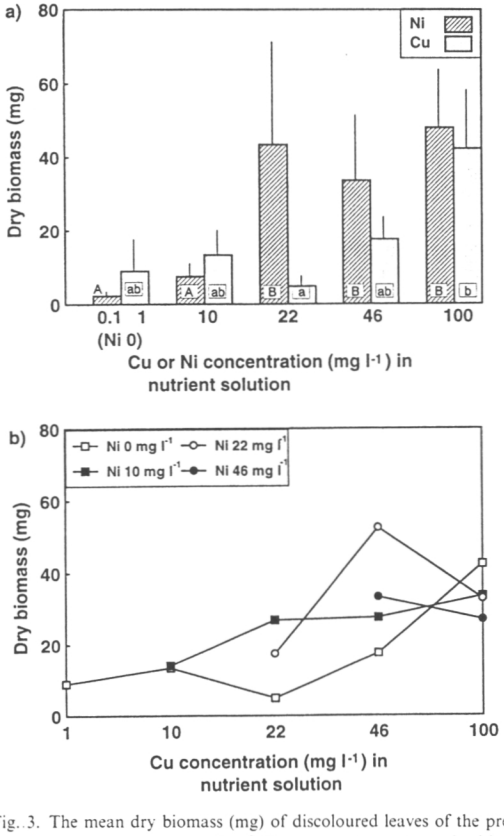


Fig. 3. The mean dry biomass (mg) of discoloured leaves of the previous and current-year growth of *Empetrum nigrum* treated with a nutrient solution containing different concentrations of (a) nickel (Ni) or copper (Cu) (mg $l^{-1}$) or (b) both Ni and Cu in combination. The bar indicates the standard error ($n = 8$). Means which do not differ significantly between treatments ($p > 0.05$) are marked with similar capital letters for the Ni treatment and small letters for the Cu treatment.

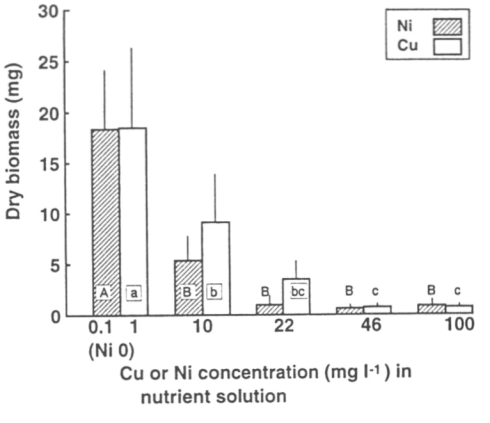


Fig. 4. The mean new root biomass (mg) of *Empetrum nigrum* treated with a nutrient solution containing Cu or Ni (mg $l^{-1}$). The bar indicates the standard error ($n = 8$). Means which do not differ significantly between treatments ($p > 0.05$) are marked with similar capital letters for the Ni treatment and small letters for the Cu treatment.

### 3.3. Cu and Ni accumulation in E. nigrum

Not treated cuttings contained Ni below detection limit and Cu less than 13 and 21 mg $kg^{-1}$ in green leaves and stems, respectively. During the 6-week course of the experiment *E. nigrum* accumulated increasing amounts of Cu and Ni with increasing Cu or Ni levels in the nutrient solution (Table 3). Metal accumulation increased with age, with younger parts containing less Cu and Ni than older ones. Discoloured leaves contained more Cu and Ni than the green leaves of corresponding age classes. The highest concentration of Cu in the stem tissue was 2033 mg $kg^{-1}$ and of Ni 2132 mg $kg^{-1}$ of plants treated with 100 mg $l^{-1}$ of Cu or Ni alone. The total uptake of Cu and Ni increased linearly as Cu and Ni concentrations in the solution increased (Table 4). The proportion of Cu and Ni taken up out of the amount applied [100 × total Cu or Ni uptake (mg)/added Cu or Ni (mg)] decreased as the solution concentrations increased, being at its maximum about 2% (Table 4).

If treated with Cu and Ni in combination, *E. nigrum* accumulated very high concentrations of both metals in the living parts. Older stems contained over 2700 mg $kg^{-1}$ Cu and about 2600 mg $kg^{-1}$ Ni if treated with 46 mg $l^{-1}$ Cu and 46 mg $l^{-1}$ Ni together. If the Cu concentration in the nutrient solution was 100 mg $l^{-1}$ and the Ni concentration 46 mg $l^{-1}$, older stems contained 3038 mg $kg^{-1}$ Cu and 1267 mg $kg^{-1}$ Ni (Table 3). The accumulation pattern was similar to that if only Cu or Ni was applied.

The concentrations of Cu and Ni in different plant parts varied depending on whether Cu and Ni were given in combination or separately. However, the sum of the Cu and Ni concentrations were generally higher when given separately than in combination in leaves (Table 3). In older stems the sum of the Cu and Ni concentrations applied separately were higher than concentrations of these metals given in combination in the following treatments: Cu/Ni 10/10, 22/10, 46/10. As the amount of Cu and Ni applied increased, the sum

Table 3
Copper (Cu) and nickel (Ni) concentrations (mg $kg^{-1}$ dry wt.) in young (current and previous-year growths) and older parts (older than current and previous-year growths) of *Empetrum nigrum* treated with different concentrations of Cu and Ni (one bulk sample comprises eight plants)[a]

| Applied conc. (mg $l^{-1}$) | Green leaves, young | | Discoloured leaves, older | | Discoloured leaves, young | | Stems, older | | Stems, young | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cu/Ni | Cu | Ni | Cu | Ni | Cu | Ni | Cu | Ni | Cu | Ni |
| 1/0 | 26 (30)[b] | | – | | 16 | | 76[c] | | 22[d] | |
| 10/0 | 52 | | – | | 53 | | 514 | | 44 | |
| 22/0 | 42 | | 465 | | – | | 875 | | 28 | |
| 46/0 | 78 | | 265 | | 98 | | 1755 | | 58 | |
| 100/0 | 52 | | – | | 141 | | 2033 | | 183 | |
| 0.1/0 | | 15[e] | | – | | – | | 31[f] | | 7[g] |
| 0.1/10 | | 34 | | – | | < | | 252 | | 40 |
| 0.1/22 | | 17 | | 313 | | 90 | | 522 | | 32 |
| 0.1/46 | | 97 | | – | | 335 | | 1217 | | 203 |
| 0.1/100 | | 56 | | – | | 192 | | 2132 | | 583 |
| 10/10 | 28 | 22 | – | – | 24 | < | 180 | 167 | – | – |
| 22/10 | – | – | – | – | – | – | 603 | 259 | 57 | 32 |
| 46/10 | 36 | 11 | 353 | 205 | 51 | < | 1272 | 247 | – | – |
| 100/10 | 40 | < | 327 | 196 | 124 | 14 | 2567 | 237 | 60 | 14 |
| 22/22 | – | – | 318 | 374 | 52 | 24 | – | – | 90 | 102 |
| 46/22 | 29 | 25 | 310 | 385 | 82 | 32 | – | – | – | – |
| 100/22 | 33 | < | – | – | 690 | 131 | – | – | – | – |
| 46/46 | 26 | < | 484 | 613 | 83 | 62 | 2705 | 2594 | 32 | 136 |
| 100/46 | 28 | 16 | – | – | 54 | 20 | 3038 | 1267 | 30 | < |

[a] <, Below detection limit; –, not enough sample material for the analyses or a temporary fault in the ashing oven. Cu concentration in older green leaves is given in parentheses. Regression equations for concentration in plant part ($y$) as a function of applied concentration ($x$) are given for data sets (five samples) marked with letters b–g.

[b] $y = 26.43 + 1.63x - 0.01x^2$, $r^2 = 0.98$, $p < 0.05$.

[c] $y = 50.0 + 157.73x^{0.56}$, $r^2 = 0.98$, $p < 0.05$.

[d] $y = 9.81 + 1.60x$, $r^2 = 0.91$, $p < 0.05$.

[e] $y = 12.18 + 9.30x^{0.40}$, $r^2 = 0.81$, ns.

[f] $y = 30.0 + 36.46x^{0.88}$, $r^2 = 0.99$, $p < 0.01$.

[g] $y = 4.73 + 0.60x^{1.49}$, $r^2 = 0.99$, $p < 0.05$.

Table 4
Total copper (Cu) and nickel (Ni) uptake (mg) during the experiment measured in the total aboveground biomass[a]

| Applied Cu (mg) | Total Cu uptake (mg)[b] | Uptake (%) | Applied Ni (mg) | Total Ni uptake (mg)[c] | Uptake (%) |
|---|---|---|---|---|---|
| 0.6 | 0.0117 | 1.95 | 0 | 0.0059 | – |
| 6 | 0.0528 | 0.88 | 6 | 0.0284 | 0.47 |
| 13.2 | 0.0982 | 0.74 | 13.2 | 0.0575 | 0.44 |
| 27.6 | 0.0964 | 0.35 | 27.6 | 0.0989 | 0.36 |
| 60 | 0.2093 | 0.35 | 60 | 0.2225 | 0.37 |

[a] This is based on the equation: the total aboveground biomass (kg)×total Cu or Ni concentration in the aboveground parts of *Empetrum nigrum* (mg $kg^{-1}$). The Cu and Ni concentrations in the aboveground parts used in the calculations (see Table 3). Regression equations for the total Cu or Ni uptake in aboveground parts ($y$) as a function of applied Cu or Ni ($x$) are given for data sets (five samples) marked with the letters b and c.
[b] $y = 0.029 + 0.003x$, $r^2 = 0.92$, $p < 0.05$.
[c] $y = 0.006 + 0.004x$, $r^2 = 1$, $p < 0.001$.

of the Cu and Ni concentrations in stems were lower if these metals were applied separately than if they were applied in combination (100/10, 46/46, 100/46) (Table 3).

### 3.4. Discussion

The growth inhibition of *E. nigrum* with increasing Cu and Ni concentrations in the nutrient solution indicated that Cu and Ni had toxic effects on plants. The clearest responses to Cu and Ni were in the dry weights of the shoots and roots, as has also been reported earlier for other plant species (e.g. Roth et al., 1971; Taylor and Crowder, 1983; Krämer et al., 1996; L'Huillier et al., 1996). Root growth was affected at relatively low levels of these metals, and it decreased already by more than half of the lowest treatments (0.1 or 1 mg $l^{-1}$ Cu and 0 mg $l^{-1}$ Ni) compared to the treatment of 10 mg $l^{-1}$ Cu or Ni. In field conditions the growth of *E. nigrum* has also clearly decreased in Cu- and Ni-polluted areas compared to the populations growing in a clean background area (Shevtsova, 1998). The growth suppression is the cost of tolerance and, although growth was affected, the survival did not decrease during the short experimental period. The 100% survival in high Cu and Ni treatments would indicate that populations originating from clean areas can also tolerate these high metal concentrations. However, survival at the highest Cu and Ni concentrations would have probably been affected if the experiment had continued.

*E. nigrum* tolerated high accumulated concentrations of Cu and Ni, with the highest concentrations being in the old stems. The Cu and Ni concentrations in discoloured leaves were higher than those in green leaves of the same age. The accumulation pattern of these metals within the plant was in good agreement with the values reported for *E. nigrum* growing in the field (Helmisaari et al., 1995; Uhlig et al., 1996). The current annual living shoots of *E. nigrum* have been reported to contain 180 mg $kg^{-1}$ Cu, the older living parts 1450 mg $kg^{-1}$, and dead biomass 4130 mg $kg^{-1}$ at a distance of 0.5 km from the Harjavalta smelter (Helmisaari et al., 1995). In aboveground parts, Ni, Fe, Pb and Zn have also been reported to accumulate with age, the highest concentrations occurring in old stems. Relatively lower concentrations of Cu are found in the roots compared to the aboveground parts (Uhlig et al., 1996). The accumulation pattern was similar throughout the whole concentration range. Therefore, high concentrations of metals in the older stem would indicate root-to-shoot transport. The results suggest that *E. nigrum* possesses an internal tolerance mechanism, which is supported by greenhouse and previous field studies (Uhlig et al., 1996). Krämer et al. (1996) suggested that root-to-shoot transport is important in Ni tolerance and hyperaccumulation.

*E. nigrum* accumulated over 2000 mg $kg^{-1}$ Cu or Ni in the living tissue treated with 100 mg $l^{-1}$ Cu or Ni alone and more than 2700 mg $kg^{-1}$ Cu and about 2600 mg $kg^{-1}$ Ni if treated simultaneously with 46 mg $l^{-1}$ Cu and 46 mg $l^{-1}$ Ni. These concentrations were higher than those measured earlier in the living tissue of *E. nigrum*, the highest reported Cu concentration in older living parts being 1500 mg $kg^{-1}$ (Helmisaari et al., 1995) and the Ni concentration in leaves 1510 mg $kg^{-1}$ (Chertov et al., 1993). The experiment indicates that the high concentrations found in the field only partly can be due to surface contamination. The concentrations measured in living parts in the field and greenhouse are higher than the limit suggested for hyperaccumulation (1000 mg $kg^{-1}$) (Brooks et al., 1977, 1980). An other ericaceous dwarf shrub, *Vaccinium uliginosum* L., has also been found to accumulate as high Cu concentrations in the field (DiLabio and Rencz, 1980).

The effect of adding Ni to the Cu solution suppressed the dry weights of the roots and shoots slightly more than Cu alone. However, the combined application of Cu and Ni suppressed the dry weights less than the summed effects of Cu and Ni alone. Also the sum of the Cu and Ni concentrations in the plant parts were generally higher in the plants treated with Cu and Ni separately than those treated with Cu and Ni in combination. Only in plants treated with the highest

concentrations of Cu and Ni, were the sum of these metals in the older stems higher when applied in combination than separately. The plant concentrations indicate that, at lower concentrations, there is competitive interaction between the uptake of these two metals. When the concentration of a toxic metal increases, adding another toxic metal does not further limit growth. However, interactions between metals are often complex, and they are dependent on the metal concentration and pH in the growth medium (Balsberg-Påhlsson, 1989).

The concentrations of Cu in the plant parts treated only with the control Cu levels (0.1 or 1 mg $l^{-1}$) were high compared to background values measured in the field (Helmisaari et al., 1995; Uhlig et al., 1996). Field conditions are different from those in the greenhouse where, for instance, organic matter and soil organisms are missing. Earlier manipulation studies have shown that roots growing in a medium with only low concentrations of metals are able to take up large amounts of these metals into the plant. Medappa and Dana (1970) showed that cranberry (*Vaccinium macrocarpon* Ait.) contained 600 mg Al $kg^{-1}$ in the roots when the external solution concentration was only 2.5 mg $l^{-1}$. The roots of *Salix caprea* L. exposed to trace concentrations of Fe in a nutrient solution contained 1680 mg $kg^{-1}$ Fe root dry wt. (Talbot and Etherington, 1987). Taylor and Crowder (1983) reported that *Typha latifolia* L. accumulated higher Cu and Ni concentrations in the greenhouse than in the field than would have been expected on the basis of the Cu and Ni concentrations in the growth substrate.

The use of activated carbon to determine the biomass of roots grown during this experiment may have influenced the experimental conditions. Activated carbon has a detoxifying effect on toxic compounds (Shaw et al., 1990; Zackrisson and Nilsson, 1992), and charcoal have increased the colonization of ericoid mycorrhizas in *Vaccinium* sp. (Duclos and Fortin, 1983). The exact plant-available concentrations of the elements are not known because activated carbon has most probably immobilized some of the Cu and Ni.

## 4. Conclusions

This greenhouse experiment showed that *E. nigrum* tolerated high Cu and Ni concentrations in its living parts, and helps to explain occurrence of this plant in highly polluted areas near the smelters. Although growth was suppressed, survival was not affected by the metals. The greenhouse studies showed that Cu and Ni concentrations were the highest in the stems indicating root-to-shoot transport. Transport to the green leaves appears to have been restricted, as reflected by the relatively constant Cu and Ni concentrations in the green leaves. Therefore, it is suggested that *E. nigrum* possesses an internal Cu and Ni tolerance.

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# II

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# Copper resistance of *Calluna vulgaris* originating from the pollution gradient of a Cu–Ni smelter, in southwest Finland

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**"Capsule":** *Heather seedlings from seed collected at three locations did not differ appreciably in their measured responses to copper treatments.*

**Abstract**

The copper (Cu) resistance of 1-year-old seedlings of heather (*Calluna vulgaris*) was tested in a greenhouse experiment. The plant material originated from seeds collected from three peatland sites located 1.2 km to the NW, and 2.5 and 5.5 km to the NE of the Harjavalta Cu–nickel (Ni) smelter, SW Finland. The plants were watered with a nutrient solution containing five different levels of Cu (1, 10, 22, 46 and 100 mg $l^{-1}$). Cu clearly decreased the length growth of shoots, shoot and root biomass of *C. vulgaris*. More than 50% of the seedlings exposed to the highest Cu treatment died. *C. vulgaris* accumulated high amounts of Cu, the living old roots containing a maximum of 2200 mg $kg^{-1}$ Cu and the living stems 1300 mg $kg^{-1}$ Cu. Discolouring leaves contained higher Cu concentrations than green leaves. The results indicate Cu accumulation in roots and root-to-shoot transport. Some differences were found between the responses of the three seed provenances, but none of the populations proved to be more resistant to Cu than the others in all the measured responses. 

*Keywords:* *Calluna vulgaris* (heather); Copper; Heavy-metal resistance; Cu accumulation; Cu–Ni smelter

## 1. Introduction

Heather (*Calluna vulgaris* (L.) Hull.) is a widespread and common species in Europe that usually grows on acidic and nutrient-poor soils (Gimingham, 1960, 1972). It is found on heathlands (e.g. Mohamed and Gimingham, 1970; Haapasaari, 1988), moors, bogs, fixed sand dunes and in forests (Gimingham, 1960). In Finland, *C. vulgaris* is among the 10 most common vascular plant species in forest and peatland vegetation (National Forest Inventory, 1995, unpublished results). The litter of *C. vulgaris* is rich in phenolic compounds (Jalal and Read, 1983), which decompose slowly and modify the soil causing acidification and organic matter accumulation. This leads to soil conditions which are optimal for the growth of *C. vulgaris* and impairs the growing conditions of other species (Grubb and Suter, 1971; Robinson, 1971; Haslam, 1977; Leake, 1988). The dominance of *C. vulgaris* in nutrient-poor environments has been explained on the basis of the effective use of nutrients; the ericoid mycorrhizal endophyte of *C. vulgaris* roots is also able to utilise organic sources of nitrogen and carbon (Bajwa et al., 1985; Leake and Read, 1989).

Resistance to heavy metals can be achieved by two strategies: tolerance and avoidance (Baker, 1987). Tolerant populations of many plant species have been found in heavy-metal-polluted mine and smelter areas (e.g. Schat and Ten Bookum, 1992). Tolerance to heavy metals may be either based on the evolution of tolerant genotypes (ecotypes) or may be environmentally induced (phenotypic plasticy) (e.g. Antonovics et al., 1971; Baker, 1987). There are indications that *C. vulgaris* possesses constitutive tolerance, and ecotypic

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differentiation of *C. vulgaris* populations growing in polluted or non-polluted areas has been suggested (Burt, 1984). In the middle of its distribution area, e.g. in Great Britain and Germany, *C. vulgaris* has been found to be very tolerant to heavy metals occurring on a variety of substrates, e.g. serpentine and polluted soils (Marrs and Bannister, 1978; Eltrop et al., 1991). *C. vulgaris* is one of the species colonizing waste heaps in metal-polluted areas (Burt, 1984). In the northern parts of its distribution, however, *C. vulgaris* is absent from severely heavy-metal-polluted areas in Fennoscandia (Gilbert, 1975; Laaksovirta and Silvola, 1975; Väisänen, 1986; Salemaa and Vanha-Majamaa, 1993) and the Kola Peninsula (Mikhail Kozlov, unpublished results), and it is considered to be a sensitive species to metals (Laaksovirta and Silvola, 1975).

In general, plants possess physiological mechanisms that enable them to resist high substrate heavy-metal concentrations (Antonovics et al., 1971; Baker, 1981, 1987; Woolhouse, 1983). Plants can either detoxify metals by binding them with organic acids, proteins, etc. (Lee et al., 1978; Rauser and Curvetto, 1980), or accumulate the metals in the different plant parts or cell organelles (Reilly, 1969; Bringezu, et al., 1999). Copper (Cu) is essential for plant growth and is involved in many metabolic processes, e.g. photosynthesis. However, high concentrations are toxic to plants (Marschner, 1995). Cu may accumulate in older plant parts (Helmisaari et al., 1995), such as stems (Uhlig et al., 1996) or senescing leaves (Reilly, 1969; Ernst, 1972). Bradley et al. (1981, 1982) and Burt (1984) showed that the ericoid mycorrhiza of *C. vulgaris* accumulates elevated concentrations of Cu and prevents translocation of metals to the growing parts of the plant. Even if the rate of heavy metal uptake can be controlled by plants, total avoidance of metal uptake is not possible (e.g. Baker and Walker, 1990).

The ecology (Gimingham, 1972) and heavy-metal resistance (Bradley et al., 1981, 1982; Burt, 1984) of *C. vulgaris* have been extensively studied in oceanic heathlands, but its response to heavy metals in boreal forests and peatlands is not known. The aim of this study was to investigate: (1) the Cu resistance of northern populations of *C. vulgaris* under controlled conditions; (2) whether the populations originating from sites closest to a copper–nickel (Cu–Ni) smelter are the most resistant ones; and (3) the pattern of Cu accumulation within different parts of *C. vulgaris*.

## 2. Materials and methods

### 2.1. Seed material of C. vulgaris

The seeds of *C. vulgaris* originated from peatland seedbanks at three sites, located at different distances from the Cu–Ni smelter at Harjavalta, southwest (SW) Finland. The Cu smelter was established in 1945 and the Ni smelter in 1960, and sulphur dioxide and heavy metals have been emitted into the environment for the past 40–50 years. The deposition of metals near the smelter was considerably reduced in the 1990s after a new, taller stack and electrostatic filters were built (Rantalahti, 1995). The prevailing wind direction is from the SW and the emissions are primarily dispersed to the northeast (NE).

The seedbank samples were collected from three peatland sites located 1.2 km to the northwest (NW) (Lammaistensuo) and 2.5 km (Kotosuo) and 5.5 km to the NE (Pyhäsuo) of the smelter. Metal concentrations in the surface peat decreased with increasing distance from the pollution source; at 0.9 km the total Cu concentration was 3560 mg $kg^{-1}$ dry wt. and total Ni concentration 470 mg $kg^{-1}$ dry wt., and at 2.4 km 1870 mg $kg^{-1}$ dry wt. Cu and 340 mg $kg^{-1}$ dry wt. Ni. The corresponding concentrations at a distance of 5.0 km were 770 mg $kg^{-1}$ Cu and 180 mg $kg^{-1}$ Ni. Metals were extracted with acid (dry digestion) by inductively coupled plasma atomic emission spectrometry (ICP–AES). Soil pH was 3.5–3.7 (Veijalainen, 1998).

The abundance of *C. vulgaris* in the understorey vegetation increases with increasing distance from the pollution source. It appears for the first time at 2.3 km with a point frequency of 0.6%. At 2.8 km it is 2.4% and at 5.7 km 11.3% (Antti Reinikainen, unpublished results). However, results from seedbank experiments show that viable seeds of *C. vulgaris* are found at 1.2 km, which do not germinate in the field, but germinate in optimal light and moisture conditions, in greenhouse. Seedling mortality increases with decreasing distance from the pollution source (Salemaa and Uotila, 1996; Huopalainen, 1998).

The seedbank samples (0–5 cm soil layer) of *C. vulgaris* were collected on 27–29 September 1995 using a 5×10-cm corer, using methods described by Huopalainen (1998). The samples were stored in the dark at +5°C for 5 weeks (chilling). After chilling, the samples were transferred to a greenhouse where they were spread evenly in a 1-cm-thick layer on a mixture of horticultural peat and quartz sand in trays to maintain the optimal light conditions (Wesson and Wareing, 1969). When the seeds had germinated and seedlings grown sufficiently, they were planted in a mixture of peat and quartz sand and left to grow until the experiment started.

### 2.2. Plant culture system

Nine-month-old seedlings of *C. vulgaris* were used in the experiment. The roots of the seedlings were washed with tap water and cut back to a length of 12 cm in order to standardize the experiment material and to give

the roots sufficient growing space. The roots were dipped in activated carbon so as to facilitate separation of the stained old roots from white new ones at the end of the experiment. The plants were transferred to plastic pots containing quartz sand (0.5 l) and randomised for different Cu treatments.

The cuttings were watered twice weekly (50 ml/pot) with a nutrient solution modified by Stribley and Read (1976) containing the following nutrients (mg $l^{-1}$): P, 11.3 ($KH_2PO_3$); K, 28.5 (KCl); Ca, 29.2 ($CaCl_2.2H_2O$); Mg, 8.8 ($MgCl_2.6H_2O$); Fe, 3 ($FeCl_3.6H_2O$); Mn, 0.5 ($MnCl_2$); B, 0.5 ($H_3BO_3$); Zn, 0.1 ($ZnCl_2$); Mo, 0.1 ($Na_2MoO_4$); N, 65 (ammonium and nitrate as $NH_4NO_3$ and ammonium as $NH_4Cl$ in the ratio $NH_4$:$NO_3$, 70:30). Five concentrations of Cu (1, 10, 22, 46 and 100 mg $l^{-1}$), applied as $CuSO_4$, were used with 10 replicates per treatment. The first treatment (1 mg $l^{-1}$) represented the control. The total number of seedlings was 150.

The experiment was carried out in the greenhouse of the Finnish Forest Research Institute at Ruotsinkylä (60°21′ N, 25°00′ E) over the period 12 June to 25 July 1996, under natural photoperiods (light period approximately 18 h, dark period 6 h). The mean temperature was regulated to +20°C during the day and +15°C at night, and the relative humidity above 60%.

### 2.3. *The response variables of* C. vulgaris

The parameters used to assess metal toxicity were elongation of the leading shoot and two side branches, and the biomass (dry wt.) of the leaves, stems and roots. The root and shoot uptake of Cu and the allocation of different elements to the leaves, stems and roots were also determined.

The total initial length of the leading shoot and two side branches were measured before planting, and the growth during the experiment determined as the difference between the initial and final lengths of the stems of the leading shoots and side branches. The material was divided into six parts for biomass analyses: (1) green leaves; (2) discoloured leaves; (3) stems of leading shoots; (4) stems of side branches; (5) new roots; and (6) old roots. The samples were homogenized, dried at +60°C, weighed and dry digested (+550°C). The extraction of the ash was done with 2–3 ml 6 M HCl (pro-analys) in water bath (approximately +80°C). The dry residue was diluted with 10 ml 1 M HCl for 20 min and filtered (filter paper; Schleicher & Schuell 589[3]) with 0.1 M HCl. The final volume of the solution was 25–50 ml depending on the sample weight. The total element (Cu, Ni, P, K, Mg, Ca, Fe) concentrations of the plant parts were determined by inductively coupled plasma atomic emission spectrometry (ICP–AES). Ten replicate samples were combined in order to obtain sufficient material for the chemical analyses.

### 2.4. *Statistical analysis*

Two-factor analysis of variance (ANOVA) was used to analyse the effects of Cu treatment, the origin of the seedlings and their interaction with the response variables of *C. vulgaris* (GLM procedure, SAS Institute Inc., 1994). The initial length of the leading shoot was used as the covariate in the variance model. Logarithmic transformations lg ($x+1$) were performed in order to normalise the data. Pairwise comparisons between the treatments were performed by the *t*-test. Pearson correlations were calculated between the concentrations of Cu and other elements in the different plant parts. The model-predicted length growth of the leading shoot and two side branches, shoot and leaf biomass are shown in Figs. 1–3, in which the initial length of the leading shoot is used as a covariate.

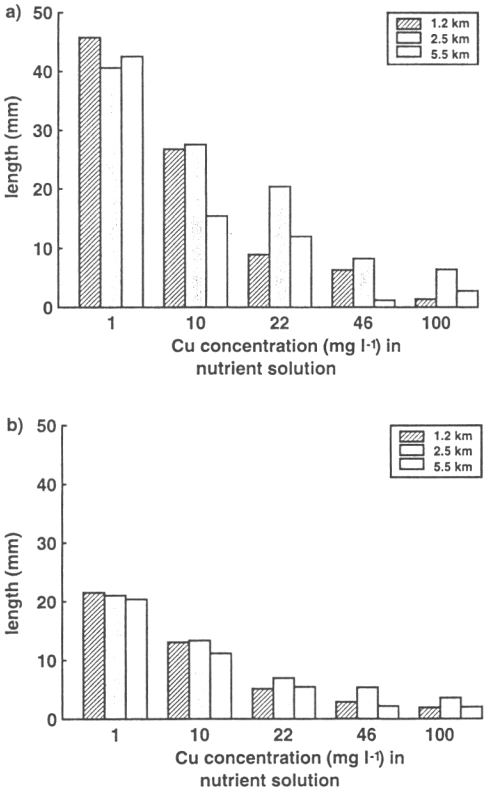


Fig. 1. The mean length growth (mm) of (a) leading shoot and (b) side branches of *Calluna vulgaris* predicted by the variance model in which the initial length of the seedlings was used as the covariate ($n = 10$/Cu treatment). Seedlings were collected at three distances from the smelter and treated with a nutrient solution containing different concentrations (mg $l^{-1}$) of Cu.

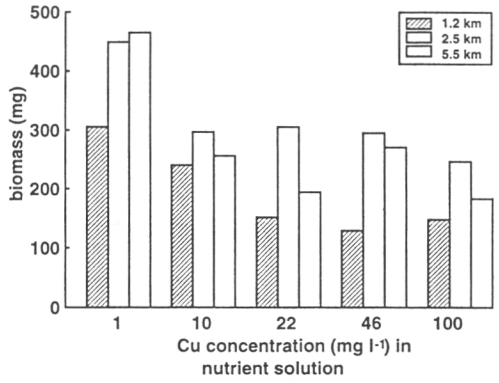


Fig. 2. The mean aboveground biomass (mg) of *Calluna vulgaris* predicted by the variance model in which the initial length of the seedlings was used as the covariate ($n = 10$/Cu treatment). See details in Fig. 1.

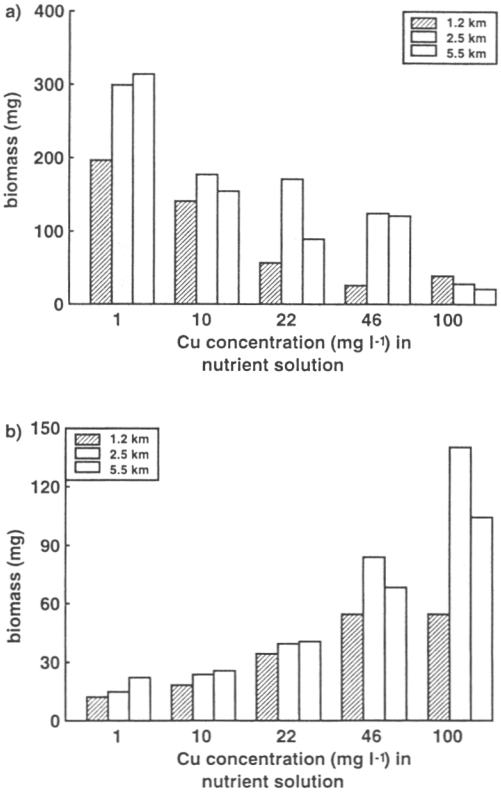


Fig. 3. The mean biomass (mg) of (a) green and (b) discoloured leaves of *Calluna vulgaris* predicted by the variance model in which the initial length of the seedlings was used as the covariate ($n = 10$ Cu treatment). See details in Fig. 1.

## 3. Results

### 3.1. The influence of Cu on length growth and biomass

Increasing Cu concentrations caused a clear decrease in the length growth of leading shoot and side branches of *C. vulgaris* (Fig. 1a, b). The highest Cu treatment almost completely inhibited growth (Fig. 1), the maximum growth reduction of the leading shoots being 86–99% and that of the side branches 84–92% compared to that for the control treatment (1 mg $l^{-1}$ Cu). The growth of the leading shoot was much higher than that of the side branches at low concentrations, but not at the two highest Cu concentrations (Table 1). The mean length growth of leading shoot and side branches differed significantly ($p < 0.001$) between the Cu treatments (Table 2). The origin of the seedlings also had a statistically significant effect on the growth of the leading shoots ($p < 0.001$) (Table 2). The model-predicted length growth of the seedlings originating from 2.5 km of the smelter was the highest in almost all of the Cu treatments (Fig. 1a, b).

The shoot biomass decreased as the Cu concentration in the solution increased, the difference between the treatments being significant ($p < 0.001$) (Table 2). The decrease was significant when the Cu concentration increased from 1 to 10 mg $l^{-1}$ in one origin, but the decreasing trend was not clear at higher concentrations (Table 1). The shoot biomass decreased less than the length growth, the maximal reduction in biomass varying from 52 to 67% in the different seedling origins. The effect of origin was significant ($p < 0.001$) (Table 2). The model-predicted shoot biomass values of the seedlings from a distance of 2.5 km were again generally the highest (Fig. 2).

The green leaf biomass decreased and leaf discolouration increased clearly as the Cu concentrations in the nutrient solution increased (Table 1). The effect of the Cu treatments on both leaf biomasses was significant ($p < 0.001$) (Table 2). The proportion of discoloured leaves out of the total leaf biomass was 5–8% at the lowest Cu concentrations, but 74–85% at the highest Cu concentration. The mean green leaf biomasses differed significantly between the origins ($p < 0.05$) (Table 2). The model-predicted biomasses of the green and discoloured leaves were generally lower in the 1.2 km origin, but no trends were found in the other origins (Fig. 3a, b). The statistical interaction term between the origin and the Cu concentration in green leaf biomass was significant ($p < 0.01$) (Table 2).

Increasing Cu concentrations caused a stronger decrease in the new root biomass than in shoot biomass (Figs. 2 and 4). However, shoot/root ratio stayed relatively constant throughout the whole concentration range (Table 1). The mean root biomass was significantly different between the Cu treatments ($p < 0.001$) (Table 2).

Table 1
The means ($\bar{x}$) and standard errors (SE) of growth (mm) and biomass (mg), shoot/root (S/R) ratio and survival (%) of *Calluna vulgaris* treated with different concentrations of copper (Cu)[a]

| Cu applied (mg $l^{-1}$) | | Elongation | | | | Dry weight | | | | | | | | | Survival (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cu | Origin (km) | Leading shoot (mm) | | Side branches (mm) | | Aboveground (mg) | | Green leaves (mg) | | Discoloured leaves (mg) | | New roots (mg) | | S/R ratio | |
| | | $\bar{x}$ | SE | $\bar{x}$ | SE | $\bar{x}$ | SE | $\bar{x}$ | SE | $\bar{x}$ | SE | $\bar{x}$ | SE | | |
| 1 | 1.2 | 49[a] | 6.8 | 22 a | 4.5 | 383 abc | 43 | 237 acd | 28 | 21 ab | 4.8 | 69 a | 21.1 | 2.7 | 100 |
| 1 | 2.5 | 41[a] | 6.9 | 21 a | 3.9 | 463 ad | 97 | 306 a | 61 | 16 b | 4.1 | 39 a | 7.1 | 2.6 | 100 |
| 1 | 5.5 | 44[a] | 8.0 | 21 a | 4.6 | 516 ab | 95 | 340 a | 63 | 28 abe | 5.8 | 38 ab | 10.0 | 2.7 | 100 |
| 10 | 1.2 | 25 a | 6.3 | 13 ae | 3.6 | 201 cf | 37 | 120 acd | 24 | 14 ab | 2.4 | 20 ab | 4.2 | 3.1 | 100 |
| 10 | 2.5 | 25 ah | 7.5 | 13 a | 2.0 | 218 bcd | 37 | 137 ad | 26 | 15 ab | 4.7 | 14 ab | 3.4 | 2.0 | 100 |
| 10 | 5.5 | 15 bh | 6.0 | 11 ab | 2.3 | 253 ce | 47 | 153 ab | 34 | 26 abc | 6.0 | 14 bc | 3.5 | 2.1 | 100 |
| 22 | 1.2 | 11 bef | 2.3 | 6 cdf | 2.0 | 212 efh | 22 | 89 bd | 9 | 41 ac | 9.8 | 5 de | 1.7 | 3.0 | 100 |
| 22 | 2.5 | 18 aeh | 3.7 | 7 bce | 1.6 | 237 bc | 60 | 136 acd | 38 | 32 cef | 6.6 | 11 bcf | 3.4 | 2.2 | 80 |
| 22 | 5.5 | 11 bf | 3.5 | 5 cd | 1.5 | 167 egf | 30 | 75 bc | 18 | 38 cfh | 6.0 | 3 de | 1.2 | 3.0 | 90 |
| 46 | 1.2 | 8 cdf | 3.9 | 3 cdf | 0.7 | 168 gh | 29 | 46 e | 18 | 59 df | 7.7 | 2 de | 0.9 | 3.0 | 50 |
| 46 | 2.5 | 7 bdg | 2.2 | 5 cdf | 2.1 | 267 bc | 53 | 110 bd | 33 | 81 di | 14.8 | 9 ce | 3.8 | 2.4 | 90 |
| 46 | 5.5 | 3 c | 1.3 | 2 dg | 0.5 | 307 c | 56 | 140 ab | 36 | 72 dgh | 12.2 | 11 def | 7.0 | 2.4 | 100 |
| 100 | 1.2 | 1 c | 0.3 | 2 df | 0.6 | 125 gh | 18 | 28 e | 9 | 52 df | 6.5 | 2 de | 1.6 | 3.3 | 50 |
| 100 | 2.5 | 6 bd | 1.4 | 3 cdf | 1.1 | 224 ce | 35 | 17 e | 7 | 138 gi | 30.2 | 2 d | 1.3 | 2.4 | 40 |
| 100 | 5.5 | 3 cg | 1.4 | 2 fg | 0.9 | 196 egf | 37 | 28 e | 12 | 106 di | 23.8 | 1 d | 0.7 | 2.8 | 40 |

[a] The means which did not differ significantly between treatments ($p > 0.05$) are marked with similar letters.

Table 2
*F* and *p* values of two-factor analysis of variance (ANOVA) for the log-transformed response variables

| Source of variation | df | Elongation | | | | Dry weight | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Leading shoots | | Side branches | | Aboveground | | Green leaves | | Discoloured leaves | | New roots | |
| | | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| Cu | 4 | 38.36 | 0.0001 | 34.68 | 0.0001 | 11.14 | 0.0001 | 30.04 | 0.0001 | 32.42 | 0.0001 | 31.53 | 0.0001 |
| Origin | 2 | 7.56 | 0.0008 | 1.55 | 0.2171 | 12.37 | 0.0001 | 3.50 | 0.0330 | 2.92 | 0.0572 | 1.36 | 0.2606 |
| Cu*origin | 8 | 1.3 | 0.2491 | 0.08 | 0.9996 | 0.99 | 0.4455 | 2.81 | 0.0065 | 1.00 | 0.4410 | 0.81 | 0.5983 |
| Length | 1 | 8.43 | 0.0043 | 0.93 | 0.3368 | 88.51 | 0.0001 | 27.30 | 0.0001 | 18.03 | 0.0001 | 7.85 | 0.0058 |

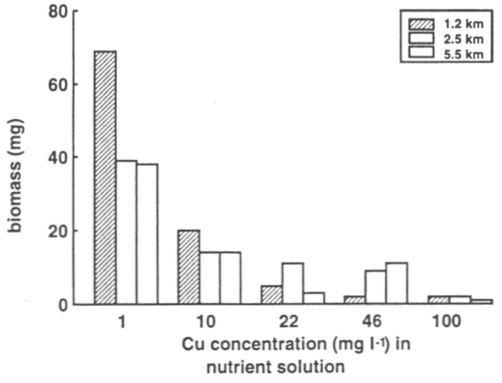


Fig. 4. The mean new root biomass (mg) of *Calluna vulgaris* treated with a nutrient solution containing different concentrations of Cu (mg $l^{-1}$) ($n = 10$ Cu treatment). See details in Fig. 1.

Root growth was almost totally inhibited by 100 mg $l^{-1}$ Cu, the proportion of new roots being only 2–3% at the highest Cu concentrations.

### 3.2. *Survival of* C. vulgaris *seedlings*

Cu clearly caused a decrease in the survival of the *C. vulgaris* seedlings. The average mortality was 10, 20 and 60% for Cu concentrations of 22, 46 and 100 mg $l^{-1}$. The mortality of the seedlings varied between the origins in the different treatments, but none of the origins had the highest survival rate in all the treatments (Table 1).

### 3.3. *Cu accumulation in* C. vulgaris

Cu concentrations increased in all parts of *C. vulgaris* with increasing Cu levels in the nutrient solution (Fig. 5).

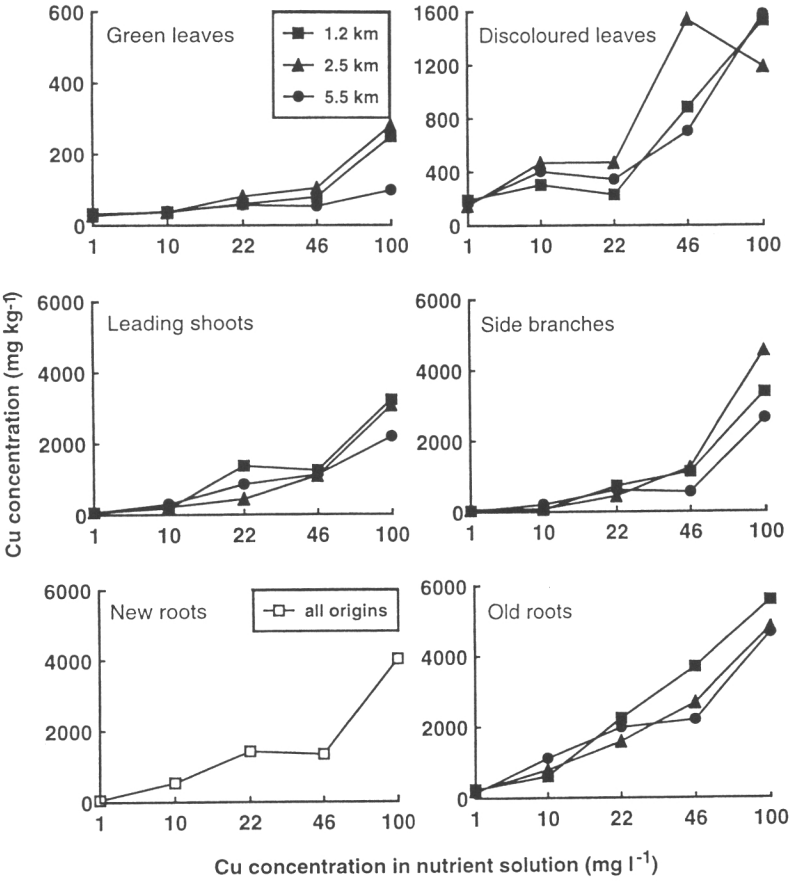


Fig. 5. The mean Cu concentrations (mg $kg^{-1}$) in the leaves, stems and roots of *Calluna vulgaris* from the three collection sites. Plants were exposed to different Cu concentrations in a nutrient solution (10 plants in one composite sample).

The individual plant parts had varying Cu concentrations and the concentrations decreased generally in the following order: old roots > new roots > stems > discoloured leaves > green leaves (Fig. 5). The Cu concentrations were extremely high in old roots and stems, exceeding 5500 and 4500 mg $kg^{-1}$, respectively. Because the dead and living plants were not analysed separately, these results also include dead parts. The highest Cu concentrations in the living parts of old roots exceeded 2200 mg $kg^{-1}$ and in the stems of the leading shoots 1300 mg $kg^{-1}$. The lowest concentrations were found in the green leaves (maximum 300 mg $kg^{-1}$), while the discoloured leaves contained up to 1600 mg $kg^{-1}$ Cu (Fig. 5).

The proportion of Cu taken up from the nutrient solution (100×root and shoot uptake/added Cu) was the highest at the lowest Cu concentrations and it was about 8% in the lowest Cu treatment (Table 3). It decreased with increasing Cu concentration in the nutrient solution, and the relative shoot and root uptake was only about 2% in the concentration of 10 mg $l^{-1}$ Cu in nutrient solution. Relative root uptake decreased almost as much as relative shoot uptake with increasing Cu concentration in the nutrient solution. Also approximately the amount of Cu in the shoots was as high as that in the roots (Table 3).

### 3.4. *Correlations between Cu and other elements in the plant parts of* C. vulgaris

There were interactions between Cu and the other elements. The uptake of P, Ca, K and Mg decreased as the Cu concentration increased in the stems of the leading shoots and side branches indicating that Cu interfered with nutrient uptake. The uptake of K increased and that of Ca and Fe decreased as the Cu concentration increased in the discoloured leaves of *C. vulgaris* (Table 4).

Table 3
Shoot and root Cu uptake (μg) and relative uptake of Cu (percentage of the uptaken amount of Cu applied) of *Calluna vulgaris* originating from three distances from the smelter

| Applied Cu ($10^3$ μg) | Origin distance from the smelter (km) | Root uptake (μg) | Shoot uptake (μg) | Total uptake (μg) | Relative root uptake (%) | Relative shoot uptake (%) | Relative total uptake (%) |
|---|---|---|---|---|---|---|---|
| 0.6 | 1.2 | 21 | 18 | 39 | 3.5 | 3.0 | 6.5 |
| 0.6 | 2.5 | 30 | 22 | 52 | 5.0 | 3.7 | 8.7 |
| 0.6 | 5.5 | 24 | 21 | 45 | 4.0 | 3.5 | 7.5 |
| 6.0 | 1.2 | 38 | 17 | 55 | 0.6 | 0.3 | 0.9 |
| 6.0 | 2.5 | 81 | 22 | 103 | 1.3 | 0.4 | 1.7 |
| 6.0 | 5.5 | 124 | 35 | 159 | 2.1 | 0.6 | 2.7 |
| 13.2 | 1.2 | 156 | 99 | 255 | 1.2 | 0.7 | 1.9 |
| 13.2 | 2.5 | 173 | 57 | 230 | 1.3 | 0.4 | 1.7 |
| 13.2 | 5.5 | 107 | 58 | 165 | 0.8 | 0.4 | 1.2 |
| 27.6 | 1.2 | 203 | 130 | 333 | 0.7 | 0.5 | 1.2 |
| 27.6 | 2.5 | 289 | 225 | 514 | 1.0 | 0.8 | 1.9 |
| 27.6 | 5.5 | 277 | 132 | 409 | 1.0 | 0.5 | 1.5 |
| 60.0 | 1.2 | 209 | 238 | 446 | 0.3 | 0.4 | 0.7 |
| 60.0 | 2.5 | 452 | 435 | 887 | 0.8 | 0.7 | 1.5 |
| 60.0 | 5.5 | 332 | 321 | 653 | 0.6 | 0.5 | 1.1 |

Table 4
The Pearson correlations between copper (Cu) concentrations (mg $kg^{-1}$) and the other elements in different plant parts of *Calluna vulgaris*[a]

| Elements in the different parts of the plant | P | Ca | K | Mg | Fe |
|---|---|---|---|---|---|
| Cu in the stems of leading shoots | −0.86* | −0.75* | −0.76* | −0.74* | 0.03 |
| Cu in the stems of side branches | −0.58* | −0.55* | −0.58* | −0.61* | 0.27 |
| Cu in the green leaves | 0.20 | 0.50 | 0.34 | 0.40 | −0.10 |
| Cu in the discoloured leaves | 0.33 | −0.70* | 0.65* | −0.19 | −0.58* |

[a] $n = 15$, equals the number of Cu concentrations applied to three origins (5×3).
*$p < 0.05$.

## 4. Discussion

Increasing Cu concentrations in the nutrient solution had clearly an adverse effect on the growth parameters of *C. vulgaris* compared to plants treated with the control solution (1 mg $l^{-1}$ Cu). Burt (1984) found that in the treatment of 80 mg $l^{-1}$ Cu, the root growth of *C. vulgaris* was completely inhibited, while shoot biomass increased during the experiment. Also in this study, the root biomass was more strongly inhibited than the shoot biomass, indicating the toxic effects of Cu especially on the roots.

*C. vulgaris* appeared to accumulate high concentrations of Cu because the surviving roots and stems contained over 1000 mg $kg^{-1}$ Cu. The concentrations were at the same level as in the study of Burt (1984) on non-mycorrhizal plants. Bradley et al. (1981, 1982) reported even higher shoot and root concentrations in mycorrhizal and non-mycorrhizal plants. Although *C. vulgaris* is capable of accumulating high concentrations of Cu in greenhouse conditions within a relatively short period, the Cu concentrations of plants growing in Cu-polluted soil have been considerably lower. In Great Britain, the Cu concentration in *C. vulgaris* fine roots was only about 140 mg $kg^{-1}$ and in brown shoots 90 mg $kg^{-1}$, even though the total Cu concentration in the spoil heap was 2500 mg $kg^{-1}$ (Burt, 1984). 115 mg $kg^{-1}$ Cu has been reported in green shoots (Marrs and Bannister, 1978).

The greenhouse experiment clearly showed that northern populations of *C. vulgaris* also resisted high Cu concentrations. However, because the duration of the experiment was relatively short and mortality increased strongly at the highest Cu concentrations, it is probable that long-term exposure is detrimental to *C. vulgaris* seedlings. It has been demonstrated that, although the seeds are capable of germination, seedling survival is short in seedbank soil sampled from polluted areas (Salemaa and Uotila, 1996; Huopalainen, 1998). Not only the origin of *C. vulgaris*, but also the environmental conditions may regulate the occurrence of *C. vulgaris* and explain why it grows in heavy-metal polluted areas on oceanic heathlands but not in boreal forests. In addition to high metal concentrations (Derome and Lindroos, 1998; Veijalainen, 1998), also drainage and deficiency of base cations near the Cu–Ni

smelter at Harjavalta (Derome and Nieminen, 1998) may explain the absence of *C. vulgaris*. The water supply is known to be critical for seedling establishment of *C. vulgaris* (e.g. Gimingham, 1972).

It has been suggested that the Cu resistance of *C. vulgaris* is based on an exclusion mechanism, in which the ericoid mycorrhiza of *C. vulgaris* roots accumulates the metals and thus prevents Cu transportation to the shoots (Bradley et al., 1981, 1982; Burt, 1984). Indications of true Cu tolerance in *C. vulgaris* have also been reported (Burt, 1984). According to our data the role of mycorrhiza in heavy metal resistance cannot be evaluated. However, the tolerance of *C. vulgaris* growing in the polluted soil may develop before infection of the fungus in the roots (Burt, 1984), thus suggesting that the plant itself also tolerates high metal concentrations. The higher Cu concentrations found in the roots than in the shoots support the hypothesis of Bradley et al. (1981, 1982). However, based on the high Cu concentrations in the stems, our results indicate root-to-shoot transport suggesting that *C. vulgaris* possesses true tolerance to Cu. The discoloured leaves had higher Cu concentrations than the green leaves, which indicated that *C. vulgaris* also transports Cu to the leaves. This is supported by Marrs and Bannister (1978) and Burt (1984), who concluded that *C. vulgaris* could detoxify and remove metals by accumulating them in older shedding leaves and stems.

In the beginning of the experiment, activated carbon was used to stain the old roots as it is a good method for distinguishing between old and new roots for the biomass measurements. However, activated carbon is known to absorb metals (Shaw et al., 1990), and this might have altered the experimental conditions. For instance, the Cu concentrations in the old roots might have been overestimated to some extent, even though the roots were washed before the chemical analyses. Some Cu may also have remained on the root surface. In contrast, the shoot Cu concentrations more certainly reflect the Cu taken up by the roots from the nutrient solution.

Compared to another evergreen dwarf shrub, *Empetrum nigrum*, which commonly grows close to smelters (Helmisaari et al., 1995), the growth of *C. vulgaris* decreased to a relatively greater extent under comparable concentrations. When exposed to a concentration of 100 mg $l^{-1}$ Cu in the nutrient solution, the survival of *E. nigrum* was not affected even though the Cu concentrations in the living stem tissue were more than 3000 mg $kg^{-1}$ (Monni et al., 1999). Because the survival of *C. vulgaris* was strongly affected at the highest concentrations, it appears to be more sensitive to Cu than *E. nigrum*. The different age of the seedlings may, however, affect the result.

In contrast to the findings of this study, Burt (1984) suggested ecotypic differences in Cu tolerance between *C. vulgaris* seedlings originating from metal-contaminated mine spoil and a non-polluted background area. Significant differences between the origin of the seedlings were only found in certain growth parameters, but no systematic tendency was seen. Neither did Cu accumulation give any indications of differences between the origins. Because the most distant origin of the seedlings was 5.5 km from the smelter and not from a non-polluted background area, this might be the reason why no clear ecotypic differences were found. In addition, there are suggestions that the evolution of tolerant races of woody plants does not occur as frequently as for grasses and herbs (Antonovics et al., 1971).

## 5. Conclusions

The exposure of *C. vulgaris* seedlings to Cu caused clear toxicity symptoms in the roots of the plants, and survival was strongly affected. The shoot biomass also decreased, though it was not so clearly affected as root growth. However, *C. vulgaris* accumulated high concentrations of Cu in the living parts during the short exposure period, and the accumulation pattern was similar throughout the concentration range. We can conclude that *C. vulgaris* tolerated Cu by accumulating it especially in the roots and stems, as well as in the discolouring leaves. There were some indications of ecotypic differences between the origins but the responses were not related to the distance from the smelter.

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# Chemical composition and ecophysiological responses of *Empetrum nigrum* to aboveground element application

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**"Capsule":** *Heavy metals in solutions sprayed on* Empetrum nigrum *remained on plant surfaces and were not taken up.*

**Abstract**

*Empetrum nigrum* L. (crowberry) is one of the plants surviving near the Cu–Ni smelters in Finland and Russia. According to field observations, the roots of *E. nigrum* are situated below 40 cm depth and the root biomass is reduced in the polluted sites. This could cause a reduced root uptake of macronutrients and trace elements in the field and, therefore, the possible element uptake by aboveground parts of *E. nigrum* was studied in a greenhouse. Six different treatment solutions containing various heavy metal and macronutrient concentrations were applied to the stems and leaves of *E. nigrum* and the chemical composition and ecophysiological parameters were measured. Heavy metal concentrations in the leaves and stem bark, and Cu concentrations in the stems, increased with increasing metal concentrations in the spraying solutions. The bark and leaves had higher heavy metal concentrations than the stems of comparable age classes. The macronutrient and Mn concentrations in *E. nigrum* did not change significantly with increasing element concentrations in the spraying solution. Neither the stem water potential nor the leaf chlorophyll concentrations showed any clear response to element applications. Therefore, the element uptake by aboveground parts of *E. nigrum* was not confirmed by this study. However, there was a tendency to a decrease in $CO_2$ exchange rate and increase in foliar abscisic acid content in plants treated with the highest element concentrations. 

*Keywords:* *Empetrum nigrum* L.; Aboveground uptake; Chlorophyll; $CO_2$ exchange; Drought stress; Heavy metals

## 1. Introduction

*Empetrum nigrum* L. is a xeromorphic (Carlquist, 1989; Wollenweber et al., 1992), evergreen dwarf shrub that occurs on a variety of substrates (Bell and Tallis, 1973). In the northern hemisphere characteristic environments are nutrient-poor, well-illuminated dry forests (Sarvas, 1937) and ombrogenous peat and peaty podsols (Good, 1927; Bell and Tallis, 1973). However, *E. nigrum* also occurs on serpentine soils (Proctor and Woodell, 1971). It is one of the few understorey species that grows on severely heavy metal-contaminated sites in the vicinity of Cu–Ni smelters (Laaksovirta and Silvola, 1975; Chertov et al., 1993; Helmisaari et al., 1995; Uhlig et al., 1996; Mälkönen et al., 1999). In polluted areas and in a greenhouse experiment *E. nigrum* has been found to accumulate in excess of 1000 mg $kg^{-1}$ of Cu and Ni in older stem tissue and is considered to be a species resistant to heavy metals (Uhlig et al., 1996; Monni et al., 2000a). However, it has been shown that heavy metals can be retained on the surface of the leaves (Atteia and Dambrine, 1993) and twig axes (Wyttenbach et al., 1988) despite mechanical washing or rainfall, and this may contribute to the high concentrations observed in plants exposed to pollution.

In several plant species, physiological processes such as photosynthesis and water status are sensitive to heavy metals (Lamoreux and Chaney, 1978; Rauser and Dumbroff, 1981; Becerril et al., 1989; Angelov et al., 1993; Bishnoi et al., 1993). Heavy metals have been found to inhibit electron transport in photosynthetic systems (Becerril et al., 1988) and the regenerative phase of the Calvin cycle (Weigel, 1985). In experimental

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studies, stomatal conductance and water potential of the leaves have decreased and the abacisic acid (ABA) content, which regulates the water status of the plant, increased when plants were exposed to Ni (Rauser and Dumbroff, 1981; Bishnoi et al., 1993).

Plants are able to employ several strategies for survival when exposed to heavy metals (e.g. Antonovics et al., 1971; Baker, 1987). Heavy metal resistance can be based on either avoidance or tolerance mechanisms. Plants can be protected externally against metals or they can tolerate high tissue concentrations through specific physiological mechanisms (e.g. Baker, 1987). In the field, heavy metals are generally taken up via the roots, although metal uptake via the leaves and stems has also been reported (Chamberlain, 1983; Lin et al., 1995; Koricheva et al., 1997). Nothing is known about the aboveground uptake of metals or nutrients by *E. nigrum*.

According to field observations, the roots of *E. nigrum* are situated below 40 cm depth and the root biomass is reduced in the polluted environments around the Cu–Ni smelters in Finland and Russia. This could cause a reduced root uptake of macronutrients and trace elements from upper soil horizons. Therefore, the possible element uptake by the aboveground parts of E. *nigrum* was studied by measuring (1) the element distribution and (2) the foliar chlorophyll and ABA contents, stem water potential, the dark respiration and the maximum photosynthesis of *E. nigrum* after heavy metal and macronutrient applications to leaves and stems.

## 2. Material and methods

### 2.1. Application of heavy metals and macronutrients

The aboveground element application study was carried out in the greenhouse of the Finnish Forest Research Institute, at Ruotsinkylä (60°21′ N, 25°00′ E) during 1 August–17 September 1997. The light period was natural (light period approximately 15 h, dark period 9 h) and the mean temperature was maintained at +15 and +22°C during the night and day, respectively. The relative humidity in the greenhouse varied from 30 to 90%, being at its lowest in the afternoon and the highest at night.

Nutrient solutions with six different element compositions (Table 1) were sprayed twice a week on the aerial parts of 4 to 5-year-old seedlings of *E. nigrum* grown in quartz sand (on the bottom) and peat substrate in pots. The composition of wet deposition near the Cu–Ni smelter at Harjavalta, southwest (SW) Finland, was simulated. Treatment I (control) represented approximately the same concentration of nutrients and treatment II about the same concentration of nutrients and heavy metals as rainwater at a distance of 0.5 km from the smelter reported by Helmisaari et al. (1994) and Helmisaari (personal communication). Nitrate and ammonium nitrogen were applied in the ratio 30:70. In treatments III–VI, the concentrations of all the components increased exponentially, the relative concentrations thus being constant in the different treatments. The solutions contained macronutrients Mg, Ca, P, K, N and heavy metals Cu, Ni, Pb, Zn, Fe, Cd, Cr, Mn including also plant essential trace elements (Table 1).

Contamination of the substrate and possible element uptake via the roots were avoided by protecting the roots of the seedlings with a plastic cover on each pot. The contamination of pot surface via the stem flow of *E. nigrum* was avoided by fastening the stem to plastic sheet with sticky tack. Discoloured leaves were removed to ensure that all the leaves of the plants were alive at the beginning of the experiment. The aerial parts of the seedlings were enclosed in a plastic container, the spraying treatment being applied via openings in the

Table 1
The experimental setup

| Element | Main substance used | Element conc. (mg $l^{-1}$) in spraying solution for different treatments | | | | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | VI |
| Cu | $CuSO_4$ | 0 | 0.4 | 1.2 | 3.7 | 11.4 | 35.0 |
| Ni | $NiCl_2$ | 0 | 0.05 | 0.2 | 0.5 | 1.4 | 4.4 |
| Pb | $PbCl_2$ | 0 | 0.02 | 0.05 | 0.1 | 0.4 | 1.3 |
| Zn | $ZnSO_4 \times 7H_2O$ | 0 | 0.05 | 0.1 | 0.4 | 1.3 | 3.9 |
| Fe | $FeSO_4 \times 7H_2O$ | 0 | 0.2 | 0.5 | 1.4 | 4.3 | 13.1 |
| Cd | $3CdSO_4 \times 8H_2O$ | 0 | 0.002 | 0.005 | 0.01 | 0.04 | 0.1 |
| Cr | $CrCl_2$ | 0 | 0.02 | 0.05 | 0.1 | 0.4 | 1.3 |
| Mn | $MnSO_4 \times H_2O$ | 0.005 | 0.005 | 0.02 | 0.05 | 0.1 | 0.4 |
| Mg | $MgCl_2 \times 6H_2O$ | 0.07 | 0.07 | 0.2 | 0.6 | 2.0 | 6.1 |
| Ca | $CaCl_2 \times 2H_2O$ | 0.2 | 0.2 | 0.6 | 1.9 | 5.7 | 17.5 |
| P | $KH_2PO_4$ | 0.02 | 0.02 | 0.06 | 0.2 | 0.6 | 1.8 |
| K | KCl | 0.1 | 0.1 | 0.3 | 0.9 | 2.9 | 8.8 |
| N ($NO_3$:$NH_4$, 30:70) | $NH_4NO_3$, $NH_4Cl$ | 0.9 | 0.9 | 2.6 | 7.9 | 24.2 | 74.4 |

container. The seedlings were sprayed from two opposite directions with 15 ml of solution from each direction (altogether 15 times). There were nine plants per treatment.

### 2.2. Chemical analysis of the soil and plant material

In order to determine possible contamination of the substrate, the surface peat was removed from the pots at the end of the experiment, dried at +60°C and passed through a 0.4-mm sieve. The total concentrations of Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, P, Pb and Zn in the peat were determined by dry digestion (+550°C), followed by extraction of the ash with 2–3 ml of 6 M HCl (pro analysi) at approximately +80°C. The dry residue was dissolved in 10 ml of 1 M HCl for 20 min and filtered (filter paper; Schleicher & Schuell 589[3]) with 0.1 M HCl. The solutions were analysed by induction coupled plasma atomic emission spectrometry (ICP-AES).

After 7 weeks of treatment, the plants were harvested, divided by year growth, rinsed with distilled water for 1 min to minimize the effect of surface contamination, and then oven-dried at +60°C. The plants were divided into the following fractions: (1) leaves of current-year growth; (2) leaves of previous-year growth; (3) older leaves; (4) stems of current-year growth; (5) stems of previous-year growth; (6) older stems; (7) bark of current-year stems; (8) bark of previous-year stems; and (9) bark of older stems. In control treatments, the bark of current- and previous-year stems were not separated.

The different parts were homogenized, and the element concentrations determined by dry digestion (+550°C) as described above. Total concentrations of Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, P, Pb and Zn were analysed by ICP-AES. The C and N concentrations were determined from the dry material using a CHN Leco analyser (Nelson and Sommers, 1982).

### 2.3. Analysis of chlorophyll

For the chlorophyll analyses samples of the current-year growth of *E. nigrum* were collected in liquid nitrogen in the greenhouse and the samples stored at −80°C. The shoots were freeze-dried (Hetosicc freeze dryer, type CD 52), leaves and stems separated and leaves ground with a mortar and pestle. Aliquots of 10 mg were extracted in 3 ml of 80% acetone over night at +4°C, centrifuged for 3 min, and the absorbances measured at 647 and 664 nm on a spectrophotometer (Shimadzu UV-1201, UV-VIS). Two replicates, each based on a composite sample of up to four plants, were analysed for all the treatments. Chlorophyll *a* (μmol $l^{-1}$) from leaves was calculated according to equation $13.19\times A_{664}-2.57\times A_{647}$, and chlorophyll *b* (μmol $l^{-1}$) according to the equation $22.10\times A_{647}-5.26\times A_{664}$ (Graan and Ort, 1984).

### 2.4. $CO_2$ exchange rate

Before the plants were harvested, the $CO_2$ exchange was measured using a battery-operated Li-Cor LI-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Measurements were carried out with a quarter-litre chamber. A quantum sensor was situated outside and temperature and relative humidity sensors inside the chamber. The portable light source consisted of one halogen lamp (type; electro-valo, EV-KHV-400, Na-lamp, agro, 400 W). The ambient $CO_2$ concentration in the greenhouse was between 360 and 399 ppm. Readings were collected at 2-s intervals for 42. Six different irradiance levels (0, 50, 110, 330, 600 and 820 μmol $m^{-2}$ $s^{-1}$) of photosynthetically active radiation (PAR) were used. Before the measurements, the plants were acclimatized at each light level for 5 min. All six irradiance levels were applied to the same plants. Five plants per treatment were measured. In each measurement, two to three branches of the current-year growth were placed in the chamber, the number of leaves inside the chamber being about 200. Dark respiration was measured by covering the chamber with a black plastic sheet. After taking measurements, all leaves were removed from the measured stems, the leaf area of a single leaf estimated using the formula of an ellipse and the total leaf area calculated.

### 2.5. Stem water potential

The total water potential of the xylem sap of *E. nigrum* was measured with a Scholander pressure bomb (Scholander et al., 1965) at the end of the experiment. The stems of the previous-year growth were cut with a razor blade and the stem water potential was measured in normal room light. Five plants in each treatment and two pieces of stem from each plant were measured.

### 2.6. Analysis of ABA

For ABA analyses the current-year shoots of *E. nigrum* were collected at the end of the experiment (up to four plants in the composite sample per treatment). The samples were frozen in liquid nitrogen and stored at−80°C. The shoots were freeze-dried (Hetosicc freeze dryer, type CD 52), leaves and stems separated and ground with a mortar and pestle. One-hundred milligrams of homogenized plant material was suspended in 5 ml of 0.05 M phosphate buffer (pH 8.0), and 50 ng internal standard ([$^2H_4$]ABA) was added. After extraction in darkness under shaking at 4°C for a minimum of 2 h, the samples were centrifuged at 5000 rpm for 15 min. The supernatant was removed, and the pellet was resuspended in 2 ml of 0.05 mM phosphate buffer (pH 8.0) and centrifuged at 5000 rpm for 15 min. The supernatants were combined, the pH was adjusted to 2.7

with 0.1 M HCl, and partitionated three times against equal volumes of ethyl acetate. The pooled ethyl acetate phase was reduced to dryness in vacuo in a rotary evaporator at 40°C.

The residue was dissolved in 100 µl of 80% methanol, injected into a $C_{18}$ high-performance liquid chromotagraphy (HPLC)-column, and eluated with a gradient of 30–50% methanol in 1% aqueous acetic acid over 20 min. The technical specifications of the HPLC system have been described earlier (Monni et al., 2000b). The fraction containing ABA was collected, methylated using ethereal diazomethane, and analysed by GC-MS as described by Monni et al. (2000b). The MS source was operated in electron impact mode at 70 eV, and ions of *m/z* 162, 190, 193 and 194 were monitored.

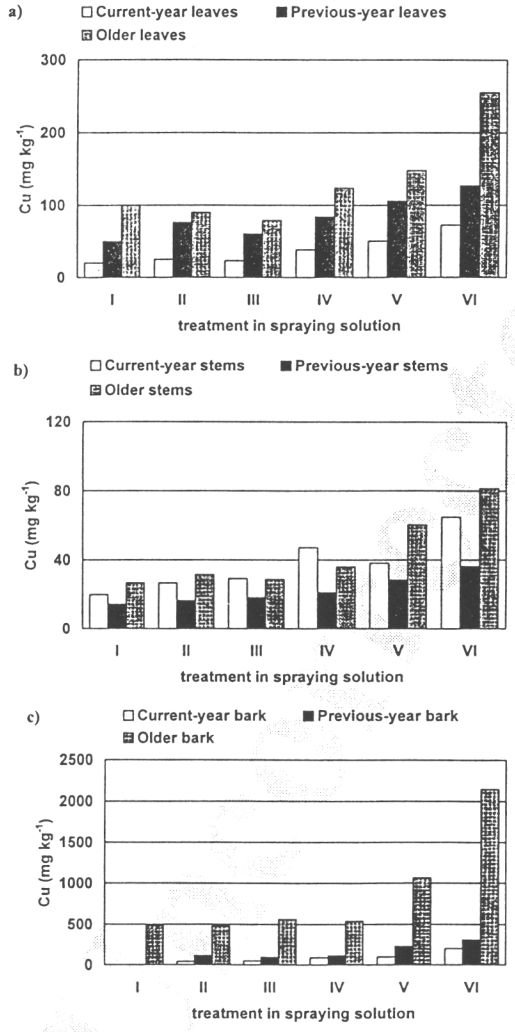


Fig. 1. Copper concentrations (mg $kg^{-1}$) in the (a) leaves, (b) stems and (c) bark of different year classes of *Empetrum nigrum* (values are based on one combined sample of six to nine plants per treatment).

### 2.7. Statistical analysis

Non-parametric Kruskal–Wallis analysis of variance (ANOVA) was used in analysing the effects of spraying treatments on the stem water potential of *E. nigrum*. Pairwise comparisons between the treatments were performed by the Kruskal–Wallis, comparison of mean ranks test. The Spearman rank correlations were calculated between Cu and Fe concentrations in different plant parts and stem water potential, chlorophyll and ABA contents of *E. nigrum* leaves. To see the element uptake via the roots from the peat and possible contamination of peat by spraying solutions, the Spearman rank correlations between the element concentrations in peat and different parts of *E. nigrum* were calculated (Sokal and Rohlf, 1995; Statistix, 1996).

The effect of treatment on $CO_2$ exchange rate of *E. nigrum* was evaluated by comparing the dark respiration at the irradiance level of 0 µmol $m^{-2}$ $s^{-1}$ and the maximum photosynthesis at the irradiance levels of 600 or 820 µmol $m^{-2}$ $s^{-1}$ in different treatments. One-way ANOVA was performed and the differences between the treatment means were compared by *t*-test (SAS Institute Inc., 1994).

## 3. Results

### 3.1. Chemical analysis of the surface peat and plant parts

Increased Ca, K, Mg and P concentrations were found in the peat in treatment III. The Cu and Zn concentrations were also higher in treatments III and VI than in the other treatments, indicating that peat contamination could not be completely prevented (Table 2). However, there was no systematic correlation of those elements in peat and plant parts indicating that the increased concentrations in peat did not affect clearly on the element concentrations in the plant parts.

Table 2
Element concentrations (mg $kg^{-1}$) in the surface peat (six to nine plant substrates in one bulk sample/treatment)[a]

| Treatment | Ca | Cu | Cd | Fe | K | Mg | Mn | Ni | P | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 6779 | 81 | – | 2396 | 870 | 2790 | 48 | 2.4 | 410 | 3.9 | 51 |
| II | 8362 | 84 | – | 2056 | 882 | 3095 | 50 | – | 459 | 5.9 | 45 |
| III | 11052 | 116 | – | 2305 | 1368 | 4298 | 58 | 2.6 | 685 | 4.7 | 63 |
| IV | 8585 | 101 | – | 2394 | 1163 | 3477 | 61 | 2.5 | 552 | 4.8 | 56 |
| V | 6734 | 94 | – | 2401 | 996 | 2802 | 55 | 2.6 | 465 | 4.4 | 60 |
| VI | 6538 | 120 | – | 1774 | 1038 | 2620 | 44 | – | 519 | 6.0 | 73 |

[a] –, below the analytical detection limit.

Table 3
Element concentrations and C/N ratio in the (1) current-, (2) previous-year and (3) older leaves of *Empetrum nigrum* (six to nine plants in one bulk sample/treatment)[a]

| Year growth | Treatment | Ca | Cd | Fe | K | Mg | Mn | Ni | P | Pb | Zn | C | N | C/N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (mg kg $^{-1}$) | | | | | | | | | | (% dry matter) | | |
| 1 | I | 6084 | – | 43 | 6080 | 2353 | 321 | – | 1661 | – | 28 | 54.7 | 1.28 | 43 |
| 1 | II | 6003 | – | 44 | 7179 | 2483 | 330 | – | 1921 | – | 28 | 54.6 | 1.25 | 44 |
| 1 | III | 5081 | – | 42 | 7929 | 2227 | 281 | – | 1895 | – | 28 | 55.0 | 1.30 | 42 |
| 1 | IV | 5683 | – | 62 | 7310 | 2218 | 297 | – | 1570 | – | 27 | 55.2 | 1.17 | 47 |
| 1 | V | 5959 | – | 95 | 6572 | 2226 | 274 | 3.5 | 1484 | – | 25 | 54.9 | 1.08 | 51 |
| 1 | VI | 5602 | – | 188 | 7194 | 2284 | 289 | 5.0 | 1596 | 9.5 | 29 | 54.8 | 1.13 | 49 |
| 2 | I | 9324 | – | 56 | 4186 | 3092 | 639 | – | 1561 | – | 43 | 53.8 | 1.25 | 43 |
| 2 | II | 8645 | – | 91 | 3999 | 2871 | 648 | 3.8 | 1423 | – | 41 | 53.9 | 1.19 | 45 |
| 2 | III | 7726 | – | 68 | 4644 | 2813 | 575 | – | 1403 | – | 42 | 54.0 | 1.28 | 42 |
| 2 | IV | 8014 | – | 83 | 4284 | 2756 | 622 | 3.8 | 1306 | – | 40 | 54.5 | 1.14 | 48 |
| 2 | V | 9433 | – | 133 | 3316 | 3016 | 617 | – | 1312 | – | 37 | 53.9 | 1.03 | 52 |
| 2 | VI | 8372 | – | 251 | 4537 | 2938 | 585 | 7.0 | 1335 | 11.9 | 40 | 53.8 | 1.12 | 48 |
| 3 | I | 16401 | – | 90 | 2665 | 4530 | 1494 | – | 1160 | – | 66 | 52.6 | 0.98 | 54 |
| 3 | II | 14824 | – | 79 | 2941 | 4198 | 1539 | – | 1312 | – | 61 | 52.5 | 1.03 | 51 |
| 3 | III | 15262 | – | 80 | 3094 | 4455 | 1424 | 5.2 | 1126 | – | 64 | 52.1 | 1.01 | 52 |
| 3 | IV | 13737 | 0.3 | 99 | 2935 | 4068 | 1321 | 5.4 | 1149 | 4.3 | 55 | 53.2 | 0.97 | 55 |
| 3 | V | 12461 | 0.3 | 136 | 2998 | 3743 | 987 | 7.9 | 1121 | 6.3 | 48 | 52.7 | 0.84 | 63 |
| 3 | VI | 14702 | 0.6 | 223 | 3267 | 4430 | 1527 | 16.8 | 977 | 12.3 | 75 | 52.7 | 0.86 | 61 |

[a] –, below the analytical detection limit.

Cu concentrations in the leaves, stems and bark of all the years increased with increasing Cu concentrations in the spraying solutions (Fig. 1). The highest Cu concentrations occurred in the bark (2147 mg kg$^{-1}$) (Fig. 1c). The maximum concentration of Cu in the leaves was 260 mg kg$^{-1}$ (Fig. 1a) and in the stems 80 mg kg$^{-1}$ (Fig. 1b). Cu accumulation increased in older leaves and bark (Fig. 1a, c). In the stems, Cu concentrations were higher in older and current-year stems than in the previous-year stems (Fig. 1b).

The concentrations of other heavy metals in the plant parts also increased due to increasing heavy metal concentrations in the spraying solution. Iron concentrations increased in all plant parts except in the previous-year and older stems (Tables 3–5), and the highest Fe concentrations occurred in the older bark (659 mg kg$^{-1}$)

Table 4
Element concentrations (mg kg$^{-1}$) in the (1) current-, (2) previous-year and (3) older stems of *Empetrum nigrum* (six to nine plants in one bulk sample/treatment)[a]

| Year growth | Treatment | Ca | Cd | Fe | K | Mg | Mn | Ni | P | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | I | 3439 | – | 30 | 3075 | 1223 | 373 | – | 824 | – | 25 |
| 1 | II | 2658 | – | 59 | 2968 | 1085 | 321 | 5.6 | 812 | – | 23 |
| 1 | III | 3160 | – | 36 | 3940 | 1283 | 355 | – | 895 | – | 26 |
| 1 | IV | 3496 | – | 135 | 2996 | 1251 | 396 | 16.3 | 796 | – | 29 |
| 1 | V | 3048 | – | 74 | 2859 | 1117 | 331 | 4.9 | 792 | – | 22 |
| 1 | VI | 2533 | – | 129 | 2947 | 969 | 306 | 5.4 | 784 | 6.1 | 24 |
| 2 | I | 1469 | – | 202 | 1838 | 558 | 296 | 28.4 | 741 | – | 14 |
| 2 | II | 1244 | – | 42 | 1878 | 470 | 258 | – | 687 | – | 12 |
| 2 | III | 1496 | – | 29 | 2369 | 560 | 265 | – | 683 | – | 16 |
| 2 | IV | 1721 | – | 79 | 1951 | 588 | 357 | 10.7 | 720 | – | 16 |
| 2 | V | 1412 | – | 37 | 1623 | 551 | 290 | – | 658 | – | 14 |
| 2 | VI | 1439 | – | 43 | 1942 | 552 | 256 | 3.4 | 683 | – | 15 |
| 3 | I | 1530 | – | 70 | 1564 | 562 | 403 | 7.5 | 616 | – | 15 |
| 3 | II | 1540 | – | 36 | 1693 | 588 | 385 | – | 600 | – | 17 |
| 3 | III | 1414 | – | 36 | 1924 | 508 | 345 | 3.0 | 581 | – | 20 |
| 3 | IV | 1491 | – | 39 | 1858 | 579 | 405 | 3.4 | 647 | – | 19 |
| 3 | V | 1514 | 0.4 | 62 | 1691 | 638 | 395 | 6.2 | 608 | – | 18 |
| 3 | VI | 1504 | 0.4 | 49 | 1711 | 528 | 385 | 6.3 | 614 | – | 21 |

[a] –, below the analytical detection limit.

(Table 5). Pb and Ni concentrations increased in the previous-year and older leaves and bark, especially. Ni concentrations, however, remained low apart from the older bark where the maximum concentration was 164 mg $kg^{-1}$ (Table 5). Cd was only detected in older tissues (Tables 3–5), primarily in the bark, where Cd levels increased with increasing metal applications (Table 5). Stem Zn levels were not affected by treatments or tissue age (Table 4). Foliar Zn and Mn concentrations increased with leaf age, but this was not related to the applied levels (Table 3). In the bark, Zn levels were the highest in the oldest bark and increased with increasing applications (Table 5).

Macro-nutrient concentrations did not increase in any plant parts in response to increasing nutrient applications. However, concentrations of K and Mg in older bark and concentrations of Mg in current-year stems, slightly decreased with increasing nutrients in spraying solutions (Tables 4 and 5). The P concentrations decreased in previous-year and older leaves, and N concentrations in the leaves, with increasing nutrient applications. Foliar N concentrations decreased and the C/N ratio increased with increasing age. In addition, the C/N ratio increased slightly with increasing treatment level (Table 3).

The Ca and Mg concentrations increased and the K and P concentrations decreased with increasing tissue age in the leaves (Table 3), while Ca, K, Mg and P decreased with increasing tissue age in the stems of *E. nigrum* (Table 4). In the bark, Ca concentration increased and K and Mg concentrations decreased with tissue age, while the age of the bark had no clear effect on P concentrations (Table 5).

### 3.2. Chlorophyll concentration

The sum of chlorophyll *a* and *b* did not change with increasing heavy metal and nutrient concentrations in the spraying solution (Fig. 2a). The highest chlorophyll concentrations were found in the leaves from treatments I, IV and VI, and the lowest in the plants from treatment V. The sum of chlorophyll $a+b$ correlated positively with the Cu concentrations in the current-year bark and Fe concentrations in the previous-year stems ($r_s = 0.94$–$1.00$, $P < 0.05$). The chlorophyll $a/b$ ratio increased slightly with increasing heavy metal and nutrient levels (Fig. 2b). The Cu concentrations in the previous-year stems, current-year and older bark and Fe concentrations in older leaves and current- and previous-year bark correlated positively with the chlorophyll $a/b$ ratio ($r_s = 0.83$–$1.00$, $P < 0.05$).

### 3.3. $CO_2$ exchange

The highest $CO_2$ exchange rate of *E. nigrum* was measured at the irradiance levels of 600 and 820 μmol $m^{-2}$ $s^{-1}$ (Fig. 3a). The increasing element concentrations in the spraying solution decreased dark respiration (Fig. 3b) and maximum photosynthesis of *E. nigrum* current-year shoots (Fig. 3c). The dark respiration was the lowest in treatments of V and VI and these differed statistically from the treatments I, II and III ($P < 0.01$; $F = 5.27$; Fig. 3b). Maximum photosynthesis was the lowest in the highest treatments (V and VI) but these did not differ statistically from the other treatments ($P = 0.0798$, $F = 2.27$; Fig. 3c).

### 3.4. Water potential

The mean water potential of *E. nigrum* varied between −11 and −13 bars. The increasing treatment levels had no consistent effects on the water potential (Fig. 4a). The water potential correlated negatively with the Cu or Fe concentrations in the current- and previous-year leaves and Cu concentrations in older stems and previous-year bark ($r_s = 0.83$–$0.90$, $P < 0.05$).

### 3.5. ABA contents

The ABA contents varied between 13 and 66 ng $g^{-1}$ dry wt. The spraying treatment appeared to affect the ABA contents, the highest contents being in treatments IV and VI. The lowest values were measured in treat-

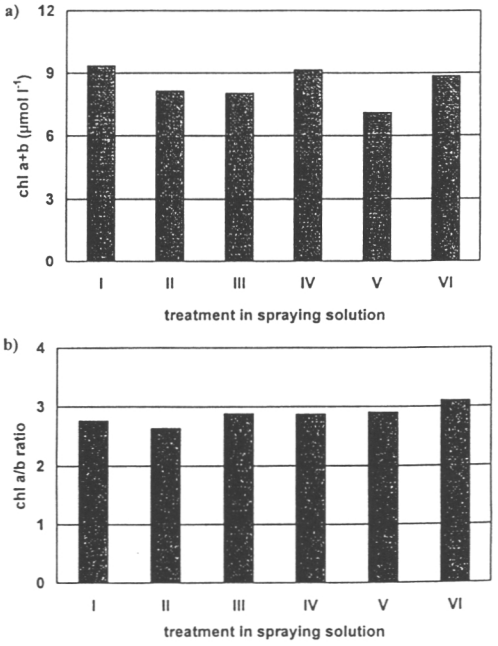


Fig. 2. (a) Chlorophyll $a+b$ concentrations (μmol $l^{-1}$) and (b) chlorophyll $a/b$ ratio in the current-year leaves of *Empetrum nigrum*. Results are based on one combined sample of up to four plants per treatment. Each sample was measured twice.

Table 5
Element concentrations (mg $kg^{-1}$) in the (1) current-, (2) previous-year and (3) older bark of *Empetrum nigrum* (six to nine plants in one bulk sample/treatment).[a]

| Year growth | Treatment | Ca | Cd | Fe | K | Mg | Mn | Ni | P | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | II | 6836 | – | 76 | 2445 | 1487 | 346 | – | 398 | – | 46 |
| 1 | III | 6720 | – | 102 | 2915 | 1666 | 435 | – | 512 | – | 42 |
| 1 | IV | 7526 | – | 100 | 2494 | 1635 | 415 | – | 422 | – | 49 |
| 1 | V | 7578 | – | 120 | 2103 | 1526 | 361 | – | 348 | – | 43 |
| 1 | VI | 7585 | – | 249 | 2349 | 1539 | 437 | – | 411 | – | 51 |
| 2 | II | 7446 | – | 127 | 1335 | 1645 | 483 | – | 366 | – | 102 |
| 2 | III | 6956 | – | 128 | 1733 | 1430 | 456 | – | 357 | – | 65 |
| 2 | IV | 7338 | – | 87 | 1272 | 1442 | 495 | – | 308 | – | 62 |
| 2 | V | 7927 | – | 157 | 1001 | 1480 | 477 | – | 302 | – | 61 |
| 2 | VI | 7175 | – | 243 | 1259 | 1435 | 470 | 20.3 | 336 | 19.9 | 70 |
| 3 | I | 9626 | 0.4 | 181 | 550 | 1133 | 353 | 5.3 | 333 | 6.7 | 178 |
| 3 | II | 8760 | 1.2 | 171 | 410 | 1006 | 317 | 8.4 | 289 | – | 195 |
| 3 | III | 9751 | 0.7 | 180 | 587 | 1122 | 341 | 16.8 | 365 | – | 213 |
| 3 | IV | 8664 | 3.6 | 188 | 567 | 1049 | 368 | 30.8 | 320 | 7.5 | 218 |
| 3 | V | 9136 | 6.1 | 340 | 449 | 992 | 350 | 78.8 | 314 | 20.5 | 235 |
| 3 | VI | 10193 | 5.5 | 659 | 380 | 904 | 341 | 163.9 | 347 | 51.5 | 333 |

[a] –, below the analytical detection limit.

ments II and III (Fig. 4b). The Cu and Fe concentrations in older leaves, Cu concentrations in the current-year stems and bark and Fe concentrations in older bark correlated positively with ABA content ($r_s = 0.83$–$0.94$, $P < 0.05$).

## 4. Discussion

The accumulation of Cu, Fe, Pb, Cd, Ni and Zn in leaves or bark increased along with increasing concentrations of heavy metals in the spraying solution. In contrast, there was no change in the concentrations of macronutrients and Mn. This difference may be partly related to differences in the surface binding of the different elements. Atteia and Dambrine (1993) reported that the Ni, Zn and Pb concentrations were unaffected and that Fe was retained by the needles when rainfall passed down through the tree crowns. Fe was also retained on the surface of spruce twig axes, even though the twigs were washed with toluene and tetrahydrofuran (Wyttenbach et al., 1988). In contrast, Little (1973) suggested that a high proportion of Zn, Pb and Cd can be removed by deionised water from broadleaved species. However, surface contamination may significantly contribute to the levels of heavy metals in the aboveground parts of plants exposed to aerial pollution (Alfani et al., 1996). In contrast, macronutrients are easily washed from leaf and bark surfaces, which is supported by earlier results with leaves (Cercasov et al., 1987) and stems (Wyttenbach et al., 1988). Wyttenbach et al. (1988) found that washing resulted in the removal of particulate material containing Ca, K, Mg, Mn, Na and P from the surface of bark. The results of this study showed that the leaves and bark of *E. nigrum* have a high capacity to bind heavy metals. Furthermore, this capacity seems to increase with increasing age of the tissue. If accumulation takes place primarily at the surface it may have only limited physiological effects on *E. nigrum*, but the abscission of older leaves and bark tissue will result in significant accumulation of heavy metals in the soil under the plants.

Increasing heavy metal concentrations with age in the leaves and stems of *E. nigrum* growing in the field and greenhouse have been found, and suggests that *E. nigrum* accumulates metals in the older tissues (Helmisaari et al., 1995; Uhlig et al., 1996; Monni et al., 2000a). The accumulation pattern was similar whether the metals were applied to the roots (Monni et al., 2000a) or to the aboveground parts in the greenhouse. Therefore, these studies suggest that both accumulation and surface contamination contribute to the high metal concentrations in the older parts of *E. nigrum* in the field. On the contrary, there was no age-dependant accumulation in the stems; Cu, Zn and, to some extent, the Fe concentrations were higher in the current-year stems than previous-year stems. The bark is very difficult to separate from the other stem tissue in current-year stems, whereas removal is easier in previous-year and older stems. For this reason, it is very likely that the current-year stem samples contained some bark tissue, thus increasing the concentrations of heavy metals in these samples.

The metal concentrations in the different aboveground parts of *E. nigrum* decreased generally in the following order: bark > leaves > stems. In a study where

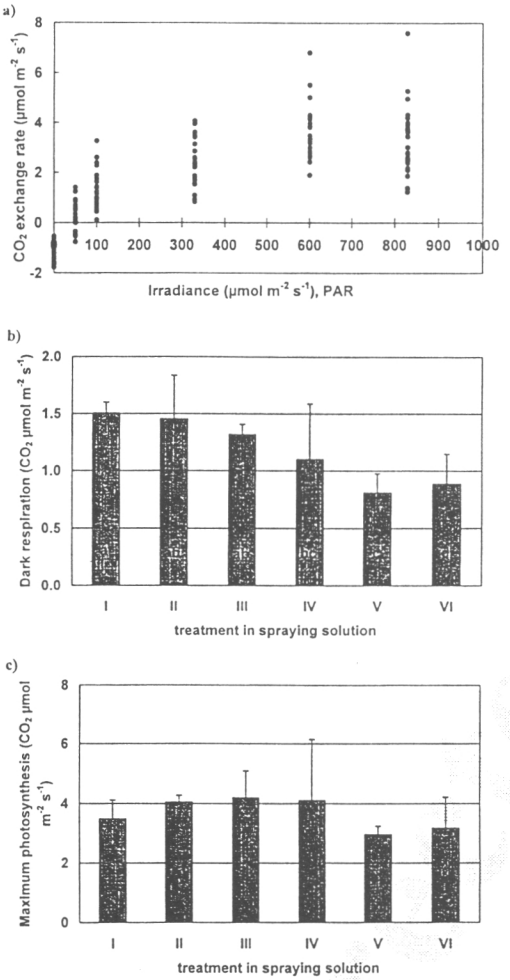


Fig. 3. (a) The measured values of $CO_2$ exchange rate ($CO_2$ μmol $m^{-2}$ $s^{-1}$) of *Empetrum nigrum* current-year shoots in all treatments ($n=4$–5/treatment) in varying light intensities of photosynthetically active radiation (PAR; μmol $m^{-2}$ $s^{-1}$), (b) the dark respiration ($CO_2$ μmol $m^{-2}$ $s^{-1}$) at irradiance level of 0 μmol $m^{-2}$ $s^{-1}$ ($n=5$/treatment) and (c) the maximum photosynthesis ($CO_2$ μmol $m^{-2}$ $s^{-1}$) at irradiance levels of 600 or 820 μmol $m^{-2}$ $s^{-1}$ ($n=5$/treatment) of *Empetrum nigrum* in different treatments ($P=0.0798$). Bar indicates the standard deviation and different letters statistical differences between the treatment means according to the *t*-test ($P<0.05$).

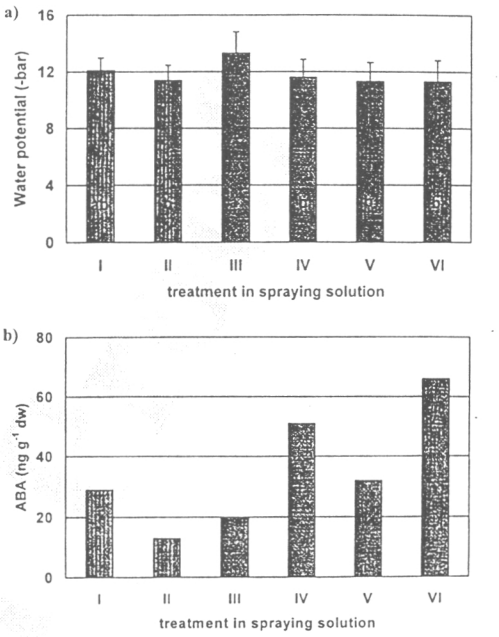


Fig. 4. (a) Water potential (−bar) of *Empetrum nigrum* ($n=9$–10). Bar indicates the standard deviation. Different letters indicate statistical differences between the treatment means according to the Kruskall–Wallis, comparison of mean ranks test ($P<0.05$). (b) Abscisic acid (ABA) content ng $g^{-1}$ dry weight in current-year leaves of *Empetrum nigrum*. Results are based on one combined sample up to four plants per treatment.

the bark was not removed, metal accumulation was reported to be higher in the stems than in the leaves in field-grown plants (Uhlig et al., 1996). The pattern was the same in a greenhouse experiment, where the metals were applied to the roots and surface contamination was excluded (Monni et al., 2000a). Helmisaari and Siltala (1989) found that the inner bark (living bark and phloem) of pine acted as a sink for elements, whereas smaller amounts accumulated in the wood and outer bark (Helmisaari and Siltala, 1989). Because the metals in this study were applied to the aerial parts of *E. nigrum*, and the stem samples were only divided to bark (including mainly epidermis and cork) and other stem tissue, more specific place in heavy metal accumulation in stem tissue cannot be evaluated.

Several studies have shown a negative influence of heavy metals on the photosynthetic pigments (Angelov et al., 1993; Bishnoi et al., 1993). Also more negative water potential by heavy metal treatments has been found (Bishnoi et al., 1993). However, in this experiment, the aerial application of heavy metals had no clear effect on the water potential and chlorophyll contents of *E. nigrum*. Chlorophyll *a*/*b* ratio, which is used as a stress indicator, increased slightly with increasing metal treatments, which was also seen in *E. nigrum* leaves near the Cu–Ni smelter in the field (Monni et al., 2000b). The chlorophyll *a*/*b* ratio has been reported to increase due to environmental stress (Delfine et al., 1999). The ABA contents increased slightly with increasing metal treatments, which has also been found earlier in experimental studies. In bean leaves the ABA content increased two-fold due to excess Ni (Rauser and Dumbroff, 1981), which is about as high increase as in *E. nigrum* leaves when exposed to the

highest heavy metal treatment. The overall ABA contents of *E. nigrum* leaves were lower than the values measured in the field (Monni et al., 2000b) and in conifer trees (Tan and Blake, 1993; Yang et al., 1993; Bianco and Dalstein, 1999).

The response of respiration of *E. nigrum* to temperature has been measured before and found to increase with increasing temperature (Wager, 1941). Otherwise, $CO_2$ exchange of *E. nigrum* has not been reported, but the observed rates were about the same level as those of *Pinus sylvestris* in Europe (Luoma, 1997). There was also an indication of decrease in dark respiration and maximum photosynthesis due to increasing treatment levels. The decrease in net photosynthesis due to heavy metals is supported by earlier studies (Lamoreux and Chaney, 1978; Angelov et al., 1993; Bishnoi et al., 1993). In contrast to this study, Lamoreux and Chaney (1978) found an increase in dark respiration by Cd, though the dark respiration was not clearly correlated with Cd content in tissue.

The total N and C concentrations in the leaves of *E. nigrum* were about the same level as those reported for the leaves and shoots of *Empetrum hermaphroditum* growing in North Scandinavia (Malmer and Nihlgård, 1980; Michelsen et al., 1996a, b). The increase in the C/N ratio with increasing treatment levels was due to the decreasing N concentrations in the leaves, and is contrary to the results of Michelsen et al. (1996a), who found that NPK fertilizers increased the N concentrations in shoots. They also found that fertilizers had no effect on the chlorophyll contents of *E. hermaphroditum* (Michelsen et al., 1996a). In the study of Michelsen et al. (1996a), however, the fertilizers were applied to the roots and the plants were not affected by heavy metal pollution. No correlation was found between leaf N concentrations, $CO_2$ exchange and chlorophyll *a* and *b* concentrations, although an increased N supply generally increases the photosynthetic rate (Hoogesteger and Karlsson, 1992). These results are consistent with the suggestion that the applied macronutrients were not taken up by the aboveground parts of *E. nigrum*.

## 5. Conclusions

The results showed that Cu, Fe, Pb, Cd, Ni and Zn applied to the aerial parts, were strongly accumulated on the bark and leaves of *E. nigrum*, while the applied macronutrients and Mn were readily removed from the leaf and bark surfaces by distilled water. Therefore, metal and macronutrient uptake by *E. nigrum* leaves, as reported earlier for crops (Chamberlain, 1983) and trees (Lin et al., 1995; Koricheva et al., 1997), was not confirmed by this study. No detectable changes in the water potential and chlorophyll concentrations were observed, indicating that the treatments had no critical effects on the ecophysiology of *E. nigrum*. This is consistent with the suggestion that the applied metals were located primarily outside the symplast. However, the surface contamination was found to decrease the dark respiration. Also an indication of a decrease in the maximum photosynthesis and an increase in the leaf ABA content of *E. nigrum* was found.

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# Ecophysiological responses of *Empetrum nigrum* to heavy metal pollution

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"Capsule": *Smelter emissions had a negative effect on the ecophysiology of* Empetrum nigrum *(crowberry), a species growing on heavy metal contaminated sites.*

Abstract

Chlorophyll, organic (citric and malic acids) and abscisic acid (ABA) contents and stem water potential were measured to indicate possible physiological effects of heavy metal deposition on *Empetrum nigrum* L. (crowberry). The leaves and stems of *E. nigrum* were collected at distances of 0.5 and 8 km from the Cu–Ni smelter at Harjavalta, south-west Finland. All the investigated parameters were clearly affected by heavy metal emissions. Chlorophyll contents in the leaves and organic acid contents in the leaves and stems were lower close to the emission source. Generally found increase in organic acid contents with increasing Ni concentrations was not found, which might be due to the lower production of organic acids measured by decreased photosynthesis near the smelter. In contrast, ABA contents in stems and leaves in general, were higher in plants growing 0.5 km from the pollution source. Close to the smelter the stem water potential of *E. nigrum* was less negative during the day but more negative during the night. These results suggest that smelter emissions have a negative effect on the ecophysiology of *E. nigrum* even though it is considered to be a tolerant species to heavy metals.


*Keywords:* *Empetrum nigrum* L.; Chlorophyll; Organic acids; Abscisic acid, ABA; Stem water potential; Cu–Ni smelter

## 1. Introduction

Heavy metal emissions are reported to have serious impacts on plants growing in the surroundings of Cu–Ni smelters (Amiro and Courtin, 1981; Helmisaari et al., 1995; Shevtsova, 1998). Many dwarf shrub species are described to grow on severely heavy metal-contaminated sites in the vicinity of Cu–Ni smelters in the northern hemisphere (Laaksovirta and Silvola, 1975; Bagatto and Shorthouse, 1991; Shorthouse and Bagatto, 1995; Shevtsova, 1998; Mälkönen et al., 1999). The evergreen dwarf shrub *Empetrum nigrum* L. (crowberry) has a wide ecological amplitude (Bell and Tallis, 1973; Elvebakk and Spjelkavik, 1995), and it survives at a distance of 0.5 km from the Cu–Ni smelter at Harjavalta, south-west (SW) Finland, where almost all other plant species have disappeared (Laaksovirta and Silvola, 1975; Helmisaari et al., 1995). At this site, not only the elevated heavy metal concentrations in the soil (Derome and Lindroos, 1998), but also decreased soil water-holding capacity (Derome and Nieminen, 1998) impairs growing conditions for plants. The metal tolerance mechanism of *E. nigrum* is not fully known, but one explanation for its survival is the accumulation of high Cu and Ni concentrations in older stems. Thus, *E. nigrum* is able to restrict the accumulation of Cu and Ni in its younger, growing parts (Helmisaari et al., 1995; Uhlig et al., 1996; Monni et al., 2000). *E. nigrum* is ericoid mycorrhizal plant and ericoid mycorrhiza of other dwarf shrub species *Calluna vulgaris* (L.) Hull. roots have been found to have

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an important role in Cu and Zn tolerance (Bradley et al., 1981, 1982).

The ecophysiological response of *E. nigrum* to metals has not been previously investigated, but growth reduction in response to increasing emission levels (Shevtsova, 1998) clearly indicates an ecophysiological impact of heavy metals on *E. nigrum*. In earlier investigations, chlorophyll, abscisic acid (ABA) and organic acid contents and water potential of plants have been used to indicate plant response to elevated heavy metal levels (Lee et al., 1978; Rauser and Dumbroff, 1981; Angelov et al., 1993; Bishnoi et al., 1993). The photosynthetic rate and chlorophyll concentrations of plants have been found to be decreased by Cu, Ni, Cd and Pb (Lamoreaux and Chaney, 1978; Becerril et al., 1989; Angelov et al., 1993; Bishnoi et al., 1993; Pandolfini et al., 1996), and an effect of Ni, Zn and Cd on water relations of plants is reported (Rauser and Dumbroff, 1981; Bishnoi et al., 1993). In experimental studies, stomatal conductance and water potential of the leaves have decreased and the ABA content, which regulates the water status of the plant, increased when plants were exposed to Ni (Rauser and Dumbroff, 1981; Bishnoi et al., 1993). However, also factors other than heavy metals may influence the ABA relations in the plants; higher levels of ABA in the roots and shoots is a typical response to nitrogen deficiency (Goldbach et al., 1975). ABA is also involved in the synthesis of proteins, prevention of precociuous germination, induction of dormancy and abscission of leaves (Marschner, 1995).

In general, plants possess physiological mechanisms that enable them to resist elevated heavy metal concentrations in their substrate (Antonovics et al., 1971; Baker, 1981, 1987; Woolhouse, 1983). Heavy metal tolerance of plants is species- and metal-specific and plants can either detoxify metals by binding them with organic acids, proteins or other ligands (Lee et al., 1978; Rauser and Curvetto, 1980; Godbold et al., 1984), or accumulate the metals in different plant parts or cell organelles (Reilly, 1969; Bringezu et al., 1999). Many plant species which are tolerant to Cu or Ni (Lee et al., 1978; Rauser, 1984) or to both Cu and Ni are found (Hogan and Rauser, 1979). However, the tolerance of these two metals is usually achieved by two different mechanisms. Organic acids play a central role in detoxifying metals in Ni- and Zn-accumulating plants (Ernst, 1975; Mathys, 1977; Lee et al., 1978; Yang et al., 1997), while Cu forms complexes with proteins and amino acids (Rauser, 1984).

The aim of this study was to investigate the general ecophysiology of *E. nigrum*, and to determine the responses of *E. nigrum* to heavy metal pollution by measuring physiological parameters. Chlorophyll contents of leaves were used as an indicator for physiological stress, because photosynthesis has been found to decrease due to elevated concentrations of Cu and Ni. Organic acids were measured to find out if there is any connection between organic acid contents and Ni resistance. To indicate the previously described connection between Cu, Ni and desiccation stress, the stem water potential and abscisic acid contents of *E. nigrum* leaves and stems were studied.

## 2. Materials and methods

### 2.1. The Harjavalta smelter area

The shoots of *E. nigrum* were collected at distances of 0.5 and 8 km to the south-east (SE) of the Cu–Ni smelter at Harjavalta, SW Finland. The Cu smelter was established in 1945 and the Ni smelter in 1960. Sulphur dioxide and heavy metals have been emitted into the environment for the past 40–60 years. The deposition of metals near the smelter was considerably reduced in the 1990s after a taller stack was built and electrostatic filters installed (Rantalahti, 1995). The prevailing wind direction is from the SW, and the emissions are therefore primarily dispersed to the north-east.

At both locations (0.5 and 8 km) 10 separate *E. nigrum* patches were marked situating more than 3 m from each other, and these plants were used for all physiological measurements. The sites were chosen SE from the smelter, because it was the only direction where the two sites represented the same forest site type (*Calluna* site type) and soil type (orthic podzol). The pH of the organic layer was 3.5 at 0.5 km distance and 3.6 at 8 km distance from the smelter (Derome and Lindroos, 1998). The site at 0.5 km is located in a heavily polluted area where the total Cu and Ni concentrations in the organic layer are over 5800 and 460 mg $kg^{-1}$ dry wt., respectively, and base cations (e.g. Ca, Mg) have been displaced from the topsoil. The concentrations of other heavy metals (Fe, Zn, Cd, Pb, Cr) in the organic layer are also elevated near the smelter. The site at 8 km is only slightly polluted, the total Cu and Ni concentrations in the organic layer being 150 and 40 mg $kg^{-1}$ dry wt., respectively. The concentrations of other heavy metals are also much lower at 8 km than at 0.5 km, but are higher than background values (Derome and Lindroos, 1998).

The material collection for physiological measurements was done on 21 and 26 July, 18–19 and 26 August and 9 October 1997. Near the Cu–Ni smelter at Harjavalta (61°19′ N, 22°9′ E), the monthly mean temperature and precipitation in July were 18.4°C and 86 mm, in August 18.1°C and 38 mm, in September 10.7°C and 111 mm and in October 2.7°C and 66 mm, respectively, calculated by the model of Ojansuu and Henttonen (1983).

### 2.2. Analysis of chlorophyll a and b

Several current-year shoots of five to 10 patches of *E. nigrum* were collected at both distances (0.5 and 8 km) on 21 July, 18–19 and 26 August and 9 October in container of liquid nitrogen (−196 °C), and stored in freezer at -80°C. The current-year shoots were freeze-dried, and separated into leaves and stems and ground with a mortar and pestle. Weighed (10 mg) aliquots were extracted with 3 ml of 80% acetone overnight at +4°C, centrifuged for 3 min, and the absorbances ($A$) at 647 and 664 nm recorded on a spectrophotometer (Shimadzu UV–1201, UV–VIS). Two measurements per sample were made. Chlorophyll $a$ (μmol $l^{-1}$) was calculated according to the equation $13.19\times A_{664}-2.57\times A_{647}$, and chlorophyll $b$ (μmol $l^{-1}$) according to the equation $22.10\times A_{647}-5.26\times A_{664}$ based on MacKinney's coefficients (Graan and Ort, 1984), and the results were expressed as chlorophyll content in the tissue (μmol chlorophyll $g^{-1}$ dry weight).

### 2.3. Analysis of citric and malic acids

Several previous-year shoots of five to six *E. nigrum* patches were collected for organic acid analysis from the two sites. Due to technical reasons the sampling was carried out only on 9 October simultaneously for other sampling. To study the connection between organic acids and heavy metals, the previous-year stems were chosen instead of the most active current-year shoots (as for chlorophyll and ABA), because the metals are accumulated mainly in the older stems.

Immediately after harvesting, the samples were frozen in liquid nitrogen. After freeze-drying, the leaves and stems were separated and ground to a fine powder using a mortar and pestle or a dismembrator (Braun Melsungen, Germany). Two replicates per sample were prepared.

Citric and malic acids were determined enzymatically by a method modified from Boehringer (1989) and Hampp et al. (1984). Twenty milligrams of lyophilized tissue powder and 20 mg of polyvinylpolypyrrolidone (PVPP) were mixed in an eppendorf tube. PVPP was used to minimize the disturbing effect of phenolics, chlorophyll and other compounds. The organic acids were extracted by adding 500 μl 0.1 N HCl, mixing gently for 15 min at room temperature, then incubated at 100°C for 10 min. After cooling, samples were centrifuged (Hettich microrapid) and aliquots of the supernatant were used for the enzymatic determinations.

For the citric acid (citrate) analyses 25 μl of extract was incubated with 160 mM glycylglycine buffer (pH 7.8), 0.18 mM $ZnCl_2$, 0.2 mM NADH and 12 U $ml^{-1}$ malate dehydrogenase (all final concentrations) in a total volume of 1 ml. The decrease in optical density after addition of 0.26 U $ml^{-1}$ citrate lyase was followed at 340 nm in a spectrophotometer (Kontron). The amount of NADH oxidized is stoichiometric with the amount of citrate. In addition, blanks (without sample) and standards containing 0.5 mM citric acid were analysed.

For the quantification of malic acid (malate) 25 μl of the extract was incubated with 60 mM glycylglycine buffer (pH 10.0), 9 mM L-glutamate, 4 mM NAD, and 1.8 U $ml^{-1}$ glutamate-oxaloacetate transaminase (all final concentrations) in a total volume of 1 ml. After addition of 27 U $ml^{-1}$ malate dehydrogenase, the increase in optical density was recorded at 340 nm and was stoichiometric with the amount of malate present in the sample. Blanks (without sample) and standards containing 1.0 mM of malate were also analysed.

### 2.4. Stem water potential of E. nigrum and soil moisture

Pressure chamber determinations of *E. nigrum* were carried out to estimate the total water potential of the xylem sap, during the day on 21 July, during the night on 26 July and during the day and night on 18–19 August. The previous-year stems were separated with a razor blade, and the stem water potential measured in sunny or partly cloudy weather using a Scholander's pressure bomb (Scholander et al., 1965; Ritchie and Hinckley, 1975). Night measurements were carried out to determine the relatively constant water potential throughout the plant. Seven to 10 plant patches per site were chosen, and two to three separate stem pieces of each plant measured.

Because only one instrument was available, the measurements could not be performed simultaneously at both sites. Therefore, the water potential measurements of *E. nigrum* on 21 July were made at noon (12:30–14:30) at the site at 8 km, and in the afternoon (16:00–17:15) at the site at 0.5 km. The stem water potential measurements of 18 August (at 0.5 km distance) and 19 August (at 8 km distance) were made throughout the day (between 13:00–16:30 in every half an hour) in order to find out the differences between the values obtained at noon and in the afternoon. However, the means of the whole day values were calculated and are shown in the figures because there were no major differences related to the time of the day. The night measurements were done first at 0.5 km distance (12:00–2:20) and then at 8 km distance (3:00–4:40) on 26 July. On 18–19 August the night measurements were also done first at 0.5 km distance (11:20 p.m.–12:40 a.m.) and then at 8 km distance (1:15–2:15). The weather conditions at night were very constant, the air temperature being approximately +12°C and air moisture 20–35% at both sites.

The soil moisture measurements were made simultaneously with the stem water potential measurements. The soil moisture was measured using a ThetaProbe soil moisture sensor (type ML1). The ground vegetation was carefully removed and the measurements were made

horizontally in the three soil horizons (O, A, B). Three plots per site (0.5 and 8 km distance from the smelter) and four measurements per each plot were done (12 replicates in total). Because of the same soil type at the both locations (Derome and Lindroos, 1998), the soil measurements were done approximately from the same depths at both sites. The difference between the sites was, however, that the understorey vegetation is almost totally lacking at 0.5 km distance from the smelter (Mälkönen et al., 1999).

### 2.5. Analysis of ABA

For the ABA analyses, several current-year shoots of two *E. nigrum* patches were collected on 21 July, 18–19 and 26 August and 9 October at the two sites (0.5 and 8 km) simultaneously with other sampling. For methodological reasons the ABA contents in the stems collected on 21 July could not be analysed. The samples were frozen in liquid nitrogen and stored at −80°C. The leaves and stems were freeze-dried, separated and ground with a mortar and pestle. One-hundred milligrams of homogenized plant material was suspended in 5 ml of 0.05 M phosphate buffer (pH 8.0), and 50 ng internal standard ([$^2H_4$]ABA) was added. After extraction in darkness, shaking at 4°C for a minimum of 2 h, the samples were centrifuged at 5000 rpm for 15 min. The supernatant was removed, and the pellet was resuspended in 2 ml of 0.05 mM phosphate buffer (pH 8.0) and centrifuged at 5000 rpm for 15 min. The supernatants were combined, the pH was adjusted to 2.7 with 0.1 M HCl, and partitionated three times against equal volumes of ethyl acetate. The pooled ethyl acetate phase was reduced to dryness in vacuo in a rotary evaporator at 40°C.

The residue was dissolved in 100 μl of 80% methanol and injected into a Radial Pak™ (Waters, Milford, USA) $C_{18}$ high-performance liquid chromatography (HPLC) column (8×100 mm, 4 μm particle size). The HPLC system consisted of a Waters 510 pump, 600E control unit, 712 autosampler and 486 UV detector. ABA was eluted from the column by a linear gradient using a binary solvent system consisting of methanol and water, both containing 30 mM acetic acid, with a flow rate of 2 ml $min^{-1}$. The gradient was from 30 to 50% methanol within 20 min. The fraction containing ABA was collected (15.5–17.5 min), and reduced to dryness in vacuo.

The residue was dissolved in 100 μl of methanol and methylated in 500 μl of diazomethane dissolved in ether for 30 min. The sample was dried under a stream of $N_2$, dissolved in 12 μl of heptane, and 1 μl of this solution was injected into the gas chromatography–mass spectrometry (GC–MS). The GC–MS system consisted of a GC8060 gas chromatograph with a A200S autosampler (Fisons, Milan, Italy), and a Platform mass spectrometer (Micromass, Altrincham, UK). The injector was operated at 230°C using the splitless mode and a CP-SIL 8CB Low Bleed MS (25 m×0.25 mm i.d., 0.12 μm coating) column with a 2.5 m deactivated guard column (0.35 mm i.d., Chrompack Middelburg, The Netherlands) was used. Helium was used as the carrier gas at a flow rate of 25 cm $s^{-1}$. The column temperature was programmed to change in a three slope linear gradient. The first phase was at 50°C for 2 min, then increased from 50 to 160°C at 15°C $min^{-1}$. The second slope was from 160 to 210°C at 3°C $min^{-1}$, and the third slope was from 210 to 280°C at 20°C $min^{-1}$. The MS source was operated in electron impact mode at 70 eV at 180°C, and the interface at 250°C. Ions of $m/z$ 162, 190, 193 and 194 were monitored.

### 2.6. Statistical analysis

To evaluate the effects of heavy metal deposition on *E. nigrum* the means of the measured parameters (soil moisture, chlorophyll, organic acid, ABA contents, stem water potential of plants) deriving from the two sites were compared by $t$-test when logarithmic transformations were used to normalise the data (Sokal and Rohlf, 1995; Statistix, 1996). Otherwise the Kruskal–Wallis comparison of mean ranks test was used (Sokal and Rohlf, 1995; Statistix, 1996).

## 3. Results

### 3.1. Chlorophyll contents

Plant chlorophyll ($a+b$) contents were lower at 0.5 than at 8 km, and the differences between the means were statistically significant through the whole season ($P<0.05$) (Fig. 1a). The means of the chlorophyll ($a+b$) contents varied between 1.9 and 3.2 μmol chlorophyll $g^{-1}$ dry wt. and were the lowest in October compared to the other sampling dates. The chlorophyll $a/b$ ratio, was generally lower at a distance of 8 km than at 0.5 km, and the difference between the means at two distances was statistically significant ($P<0.05$) in mid August (Fig. 1b).

### 3.2. Citric and malic acid contents of

The citric acid contents in the leaves and stems of *E. nigrum* were higher at 8 km than at 0.5 km. In the stems, the difference between the means was statistically significant ($P<0.05$) (Fig. 2a). In the leaves, the mean pools of citric acid were 16 nmol $mg^{-1}$ dry wt. at 8 km and 13 nmol $mg^{-1}$ dry wt. at 0.5 km, and thus exceeded those in the stems of 14 nmol $mg^{-1}$ dry wt. and 11 nmol $mg^{-1}$ dry wt., respectively (Fig. 2a).

The pools of malic acid in the leaves and stems of *E. nigrum* were higher at 8 km than at 0.5 km. The

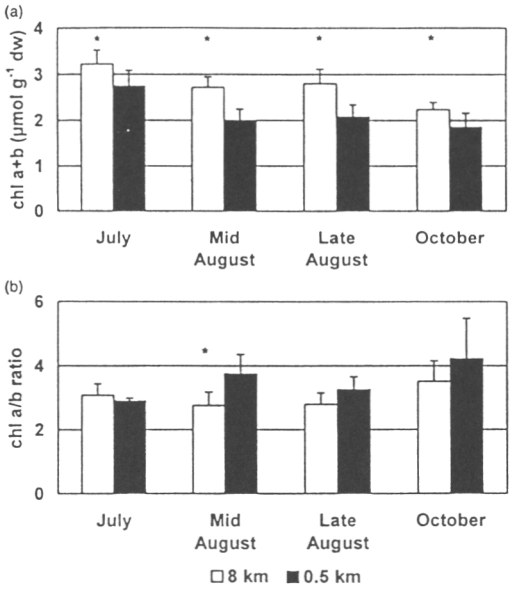


Fig. 1. (a) Chlorophyll ($a+b$) contents (μmol chlorophyll $g^{-1}$ dry weight) and (b) chlorophyll $a/b$ ratios in the leaves of *Empetrum nigrum* at 0.5 and 8 km distances from the smelter in four collection dates. Bar indicates standard deviation ($n=5$–10), and an asterisk indicates significant difference ($P<0.05$) between the means at two distances.

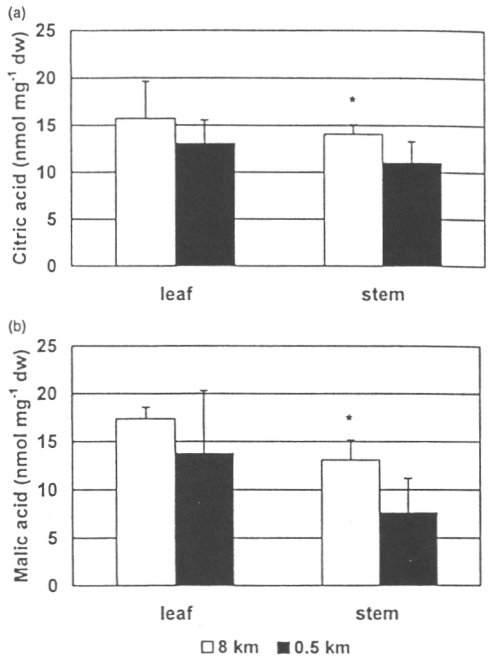


Fig. 2. (a) Citric acid and (b) malic acid contents (nmol $mg^{-1}$ dry weight) in the leaves and stems of *Empetrum nigrum* collected at 0.5 and 8 km distances from the smelter. Bar indicates standard deviation ($n=5$–6), and an asterisk indicates significant difference ($P<0.05$) between the means at two distances.

difference between the means of the malic acid content of the stems was statistically significant ($P<0.05$; Fig. 2b). In the leaves, the mean malic acid values exceeded those in the stems, and were 17 nmol $mg^{-1}$ dry wt. at 8 km and 14 nmol $mg^{-1}$ dry wt. at 0.5 km compared to 13 and 8 nmol $mg^{-1}$ dry wt., respectively, in the stems (Fig. 2b).

### 3.3. *Stem water potential of* E. nigrum *and soil moisture*

The stem water potential of *E. nigrum* was more negative during the day (−15 to −21 bars) than at night (−4 to −12 bars; Fig. 3a, b). During the day, the water potential of plants at 8 km was more negative than those at 0.5 km. However, during the night the opposite was found (more negative at 0.5 km distance). The means of the stem water potential measured during the day and at night in July and during the day in August differed significantly between the plants growing at the two sites ($P<0.05$) (Fig. 3a, b).

The soil moisture content showed seasonal variation and decreased with depth (Fig. 4a, b). In the organic layer, soil moisture was higher at 8 km than at 0.5 km both during the day and at night in July. In August, however, it was higher at the site at 0.5 km. The differences between the means were statistically significant at night ($P<0.05$). In the mineral soil (A horizon) the trend was somewhat similar, and the difference between the means at the two sites was statistically significant during the day in August ($P<0.05$) (Fig. 4a, b). In the lower mineral soil layer (B horizon), too, the trend was similar to that in the organic layer, and there were significant differences between the means measured during the day ($P<0.05$) (Fig. 4a). At night the soil moisture content was the same at both sites (Fig. 4b).

### 3.4. *ABA contents*

*Empetrum* plants growing at 0.5 km had higher contents of ABA in their stems compared to those growing at 8 km (Fig. 5a). With the exception of the July sampling, a similar pattern was also observed in the leaves (Fig. 5b). No statistical differences were found between the means ($P>0.05$). In late autumn the ABA contents decreased in the stems, but not in the leaves. The mean ABA contents in the leaves varied between 66–232 and 56–196 ng $g^{-1}$ dry wt., and in the stems between 45–113 and 33–71 ng $g^{-1}$ dry wt. at 0.5 and 8 km, respectively.

## 4. Discussion

Chlorophyll contents in the leaves of *E. nigrum* were, in general, within the range reported for unpolluted

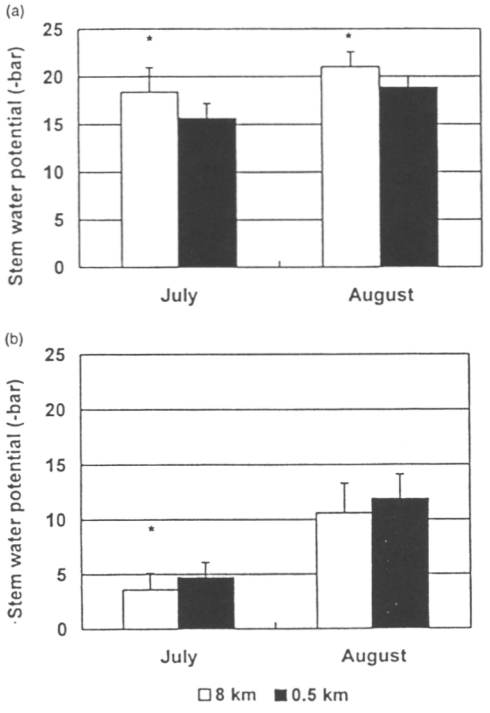


Fig. 3. Stem water potential of *Empetrum nigrum* (a) during the day and (b) at night at 0.5 and 8 km distances from the smelter. Bar indicates standard deviation ($n = 27$–30), and an asterisk indicates significant difference ($P < 0.05$) between the means at two distances.

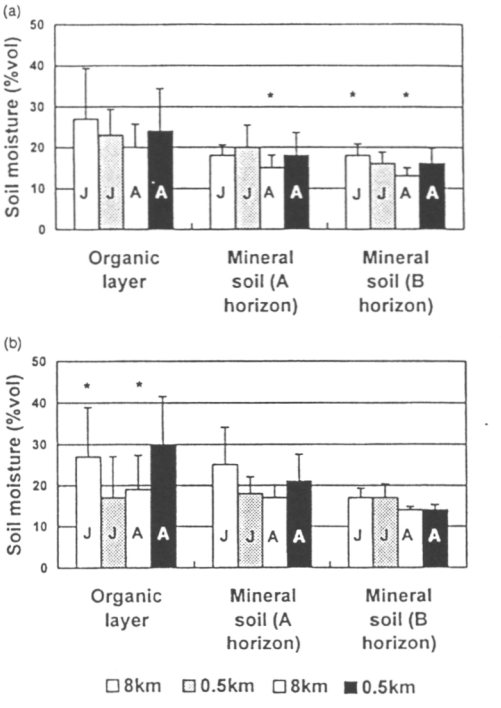


Fig. 4. Soil moisture (%vol) of organic and two mineral soil layers (a) during the day and (b) at night at 0.5 and 8 km distances from the smelter. Bar indicates standard deviation ($n = 12$) and an asterisk indicates significant difference ($P < 0.05$) between the means at two distances. J, July; A, August.

locations in Swedish Lapland for *Empetrum hermaphroditum* (Michelsen et al., 1996). However, at the 0.5 km site, the chlorophyll contents in *E. nigrum* were 15–30% lower than the values for plants growing at 8 km. Similar decreases in chlorophyll contents have been reported for several broadleaved species exposed to metals (Angelov et al., 1993; Bishnoi et al., 1993). The reduction in leaf chlorophyll contents may contribute to the stunted growth of *E. nigrum* near to the smelter. Shevtsova (1998) reported a 30% decrease in the length growth of terminal shoots of *E. nigrum* near Cu–Ni smelters in the Kola Peninsula compared to those from an unpolluted area. The growth of *E. nigrum* near to the Harjavalta smelter has also decreased (Salemaa et al., 1995). Near the smelter, the total Fe concentrations are increased and Mg concentrations decreased in *E. nigrum* tissue compared to further distance (Uhlig, unpublished results). Also, for example, pine is suffering from the Mg deficiency (Derome and Nieminen, 1998; Nieminen et al., 1999), and the pattern for exchangeable concentrations of Mg and Fe in the organic soil is similar to that of the plant parts (Derome and Lindroos, 1998). As being the constituents of chloroplasts (Marschner, 1995), the concentrations of these elements in *E. nigrum* and soil might partly explain the decreased contents of chlorophylls near the smelter. Chlorosis is the main symptom of Mg deficiency (Marschner, 1995) and excess Fe may catalyze formation of hydroxyl radicals in the chloroplasts being an early event of drought-induced damage in photosynthetic tissue in dry conditions (Price and Hendry, 1991).

Citric acid contents in the leaves and stems of *E. nigrum* were generally lower than the values reported by Lee et al. (1978). These authors found citric acid contents of up to 14–27 nmol $mg^{-1}$ dry wt. in plant shoots (*Homalium* sp.) containing 100–1000 mg $kg^{-1}$ Ni. Moreover, Ni hyperaccumulators (e.g. *Sebertia* sp., *Hybanthus* sp.) contained two times more citric acid than 'normal' plants (close to 70 nmol $mg^{-1}$ dry wt.). However, there was much variation in citric acid contents between species (Lee et al., 1978); levels up to 440 nmol $mg^{-1}$ dry wt. citric acid have been reported in grass shoots exposed to Ni (Yang et al., 1997).

The values reported in the literature for malic acid (malate) differ considerably depending on the plant species investigated (Mathys, 1977; Yang et al., 1997). In Cu- and Ni-resistant *Silene cucubalus* leaves, malate

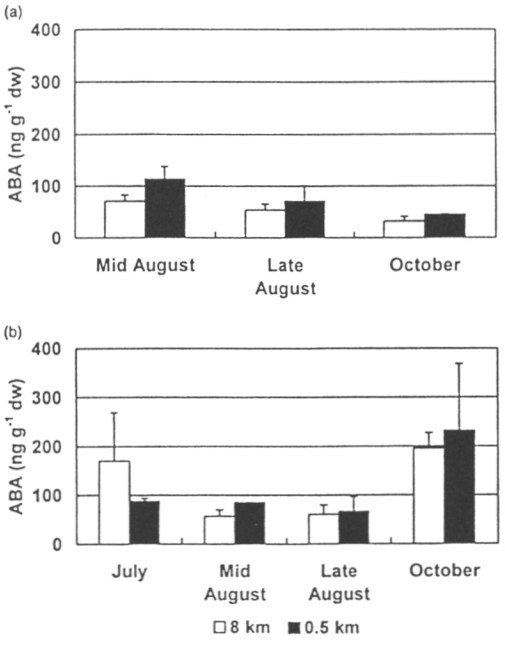


Fig. 5. Abscisic acid (ABA) contents (ng $g^{-1}$ dry weight) in (a) stems and (b) leaves of *Empetrum nigrum* at 0.5 and 8 km distances from the smelter in three to four collection dates. Bar indicates standard deviation ($n=2$). There was no statistical difference between the means ($P>0.05$).

content was up to 3 nmol $mg^{-1}$ fresh wt., when exposed to Zn (Mathys, 1977). This is about the same as values found here in the leaves and stems (8-17 nmol $mg^{-1}$ dry wt.) of *E. nigrum*. In grass species (maize, ryegrass), malic acid content increased to up to 375 nmol $mg^{-1}$ dry wt. after exposure to Ni (Yang et al., 1997).

The organic acid contents in *E. nigrum* stems and leaves were lower in the plants growing at 0.5 km than at 8 km distance from the Cu–Ni smelter. In plants, elevated pools of organic acids are usually connected to elevated heavy metal levels in the substrate (especially Ni and Zn), and positive correlations have been found between organic acid and Ni and Zn concentrations in a number of studies (Lee et al., 1978; Godbold et al., 1984; Yang et al., 1997). Organic acids are known to take part in the uptake and transport of metals, and accumulate in the cytosol or vacuoles of plants (Ernst, 1975; Ernst et al., 1992). Therefore, the concentrations of organic acids usually increase due to Ni and Zn stress (Lee et al., 1978; Godbold et al., 1984; Yang et al., 1997). However, in some studies a decline in organic acids under heavy metal stress has been reported (Foy et al., 1987).

The results of our study do not follow the generally accepted pattern. At Harjavalta, Ni and Zn concentrations in the soil are elevated at 0.5 km from the smelter (Derome and Lindroos, 1998), where citric acid contents of 11 and 13 nmol $mg^{-1}$ dry wt. in the *E. nigrum* stems and leaves were found. At this site, Ni contents in the *E. nigrum* stems were between 63 and 164 mg $kg^{-1}$, and in the leaves 32–115 mg $kg^{-1}$ (Uhlig, unpublished results). In plants growing at 8 km, the respective citric acid contents were 14 nmol $mg^{-1}$ dry wt. in the stems and 16 nmol $mg^{-1}$ dry wt. in leaves. Moreover, in plants collected from a site at 4 km from the smelter, total Ni concentrations in leaves and stems of *E. nigrum* were 13–39 and 8–16 mg $kg^{-1}$, respectively (Uhlig, unpublished results). Therefore, it would appear that the total Ni concentrations in *E. nigrum* are close to background values at 8 km from the smelter. However, the lower metabolic efficiency measured by the decreased chlorophyll contents might explain the decreased production of organic acids near the smelter.

The mean citric and malic acid contents in the leaves exceeded those in the stems of *E. nigrum*, although heavy metals are known to preferentially accumulate in the stems of *E. nigrum* (Uhlig et al., 1996; Monni et al., 2000). The highest malate contents reported by Mathys (1977) occurred in either the leaves or stems, depending on the plant species. There were not large differences between the contents of malic and citric acids in both leaves and stems of *E. nigrum*. In the leaves of herbs and grasses either malic or citric acid contents were higher, depending on the plant species (Ernst, 1975) but in *E. nigrum* berries (Kallio and Markela, 1982), the malic acid contents exceeded citric acid contents.

It has been speculated that drought resistance could help plants to survive in polluted areas (Pandolfini et al., 1996). *E. nigrum* is highly tolerant to drought and the anatomy of both its leaves (Wollenweber et al., 1992) and vascular tissue is xeromorphic (Carlquist, 1989). ABA contents in *E. nigrum* stems and leaves were partly in agreement with results of earlier studies that showed heavy metal-induced ABA accumulation (Rauser and Dumbroff, 1981; Poschenrieder et al., 1989). Plant ABA contents were higher near to the smelter in August and October, although no significant differences were found due to the low number of replicates. The reasons for the high ABA contents in the leaves in October are not known, but they may be related to environmental conditions, e.g. decreasing temperature, day length and ageing of the leaves.

The stem water potential and leaf ABA contents at both sites correlated in July, but not in August, according to an overall pattern, e.g. the ABA content in the leaves usually rises as the leaf water potential falls (Beardsell and Cohen, 1975). However, in the early stages of soil desiccation, ABA is transported as a chemical signal from the roots to the leaves where, by enhancing stomatal closure, it prevents a decline in

water potential (Davies and Zhang, 1991). Additionally, in nutrient-deficient plants, production of ABA seems to occur at less negative water potentials (Radin, 1984). Near to the smelter at Harjavalta, *E. nigrum* is suffering from decreased concentrations of Mg and Mn, but other nutrients (N, P, Ca, K) in the plant tissue are not significantly lower near the smelter (Uhlig, unpublished results). In addition to nutrient deficiency, plants might require less water for growth and photosynthesis as these metabolic processes have declined. The roots, where the ABA synthesis occurs (Marschner, 1995), are damaged near the smelter, which could in turn affect the ABA synthesis and measured ABA contents.

The soil moisture content was almost the same during day and night. The soil moisture differed from the stem water potential measurements at night, the more negative stem water potential at night near the smelter correlating in general with the soil moisture in July but not in August. The stem water potential at night is close to the soil water potential because the stomata are closed (Aber and Melillo, 1991). However, the prevailing weather conditions and heterogeneity of the soil are factors that might affect the results. The impaired water-holding capacity at 0.5 km (Derome and Nieminen, 1998), would support the more negative stem water potential at night, close to the smelter.

## 5. Conclusions

Our results indicate that heavy metal pollution has negative effects on *E. nigrum*. The decreased contents of chlorophyll pigments and organic acids and increased ABA contents indicate a reduction in the physiological activity of *E. nigrum* near to the pollution source. Although *E. nigrum* is known to be one of the most tolerant species near Cu–Ni smelters in the northern hemisphere (Helmisaari et al., 1995; Uhlig et al., 1996) and to accumulate high concentrations of Cu and Ni in its living parts in greenhouse experiments (Monni et al., 2000), the emissions have clearly decreased the vitality of *E. nigrum*.

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# V

# ULTRASTRUCTURAL ELEMENT LOCALIZATION BY EDXS IN *EMPETRUM NIGRUM*

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**Abstract**

*E. nigrum* is one of the few species growing on highly polluted areas in the northern boreal forests and accumulates considerable amounts of heavy metals especially in its older stems. Previous-year stems of *Empetrum nigrum* were collected from two different sites located at distances of 0.5 km (highly contaminated) and 8 km (low contaminated) from a Cu-Ni smelter at Harjavalta, SW Finland. The element (Al, As, Cu, Fe, Mn, Zn, Ca, K, P, S, Mg, Na) localization was performed by energy-dispersive X-ray spectroscopy (EDXS) after cryofixation, freeze-drying and pressure infiltration of the material. The results showed higher amounts of Cu, As and Fe in cell compartments of *E. nigrum* close to the smelter than in further distance. The Al and Zn amounts, in contrast, showed no clear differences between the sites. Cu was distributed homogeneously in the tissue and occurred in vacuoles, cytoplasm, cell walls as well as in lumens of the vascular tissue. The higher amounts of As were localized in the primary cell walls of living (ray cells, phloem) than dead cells (xylem, sclereids), but also vacuoles and cytoplasm contained elevated amounts of As. Ray cells, phloem and sclereids had elevated Fe amounts compared to the other tissues in the contaminated stem samples but owing to the high variation between the replicates, no significant differences were found. Also Fe was localized in the cell walls, cytoplasm and

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vacuoles. Based on the rather homogeneous localization of Cu, As and Fe in the living tissue and increased amounts of Cu, As and Fe in vacuoles, cell walls and cytoplasm near the smelter, it seems that not only one specific mechanism contribute to the heavy metal tolerance of *E. nigrum*. The role of complexing agents in heavy metal tolerance in the cytoplasm or vacuoles could not be shown by this study. Because of the more frequent localization of electron dense phenolic material in the polluted samples, it might also have a function in the heavy metal tolerance of *E. nigrum*.

**Key words**

*Empetrum nigrum* L., crowberry, heavy metal localization, nutrient localization, Cu, As, Fe, Ca, K, tolerance, transmission electron microscopy, EDXS

## 1. Introduction

The heavy metal tolerance of plants is based on different biochemical mechanisms and is often species and metal specific (e.g. Antonovics *et al.*, 1971; Baker, 1981; Woolhouse, 1983; Baker and Walker, 1990). Plants can produce intracellular metal-chelating substances, so that the intracellular availability of metals is maintained within certain limits (Verkleij and Schat, 1990). The accumulation of metal-chelating substances upon exposure to excessive metal amounts has been found. Organic acids (malic and citric acid) are important in chelating Ni and Zn (Lee *et al.*, 1978; Yang *et al.*, 1997), while Cu has been found to be associated with proteins and phenolic compounds in leaves and roots (Neumann *et al.*, 1995). Deleterious amounts of metals can also be translocated and stored in certain cell organelles, where sensitive metabolic activities do not take place (Verkleij and Schat, 1990). The translocation of metals varies, but generally cell walls, vacuoles, intercellular spaces and cytoplasm of roots and leaves contain elevated metal (Cu, Fe, Zn, Pb) amounts (Mullins *et al.*, 1985; Neumann *et al.*, 1995; Neumann *et al.*, 1997; Lichtenberger and Neumann, 1997). For example in leaves, Ni has been found to be accumulated in the chloroplasts of the bundle sheath cells (L'Huillier *et al.*, 1996).

The ecology (Bell and Tallis, 1973) and wood anatomy (Miller, 1975; Carlquist, 1989) of crowberry (*Empetrum nigrum* L.) have been extensively studied, but its heavy metal tolerance mechanism on the cellular or subcellular level is not well known. Besides wide

ecological amplitude (Bell and Tallis, 1973), *E. nigrum* grows on highly heavy metal contaminated sites in the vicinity of Cu-Ni smelters in the northern hemisphere (Laaksovirta and Silvola, 1975; Chertov *et al.*, 1993; Helmisaari *et al.*, 1995; Uhlig *et al.*, 2000). It grows also in serpentine soils (Proctor and Woodell, 1971), which typically contain elevated amounts of Ni, Cr and Mg and low levels of Ca (Proctor, 1971). *E. nigrum* has been found to accumulate relatively high concentrations of Cu and Ni in greenhouse and field especially in older plant parts (Helmisaari *et al.*, 1995; Monni *et al.*, 2000a; Uhlig *et al.*, 2000), whereas the transport of metals to the green leaves is restricted (Monni *et al.*, 2000a). The structure of the vascular tissue of the stem wood is very xeromorphic; the large number of vessels and the presence of tracheids of the imperforate tracheary element type lead to a high conductive safety of the plant (Miller, 1975; Carlquist, 1989).

The use of transmission electron microscopy (TEM) equipped with electron analyzers has made the ultrastructural localization of elements in plants possible. Heavy metal tolerance mechanisms and the localization of elements in several plant species, which contain relatively high amounts of metals in their tissues, have been studied by electron dispersive X-ray spectroscopy (EDXS) and electron energy loss spectroscopy (EELS) (Mullins *et al.*, 1985; Turnau *et al.*, 1993 a, b; Neumann *et al.*, 1995; Lichtenberger and Neumann, 1997). However, both methods have their advantages and limitations (Stelzer and Lehmann, 1993; Kottke, 1994; Bücking *et al.*, 1998).

The aim of the current X-ray microanalytical study was to investigate heavy metal and nutrient localization in stems of *E. nigrum* from a highly and a moderately heavy metal polluted area in order to obtain more information about possible tolerance mechanisms of *E. nigrum*. Previous-year stems were selected for the experiments, because the highest concentrations of metals have been measured in older tissues, especially in stems (Monni *et al.*, 2000a; Uhlig *et al.*, 2000).

## 2. Materials and methods

### *2.1. The Harjavalta smelter area*

The previous-year stems were collected at a distance of 0.5 (high contamination) and 8 km (low contamination) to the south-east from the Cu-Ni smelter at Harjavalta, SW Finland.

The Cu smelter was established in 1945 and the Ni smelter in 1960. Sulphur dioxide and heavy metals have been emitted into the environment for the past 40-60 years. The deposition of metals near the smelter was considerably reduced in the 1990's after a taller stack was built and electrostatic filters were installed (Rantalahti, 1995). The prevailing wind direction has been from the south, south-west and south-east (Derome, 2000).

The sites were chosen to the south-east of the smelter because it was the only direction with two sites representing the same forest site type (*Calluna* site type) and soil type (orthic podzol). The pH of the organic layer was 3.5 at 0.5 km and 3.6 at 8 km distance from the smelter (Derome and Lindroos, 1998). The site at 0.5 km is located in a heavily polluted area where the total element concentrations in the organic layer are 5 800 mg Cu $kg^{-1}$ dw, 460 mg Ni $kg^{-1}$ dw, 18 600 mg Fe $kg^{-1}$ dw, 520 mg Zn $kg^{-1}$ dw, 1 560 mg Al $kg^{-1}$ dw, 30 mg Mn $kg^{-1}$ dw, 970 mg Ca $kg^{-1}$ dw, 380 mg K $kg^{-1}$ dw and 440 mg Mg $kg^{-1}$ dw. Although the background values are not reached, the soil is only slightly contaminated at a distance of 8 km from the smelter, the respective concentrations being 150 mg Cu $kg^{-1}$ dw, 40 mg Ni $kg^{-1}$ dw, 2 200 mg Fe $kg^{-1}$ dw, 60 mg Zn $kg^{-1}$ dw, 1 760 mg Al $kg^{-1}$ dw, 60 mg Mn $kg^{-1}$ dw, 970 mg Ca $kg^{-1}$ dw, 400 mg K $kg^{-1}$ dw and 210 mg Mg $kg^{-1}$ dw. Near the smelter Ca, Mg and K cations are displaced from cation exchange sites by Cu and Ni cations in the organic layer, the exchangeable concentrations of those nutrients being much lower near the smelter than further distance (Derome and Lindroos, 1998).

*2.2. Preparation technique for EDXS analysis*

Previous-year stems were collected in the field, cryofixed by liquid argon gas surrounded by liquid nitrogen and stored in liquid nitrogen (-192 °C). After cryofixation the stem pieces were freeze-dried (CFD, Leica, Germany) for 14 days under high vacuum ($< 1 \times 10^{-5}$ mbar) and low temperature conditions (-100 °C) to avoid ice recrystallization in the cytoplasm of the cells (recrystallization temperature: -80 °C, Robinson *et al.*, 1985). After freeze-drying, the stem samples were pressure infiltrated directly in 100 % Spurr's epoxy resin (Spurr, 1969), using a method described by Fritz (1980). Because some of the samples could not be infiltrated directly in 100% Spurr's epoxy resin, some intermediary steps using ether were added to the embedding protocol. For EDXS the embedded samples were dry sectioned (0.5 µm), placed on filmed Ni or Cu grids and carbon coated.

*2.3. EDXS analysis*

The energy dispersive X-ray microanalytical studies were carried out under standardized conditions using a Philips EM 420 provided with the EDAX DX-4 system. EDXS spectra were collected between 0 and 20 keV with a Si(Li) X-ray detector (size: 10 $mm^2$) equipped with a thin beryllium window. An acceleration voltage of 120 kV, an objective aperture of 70 µm and a detection time of 100 live seconds were used. The calculated effective spot size ($D_{eff}$) of the measurement points was 12 nm. However, based on the interactions of the electron beam with the specimen, the real spot size was slightly larger. One spectrum between 0.6 and 8.6 keV is shown in Fig. 1. The peak centre of the Kα-line of the individual elements was 1.04 keV for Na, 1.25 keV for Mg, 1.49 keV for Al, 1.74 keV for Si, 2.01 keV for P, 2.31 keV for S, 2.62 keV for Cl, 3.31 keV for K, 3.69 keV for Ca, 5.89 keV for Mn, 6.40 keV for Fe, 7.45 keV for Ni, 8.04 keV for Cu, 8.63 keV for Zn and 10.53 keV for As. The element distribution was measured as a peak to background ratio (P/B) in order to minimize the effects of surface irregularities of the sections during analysis (Fig. 3). For the X-ray map a magnification of 6400 ×, a resolution of 128 × 100 measurement points and a dwell time of 500 ms in the live second mode were used. The image was captured with 1024 × 800 points.

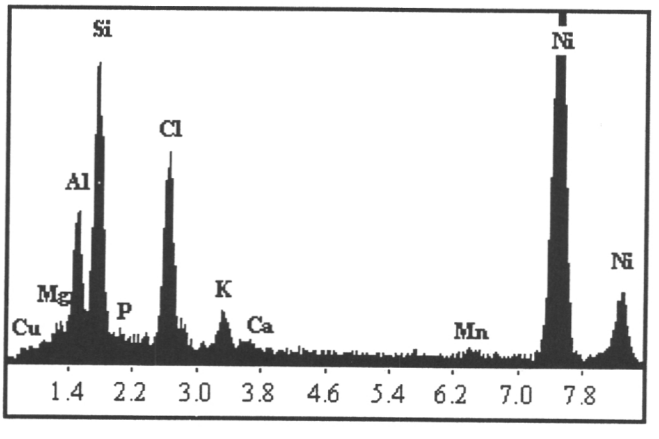


Figure 1. Typical EDX spectra (0.6 - 8.6 keV) of the electron-dense material in the ray cell of an *E. nigrum* stem collected at 0.5 km distance from the Cu-Ni smelter. The Cl peak is an artefact caused by the epoxy resin, Ni by the Ni-grid and Si by the dry-sectioning of glass knives and the Si(Li) X-ray detector system.

EDXS point measurements were carried out in xylem, phloem, ray cells and sclereids (Fig. 2) of the stems. The lumen, primary and secondary cell wall of vessels, tracheids and

sclereids, as well as cytoplasm, primary cell wall and vacuole of ray cells, phloem and parenchyma of the primary ray, were analysed. Additional electron dense material found in the lumen of xylem vessels, vacuoles of ray cells and phloem were measured in both high and low contaminated samples.

*2.4. Statistical analysis*

Altogether, four samples from both distances were studied. The measurement points per sample per tissue were generally around 10 (in some tissues between 1-17). To compare the distribution of elements in the different plant tissues at two distances, the Kruskal-Wallis test, a comparison of the mean ranks, was performed, because the logarithmic transformation did not normalize the data (Sokal and Rohlf, 1995; Statistix, 1996).

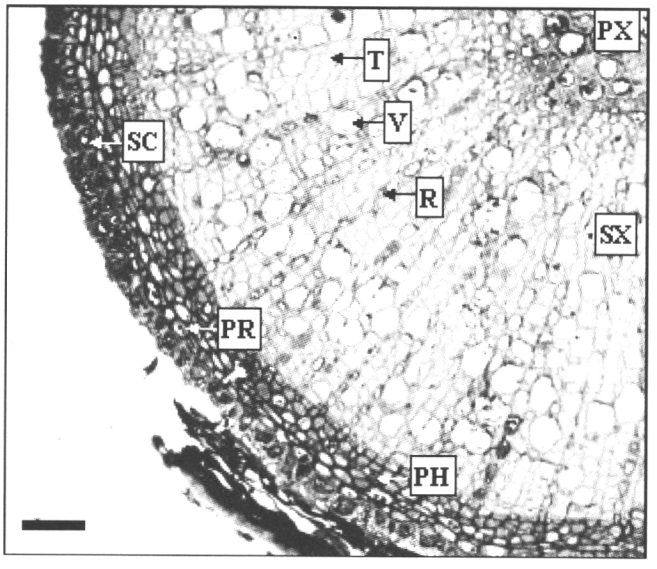


Figure 2. Cross-section of a previous-year stem of *E. nigrum* collected at 0.5 km distance from the Cu-Ni smelter. Magnification 50 ×. Scale bar 110 µm. PX = primary xylem, V = vessels, T = tracheids, R = ray cells, SX = secondary xylem, PH = phloem, PR = parenchyma of the primary ray, SC = sclereids.

## 3. Results

### *3.1. Localization of heavy metals in different tissues and cell compartments*

The heavy metal localization in stems of *E. nigrum* analysed by EDXS varied according to the metal and tissue, and the amounts of Cu, As and Fe were higher near to the smelter than in the stems from the control site (3a, b, c).

In nearly all of the analysed tissues, the Cu amount was higher in the samples from the high contamination plots compared to the low contaminated stem samples (Fig. 3a). However, the difference was statistically significant only in some cell compartments ($p \leq 0.05$). The highest amounts were measured in the vessel lumens and primary wall of the ray cells. The Cu amounts were elevated both in living (ray cells, phloem, parenchyma of the primary ray) and in dead cells (xylem, sclereids), but no clear differences were found between the Cu amounts in different cell compartments (Fig. 3a). According to the X-ray microanalytical mappings, Cu was rather homogeneously distributed among the tissue (Fig. 5c).

The As amounts were statistically higher at 0.5 km distance in almost all cell compartments ($p \leq 0.05$) (Fig. 3b). The highest As amounts were measured in outer regions of the stem cross-section, in different cell compartments of the phloem, in vacuoles of the primary ray and in lumen and secondary wall of sclereids. Lower As amounts were generally detected in vessels and tracheids of the xylem (Fig. 3b).

In general, higher Fe amounts were found in stems from the contaminated sites (Fig. 3c). The Fe amounts of the stems from the low contaminated site were under or close to the X-ray microanalytical limit of detection. Owing to the high variation between the replicates, the differences were statistically significant only in the secondary wall of vessels, primary cell walls and vacuoles of ray cells, vacuoles of phloem, cytoplasm of the parenchyma of the primary ray and lumen of sclereids ($p \leq 0.05$). In general, ray cells, phloem and sclereids had higher Fe amounts than other tissues from the contaminated stem samples (Fig. 3c). However, due to the high standard error of mean generally no significant differences were found.

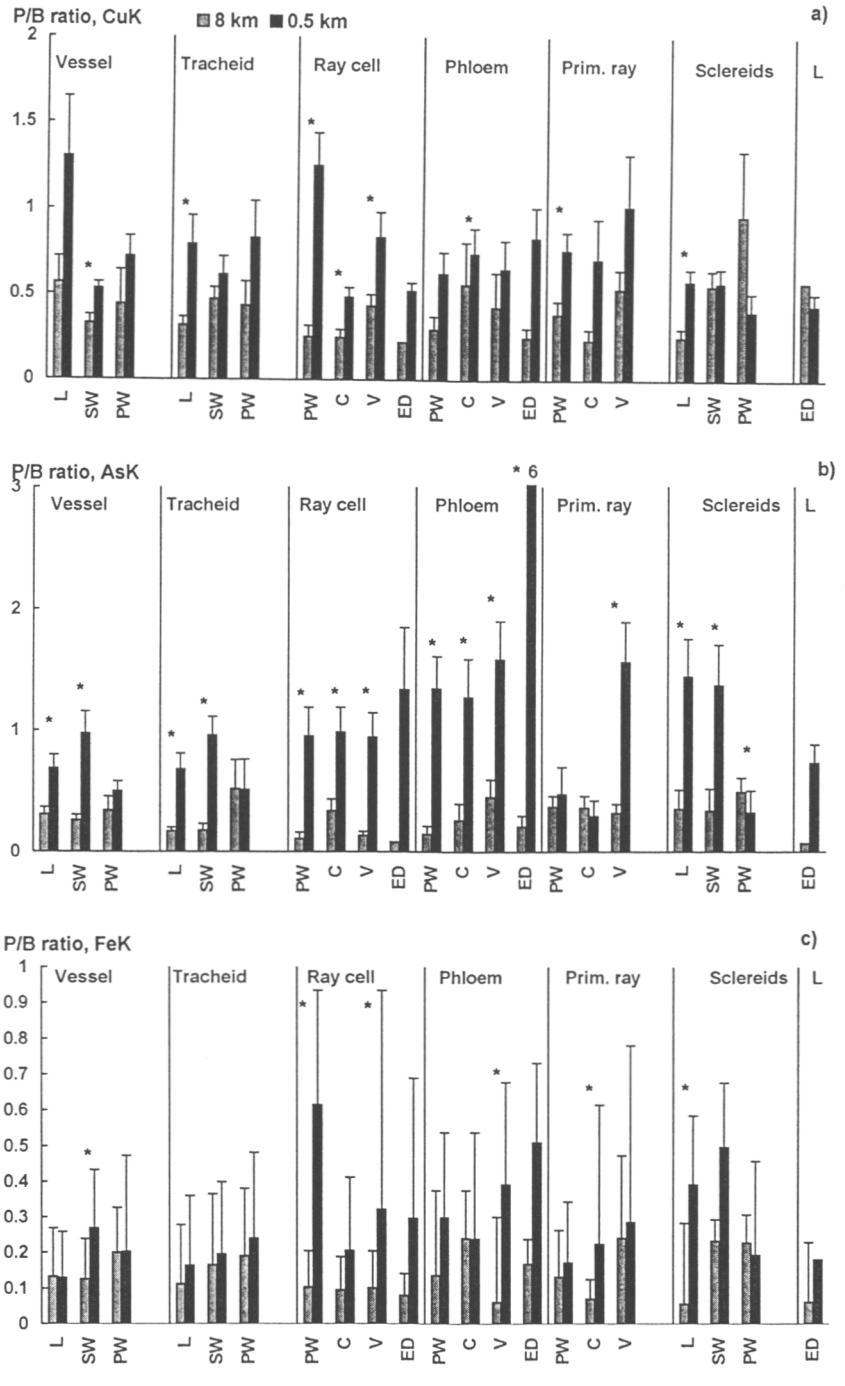


Figure 3a) - i). Peak to background (P/B) ratios of a) Cu, b) As, c) Fe, d) Al, e) Mn, f) Ca, g) K, h) P and i) S in lumen (L), secondary cell wall (SW), primary cell wall (PW), cytoplasm (C), vacuole (V) and electron-dense material (ED) of vessel, tracheid, ray cells, phloem, parenchyma of the primary ray, sclereids and lumen of dead cells (L) of *E. nigrum* previous-year stems sampled at 0.5 and 8 km distances from the Cu-Ni smelter.

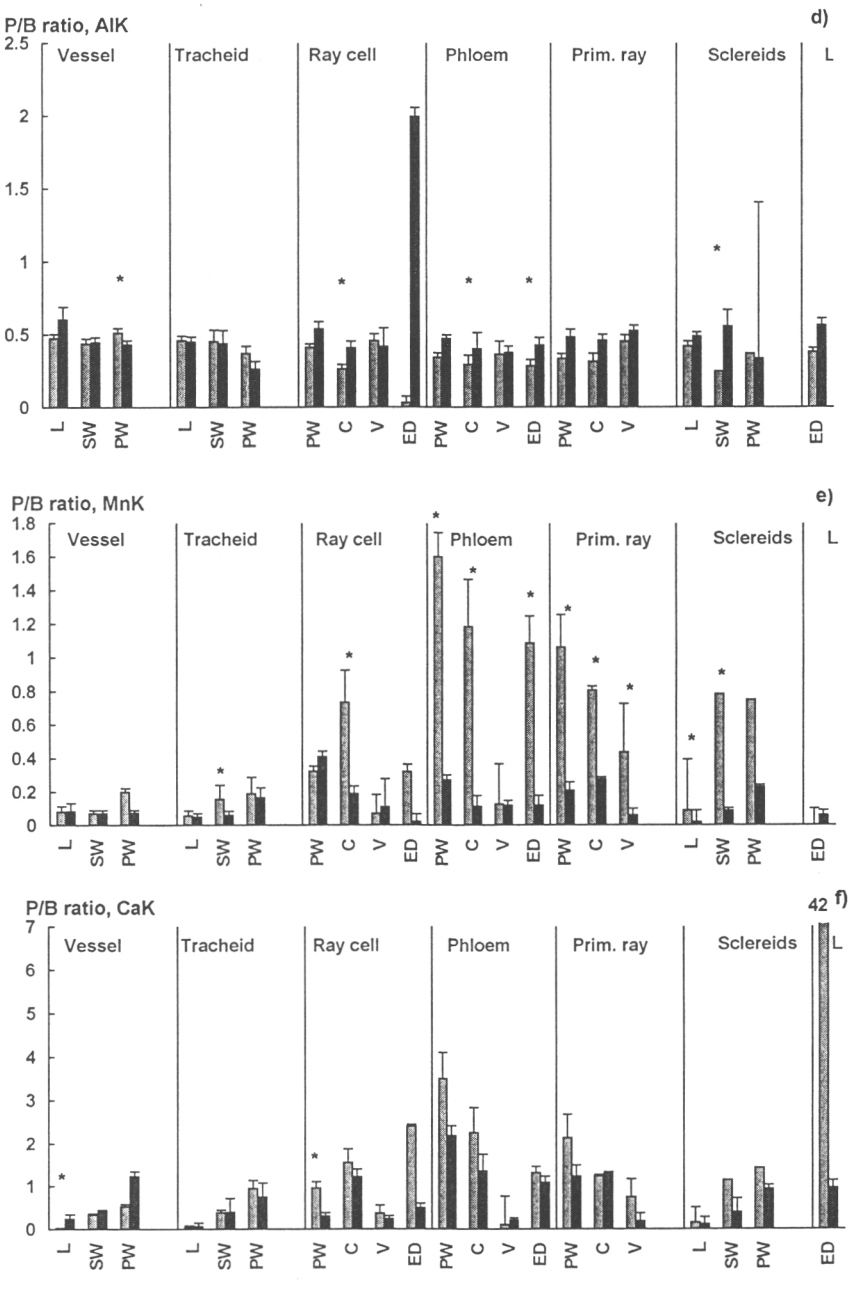


Fig. 3 (continued). The number of measurement points were: lumen (n = 18-21), secondary wall (n = 18-20) and primary wall of vessel (n = 5-10); lumen (n = 18-20), secondary wall (n = 18-20) and primary wall of tracheid (n = 3-5); primary wall (n = 11-18), cytoplasm (n = 10-18), vacuole (n = 13-18) and electron dense material of ray cells (n = 1-6); primary wall (n = 5-13), cytoplasm (n = 10), vacuole (n = 11-13) and electron dense material of phloem (n = 5);

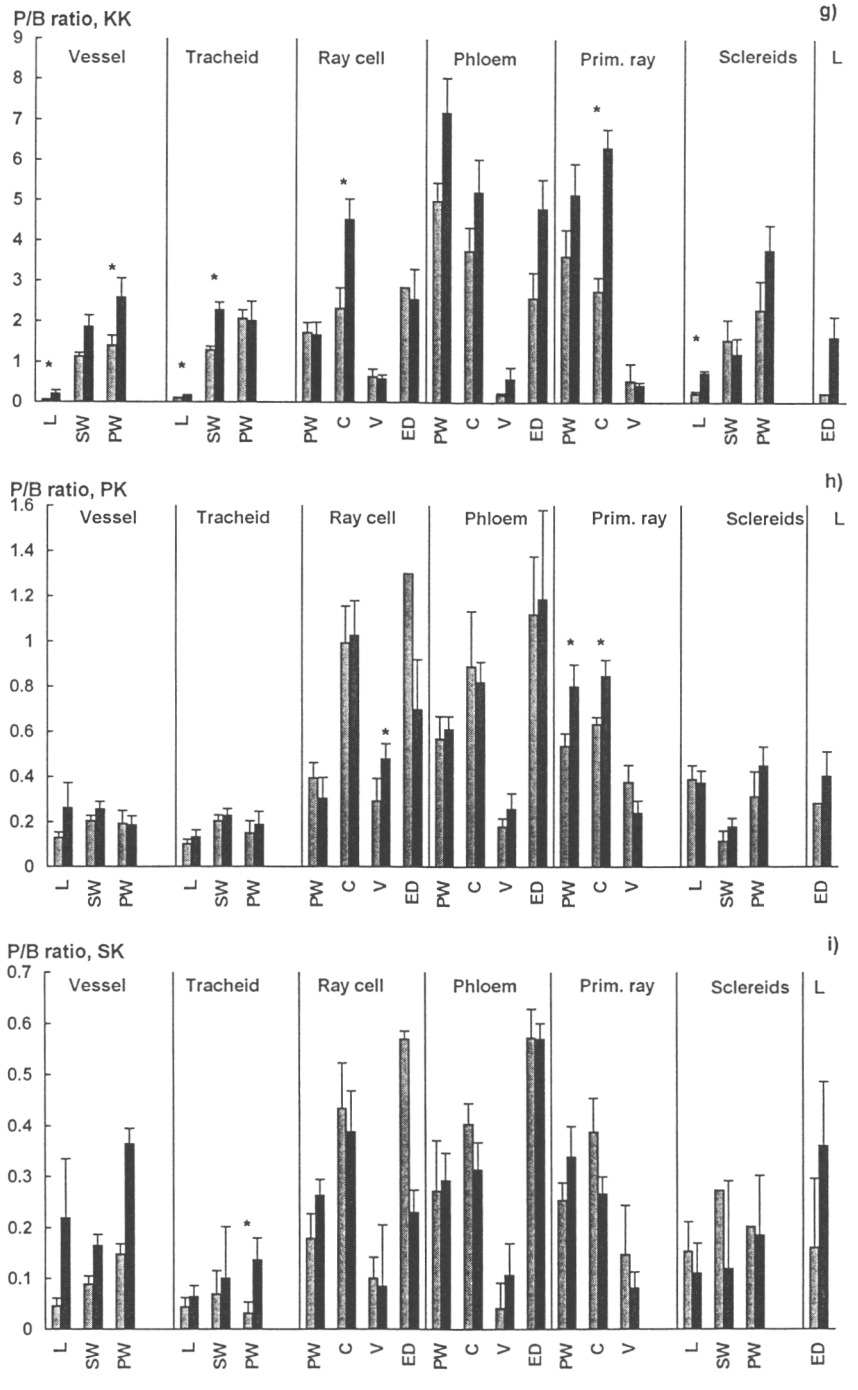


Fig. 3 (continued). Primary wall (n = 10-13), cytoplasm (n = 10-13) and vacuole (n = 10-13) of parenchyma of primary ray; lumen (n = 10-13), secondary wall (n = 10-13) and primary wall of sclereids (n = 10-12); electron dense material of lumen (n = 1-22). Bar indicates standard error of the mean (SE). Note the different scales.

The variation in the Al amounts between the different measurement points was very small (Fig. 3d; 4b). Although the Al amounts in many cell compartments were higher in the stems from the contaminated site, the difference between the two distances was very small, and the increased contents could not clearly be explained by an effect of the site. The highest Al amounts were detected in the electron-dense material of the ray cells, where particles were found with very high Al amounts (Fig. 3d, 5b).

The Zn amounts were generally near to the detection limit (P/B ratio generally below 0.25) and Zn could only clearly be detected in living cells of the phloem and the primary ray tissue in stems from the highly contaminated site.

In contrast to the elements described above, the Mn amounts in the different cell compartments of the stems were lower near to the smelter (Fig. 3e). In stems from the low contaminated site Mn was especially localized in living tissue (ray cells, phloem, primary ray cells) and the cell walls of sclereids. In contrast, in the xylem of the vascular tissue only low Mn contents were detected. In stems from the highly contaminated site, the Mn amounts were close to the detection limit. In these samples no clear differences in the Mn amounts between the different cell compartments or tissues were found (Fig. 3e).

*3.2. Localization of macronutrients and Na in different tissues and cell compartments*

The amounts of macronutrients varied between different cell compartments. The Ca amounts were generally higher at 8 km than at 0.5 km distance from the smelter, the highest amounts occurring in the electron-dense material of vessel lumens (Fig. 3f). Ca was mainly located in living parts of the tissue, in the phloem and primary ray and here especially in primary cell walls. The amounts in the primary cell walls of the dead tissue (vessels, tracheids, sclereids) were lower (Fig. 3f). The X-ray microanalytical mapping showed that Ca was mainly located in primary cell walls and here especially in the pits between two cells (Fig. 4c). However no clear statistical differences ($p \leq 0.05$) were found in the Ca amounts between primary and secondary walls. The cytoplasm of the living tissue also contained relatively high amounts of Ca, whereas the amounts in lumens and vacuoles were low in both the high and low contaminated samples (Fig. 3f).

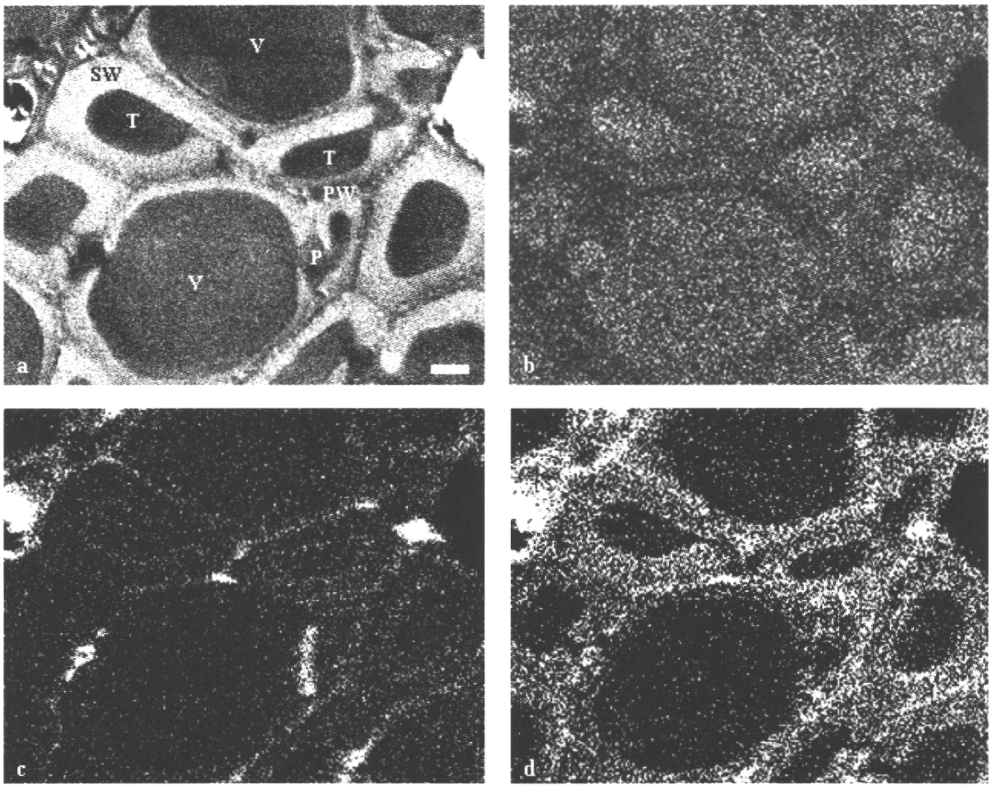


Figure 4. Vessel elements of *E. nigrum* stem at 8 km distance from the Cu-Ni smelter. a) vessel elements, b) aluminium in the lumen, c) calcium in the plasmodesmata and primary cell wall and d) potassium in the secondary cell wall of vessel elements. Magnification 6 400 ×. Scale bar 1 µm. V = vessels, T = tracheids, PW = primary cell wall, SW = secondary cell wall, P = pit.

The K amounts were higher at 0.5 km than at 8 km (Fig. 3g). The highest K amounts were measured mainly in living parts of the tissue, i.e. in phloem and primary ray cells. The primary walls of the phloem and parenchyma of the primary ray had higher K amounts than the primary walls of dead cells (vessels, tracheids, sclereids) (Fig. 3g). In contrast to Ca, relatively high K amounts were measured in primary and secondary walls of the xylem (Figs 3g, 4d) and sclereids (Fig 3g). The cytoplasm also had relatively high K amounts (Fig. 5d), whereas only low amounts were found in lumens and vacuoles of both the high and low contaminated samples (Fig 3g). In the ray cells, there were higher K amounts in the electron-dense material and the cytoplasm than in the vacuoles and primary cell walls in the highly contaminated samples (Figs 3g, 5d).

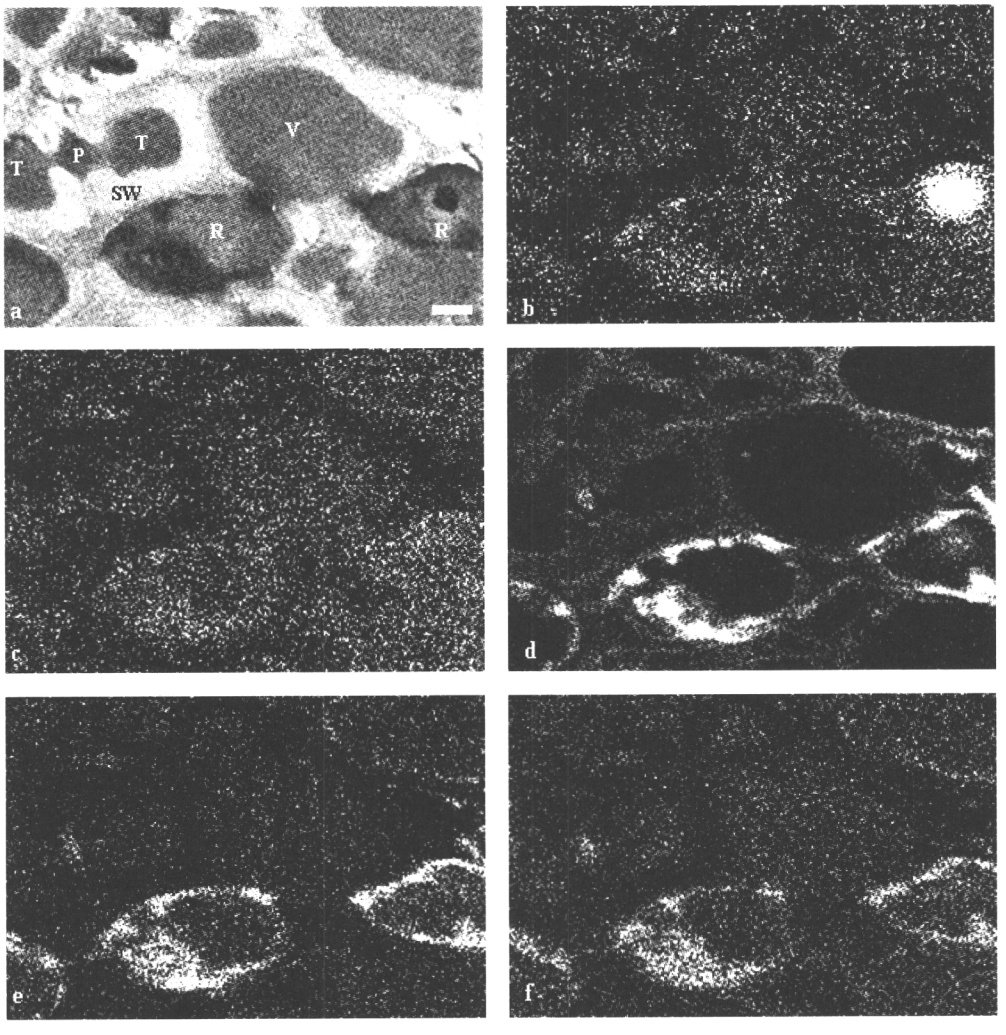


Figure 5. Vessel elements and ray cells of *E. nigrum* stem at 0.5 km distance from the Cu-Ni smelter. a) Vessel elements, b) aluminium and c) copper in the lumen of vessel elements, d) potassium, e) phosphorus and f) sulphur in the electron-dense material of vacuoles and cytoplasm of raycells. Magnification 6 400 ×. Scale bar 1 µm. V = vessels, T = tracheids, R = ray cells, SW = secondary cell wall, P = pit.

The P and S amounts did not vary according to the site (Figs 3h, i). Relatively high P and S amounts were found in ray cells, phloem and primary ray cells, whereas in dead cells, vessels and tracheids of the xylem and the sclereids lower amounts were detected (Fig. 3h, i). In the living tissues, P and S were mainly located in the cytoplasm or the electron-dense

material (Fig. 3h, i, 5e, f). High P and S amounts were also found in the primary wall of the phloem and parenchyma of the primary ray whereas vacuoles contained lower amounts of both elements (Fig. 3h, i).

The Mg and Na amounts were close to the X-ray microanalytical limit of detection, the peak to background ratio (P/B ratio) for Mg being generally below 0.4, and for Na below 0.1. The differences in the amounts were therefore most probably due to the background variation.

**4. Discussion**

The heavy metal pollution had an influence on the cellular amounts of Cu, As and Fe in the *E. nigrum* stems. The results were in good agreement with earlier results obtained near to the Harjavalta Cu-Ni smelter. At 0.5 km from the Cu-Ni smelter, the Cu and Fe concentrations in the soil (Derome and Lĭndroos, 1998) and in *E. nigrum* (Helmisaari *et al.*, 1995; Uhlig *et al.*, 2000) are much higher than at e.g. 2, 4 or 8 km distance from the smelter. Although As concentrations in the soil and plants have not been earlier reported, the emissions from the smelter do in fact also contain As (Rantalahti, 1995). The Zn concentrations in the soil and plant parts are also elevated (Uhlig *et al.*, 2000; Derome and Lindroos, 1998; Derome, 2000). The EDXS analyses did not indicate any clear differences in the Zn concentrations between the different sites, but this might be due to the detection limit of the EDXS and the difficulty to seperate the Zn peak when Cu was present in high amounts. The Mn amounts, however, were clearly higher in different cell compartments at 8 km than at 0.5 km. This supports the results of Derome and Lindroos (1998) and Uhlig *et al.* (2000) showing lower Mn amounts in the soil and plant parts at 0.5 km than further away from the Harjavalta smelter.

The results showed that the heavy metal pollution had no significant effect on the localization of P, S, K and Ca in different cell compartments, except the amounts of Ca were slightly impared near the smelter, while K amounts were elevated. Exchangeable Ca and K concentrations in the organic soil are clearly decreased near the smelter (Derome and Lindroos, 1998) but the total nutrient (Ca, K, P and S) concentrations in *E. nigrum* are approximately the same or higher near the smelter than in 8 km distance from the smelter. In contrast, the Mg concentrations in stems and leaves of *E. nigrum* (Uhlig *et al.*, 2000) and

the exchangeable Mg concentrations in the organic soil (Derome and Lindroos, 1998) are clearly depressed near the smelter and e.g. pines are suffering from Mg deficiency (Derome and Nieminen, 1998). Mg amounts obtained with EDXS in the stems were near to or below the detection limit at both sites and therefore did not indicate any Mg deficiency especially near the smelter.

In the present study the highest element (Ca, P, S, Al, As) peaks were detected in the electron-dense material, but the elemental composition and the localization of this material varied considerably. Earlier, an accumulation of metals (e.g. Cu, Zn) and nutrients (K, Ca, P) in electron-dense material in vacuoles has been reported (e.g. Vázquez, *et al.*, 1994; Neumann *et al.*, 1995). According to light microscopical investigations, these vacuolar precipitates in ray cells and phloem mainly consisted of phenolic material. Carlquist (1989), who investigated the wood anatomy of *E. nigrum*, observed that electron-dense material occurs most abundantly in rays, but also in tracheids and vessels. In this study, there was more frequently electron-dense material in the stem samples originating from 0.5 km than from 8 km from the smelter and the material was distributed throughout the stem tissue. Phenolic substances have a specific function in the ecology of *E. nigrum*. The exudation of phenolics by *E. nigrum* leaf trichomes (Wollenweber *et al.*, 1992) inhibits the establishment of tree seedlings (e.g. Nilsson and Zackrisson, 1992; Zackrisson and Nilsson, 1992; Nilsson, 1994). Due to the higher frequence of this electron-dense material in the stems from the highly polluted site and the high heavy metal amounts in this material measured by EDXS, it can be assumed that this phenolic, electron-dense material has a special function in the heavy metal tolerance of *E. nigrum*.

In this study, Cu was rather homogeneously distributed in the tissue and occurred in cell walls, vacuoles and cytoplasm of the living tissue as well as in the lumen of the vascular tissue. Recent electron microscopical studies of *Silene vulgaris* leaves showed that Cu is located in epidermal cell walls bound to glycoprotein with oxalate oxidase activity (Bringezu *et al.*, 1999). The Cu tolerance of another dwarf shrub *Calluna vulgaris* (Hull.) was supposed to be achieved by an accumulation of Cu in the ericoid mycorrhizas of the roots preventing the metal transport to the shoots (Bradley *et al.*, 1981; 1982). In contrast to that, this study as well as previous greenhouse studies (Monni *et al.*, 2000a) showed that in *E. nigrum* also a root-to-shoot transport occurs.

In the xylem sap more than 98% of the Cu is present in complexed form (Graham, 1979), where Cu has been suggested to bind to amino acids (White *et al.*, 1981). Copper has a high affinity to cysteine-rich proteins, carboxylic and phenolic groups (Marschner, 1995) and the connection of elevated Cu amount and proteins and vacuolar phenolic compounds in leaves and roots has been found (e.g. Rauser, 1984; Neumann *et al.*, 1995; Ernst *et al.*, 2000). The S amounts in this study, however, did not indicate any connection of Cu to proteins. EDXS does not detect N, which might have provided more information about the protein or phenolic amounts in cell walls, vacuoles or cytoplasm. It was not shown whether phytochelatins, as having high affinity for Cu (e.g. Marschner, 1995), or other complexing agents play a role in heavy metal tolerance of *E. nigrum*. However, because of the homogeneous distribution of Cu in the tissue, it seems that not only one specific mechanism is involved in the Cu tolerance of *E. nigrum*.

The high affinity of Fe for ligands like organic acids and inorganic phosphate makes it unlikely that ionic forms of Fe has any importance for the short- and long-distance transport in plants (Marschner, 1995). Like for Cu, also Fe accumulation in ericoid mycorrhizal roots of *C. vulgaris* has been suggested to be responsible for the reduction of Fe transport to the shoots when exposed to high Fe concentrations (Shaw *et al.*, 1990). The results of this study, however, showed that also Fe was translocated to the shoot. Fe has been reported to be bound to epidermal cell walls of tolerant *Silene vulgaris* leaves (Bringezu *et al.*, 1999), whereas cytoplasm, vacuoles and cellular organelles of tolerant *Minuartia verna* and *Silene vulgaris* leaves contained only traces or no Fe (Neumann *et al.*, 1997; Bringezu, *et al.*, 1999). In this study, the Fe amount in cell walls especially of ray cells and sclereids was higher near the smelter. Also the localization in the cytoplasm and vacuoles indicated a detoxification of Fe in these cell compartments. The specific complexing agents could not be shown according to this study.

Unlike other elements As and Al are not essential for plants. In the highly contaminated samples the primary cell walls of ray cells and phloem had higher As amounts than that of dead cells (xylem, sclereids) indicating a placement of As to the apoplast of living cells. An accumulation of metals in vacuoles of the contaminated stems was also observed. The cellular localization of As has been researched in the wood of *Pinus sylvestris* after

chromated copper arsenate (CCA) treatment. All the cell compartments were not studied but the results showed that lumen and middle lamella of tracheids and ray cell parenchyma of the peeling samples and sapwood contained As, whereas in untreated samples no As could be found (Helsen and Van den Bulck, 1998).

In tolerant *Silene vulgaris* leaves Al has been bound to epidermal cell walls (Bringezu *et al.*, 1999), whereas cytoplasm, vacuoles and cellular organelles of tolerant *Minuartia verna* and *Silene vulgaris* leaves contained no Al (Neumann *et al.*, 1997; Bringezu, *et al.*, 1999). In this study the effect of pollution was not clearly seen and Al was detected both in apoplast and symplasm, the significant differences between the sites being only seen in cells walls or cytoplasm in few tissues as well as in the electron dense material. However, the differences were very small and the overall amount was almost the same in all cell compartments. Therefore the similar pattern as above was not clearly seen according to this study.

In plants, Mn is mainly in ionic form as it forms unstable complexes with organic ligands (Marschner, 1995). As being the cofactor for different enzymes (Burnell, 1988) Mn was abundant in the cytoplasm of control plants. However, in samples near to the smelter such a pattern could not be found. Because of the extremely low amounts of Mn near the smelter, some Mn deficiency might occur. Nutritional imbalance might be the reason for the decreased metabolic efficiency, and thus the decreased contents of organic acids (malic and citric acids) and chlorophyll in *E. nigrum* near the smelter. However, also decreased Mg contents as well as increased Fe contents near the smelter might affect the chlorophyll pigment contents of *E. nigrum* (Monni *et al.*, 2000b).

In this study, Ca was mainly located in cell walls and cytoplasm of living cells (ray cells, phloem), but also in the torus of pits between tracheids and vessels indicating its significance as an important constituent of the plant middle lamella and cell wall. Ca is mainly bound to structural material, and should be most abundant in cell walls (Kirkby and Pilbeam, 1984), while the amount of Ca in the cytoplasm is usually very low (Marschner, 1995). However, e.g. environmental stresses or ABA can induce an increase in cytosolic free $Ca^{2+}$ (e.g. Marschner, 1995). However, this was not found near the smelter where ABA in current-year leaves and stems is increased (Monni *et al.*, 2000b) and cytosolic Ca decreased.

K is very mobile and mainly occurs in the symplasm, the cytosolic K content being usually relatively constant (Hsiao and Läuchli, 1986; Marschner, 1995). In *E. nigrum*, K occurred mainly in the cytoplasm, but the amounts varied due to the site or tissue. Also in primary walls of the phloem and primary ray cells high K contents were found. K amounts were low and relatively constant in vacuoles and lumens of *E. nigrum* stems independent on tissue or site. According to Hsiao and Läuchli (1986), the K amounts of vacuoles may vary whereas in the apoplast K amounts are usually very low (Läuchli and Pflüger, 1978; Hsiao and Läuchli, 1986; Marschner, 1995).

The dead cells (tracheids, vassels, sclereids) had only low amounts of P, the P being concentrated in the electron-dense material of the ray cells and phloem and cytoplasm, where it regulates metabolic pathways. It is also a constituent of nucleic acids and ATP. S, being an important component of amino acids, proteins and (Marschner, 1995) heavy metal binding phytochelatins (Marschner, 1995; Keltjens and van Beusichem, 1998), was also located in the cytoplasm in the high and low contaminated samples. Heavy metal pollution did not cause any change in the pattern of the S and P amounts in different cell organelles.

**5. Conclusions**

There were higher amounts of Cu, As and Fe in the cell compartments of *E. nigrum* at 0.5 km than at 8 km. The Al and Zn amounts, in contrast, did not differ significantly between the two sites. Cu was localized in several cell compartments (cell walls, cytoplasm, vacuoles, lumens) whereas the As amounts were higher in the primary cell walls of living (ray cells, phloem) than of dead cells (xylem, sclereids). Elevated As amounts were also found in cytoplasm and vacuoles. Ray cells, phloem and sclereids had elevated amounts of Fe compared to the other tissues in the contaminated stem samples but, owing to the high variation between the replicates, generally no significant differences were found. The vacuoles, cytoplasm and cell walls of the contaminated stems also had higher Fe amounts indicating the transport of metals to these cell compartments. Based on the rather homogeneous localization of Cu, As and Fe in the living tissue and increased amounts of Cu, As and Fe in vacuoles, cell walls and cytoplasm near the smelter, it seems that not only one specific mechanism contribute to the heavy metal tolerance of *E. nigrum*. It was not shown whether phytochelatins and organic acids are involved in heavy metal tolerance in

the cytoplasm and vacuoles, respectively, as shown in earlier investigations. According to light microscopical examinations, electron-dense material consisted partly of phenolic material, and it occurred more frequently in the polluted samples. Therefore the electron-dense material containing phenolic substances might have a function in the heavy metal tolerance of *E. nigrum*.

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