

University of Latvia

THE USE OF SERIAL ELUTION TECHNIQUE TO DETERMINE  
CELLULAR CONCENTRATIONS IN MOSS IN  
BIOMONITORING

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PhD Thesis

(Biology section - ecology)

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SURVEYS  
Guntis Brūmelis  
ANNOTATION

In biomonitoring projects, the use of the total concentrations in moss method to map the deposition of pollutant metals, assumes that most of the metal is cell wall bound and that metals are efficiently absorbed and retained, thus providing an integrated estimate of deposited ions for a given period of time. However, using the serial elution technique, which estimates the intercellular, cell wall exchangeable and intracellular concentrations, it is known that metals are transported between segments; and they can be lost by leaching or upward movement from soil can occur. Also, a significant amount of metal can occur as relatively insoluble particles. Toxicity assessment should use the intracellular component of total concentrations, which are more related to physiological effect. These arguments, proved by physiological study, show the limited interpretation possible from total concentrations. Therefore, the suitability of the serial elution technique in practical biomonitoring is assessed, to offer a method that can offer less biased interpretation of monitoring results.

The project goal was to assess the suitability of serial elution technique in the practical biomonitoring of Zn, Ca, Mg and K deposition, using concentrations in *Hylocomium splendens*.

For the first time, the serial elution technique has been applied in practical biomonitoring projects to survey the deposition around the Liepāja metal smelter and at the Saulkalne Lime Plant.

The lack of clear definition of particles in a specific elution is identified, and a method is recommended to estimate the role of soluble particles in the various fractions. It is demonstrated that the use of moss killed by boiling to provide only cell wall exchange sites, for use in uptake studies, is biased, likely due to the presence of sites on denatured intracellular material, that are less easily subject to ion association / displacement.

Seasonal patterns in cellular concentrations of Ca, Mg, K and Zn at two polluted and two unpolluted sites are identified and discussed in relation to practical monitoring. Concentrations were most variable between spring to summer intermittent dry and wet periods.

The uptake of Ca, Mg, and K are shown to depend on the pre-growth available pools, during growth in the laboratory, demonstrating the potential role of recycling between segments in the field.

Latvijas Universitāte  
SERIĀLĀS ELŪCIJAS METODE METĀLU KONCENTRĀCIJAS  
NOTEIKŠANAI SŪNU ŠŪNĀS BIOMONITORINGA VEIKŠANAI  
Guntis Brūmelis

## ANOTĀCIJA

Piesārņojuma līmeņa kartēšanai biomonitoringā bieži izmanto sūnas, nosakot tajās sumārās metālu koncentrācijas. Uzskata, ka piesārņojuma lielāko daļu saista šūnapavalki un metāli tiek efektīvi absorbēti un uzkrāti, atbilstoši kopējam izsēdušos jonu daudzumam noteiktā laika periodā. Taču, izmantojot seriālās elūcijas metodes, kas ļauj noteikt metālu koncentrāciju šūnās, starpšūnu telpā, iekššūnās un uz šūnapavalkiem, ir pierādīts, ka metāli pārvietojas sūnu ķermenī; tie var tikt izskaloti un tie var nokļūt sūnās no augsnes. Bez tam nozīmīgs metālu daudzums var būt pārstāvēts relatīvi nešķīstošu daļiņu veidā. Toksiskumu vajadzētu vērtēt nevis pēc kopējās metālu koncentrācijas, bet gan pēc to daudzuma šūnās, kas raksturo sārņu fizioloģisko iedarbību. Šos apsvērumus apstiprina fizioloģiska rakstura pētījumi un tādejādi kopējās metālu koncentrācijas sniedz nepilnīgu informāciju. Šo apsvērumu dēļ, esošo neobjektīvo metožu uzlabošanai, tika novērtēta seriālās elūcijas tehnikas piemērotība biomonitoringa praksei.

Darba mērķis ir noskaidrot seriālās elūcijas metodes piemērotības biomonitoringa praksei novērtēšana, analizējot piesārņotību ar Zn, Ca, Mg un K pēc šo elementu koncentrācijām spīdigajā stāvsūnā (*Hylocomium splendens*).

Seriālās elūcijas metode pirmo reizi tiek pielietota praktiska monitoringa veikšanai. Konstatēts, ka līdz šim nav bijuši noteikti priekšstati par daļiņu stāvokli dažādās elūcijās un tiek ieteikta metode šķīstošo daļiņu nozīmes noteikšanai dažādās elūcijās. Parādīts, ka sūnu karsēšanas rezultātā eluātā nonāk ne tikai uz šūnapavalki, kā to uzskatīja līdz šim, bet arī denaturētas protoplazmas daļas, kas apgrūtina metālu piesaistīšanas šūnapavalkiem izpēti laboratorijas eksperimentos.

Noskaidrots Ca, Mg, K un Zn koncentrācijas izmaiņu raksturs sezonas laikā divos piesārņotos un divos nepiesārņotos apvidos un iztīrāta iegūto atziņu nozīme praktiskā monitoringa veikšanai. Pierādīts, ka Ca, Mg, K un Zn uzkrāšana notiek atkarībā no šo elementu daudzuma sūnu segmentos pirms augšanas perioda sākuma, bet laboratorijas apstākļos ir parādīta elementu pārvietošanās starp sūnu dažāda vecuma segmentiem, kas potenciāli ir iespējama arī dabā.

Латвийский Университет  
ОПРЕДЕЛЕНИЕ КОНЦЕНТРАЦИИ МЕТАЛЛОВ В КЛЕТКАХ МХА  
МЕТОДОМ СЕРИЙНОГО ЭЛЮИРОВАНИЯ ДЛЯ ПРОВЕДЕНИЯ  
БИОМОНИТОРИНГА  
Брумелис Гунтис

## АННОТАЦИЯ

Для составления карт уровней загрязнения при биомониторинге часто используются мхи, позволяющие определять суммарную концентрацию металлов, накапливаемых ими. Считается, что клеточные оболочки связывают большую часть загрязнения и металлы абсорбируются и накапливаются пропорционально общему количеству, выпавших в осадок, ионов.

Используя метод серийного элюирования, можно определить концентрацию металлов, как в клетках, в межклеточных пространствах, так и на клеточных оболочках. Доказано: металлы передвигаются в растении, могут вымываться и могут попадать в мох из почвы. Кроме того, значительное количество металлов накапливается в виде нерастворимых частиц. Поэтому токсичность необходимо оценивать не по общей концентрации металлов, а по содержанию в клетках растений металлов, оказывающих физиологическое воздействие, что подтверждается исследованиями физиологического характера.

Целью работы являлось определение пригодности метода серийного элюирования при проведении биомониторинга. Анализировались загрязнения Zn, Ca, Mg и K по концентрации этих элементов в гилокомиуме блестящем (*Hylocomium splendens*).

Применение метода серийного элюирования в практике биомониторинга позволило выявить состав частиц и значимость растворимых частиц в последовательных элюациях. Доказано, что в результате нагревания мха в элюат попадают не только клеточные оболочки, как это считалось до сих пор, но и денатурированные частицы протоплазмы, которые затрудняют исследования.

В процессе мониторинга, выявлен характер сезонных изменений концентраций Ca, Mg, K и Zn на двух загрязненных и двух незагрязненных участках. Доказано, что накопление Ca, Mg, K и Zn происходит пропорционально количеству этих элементов в сегментах мха перед началом периода роста.

В лабораторных условиях доказано перемещение элементов между сегментами мха разного возраста, что потенциально возможно и в природе.

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## 1. GENERAL DESCRIPTION

### 1.1 Importance of work:

In biomonitoring projects, the total concentrations in moss have been often used to map the deposition of pollutant metals. Using this method, the areas of concern, with high concentrations of metals in Latvia, have been identified. The method assumes that most of the metal is cell wall bound and that metals are efficiently absorbed and retained, thus providing an integrated estimate of deposited ions for a given period of time. However, using the serial elution technique, which estimates the intercellular, cell wall exchangeable and intracellular concentrations, it is known that metals are transported between segments; and they can be lost by leaching or upward movement from soil can occur. Also, a significant amount of metal can occur as relatively insoluble particles. Toxicity assessment should use the intracellular component of total concentrations, which are most related to physiological effect. These arguments, proved by physiological study, show the limited interpretation possible from total concentrations. Therefore, the suitability of the serial elution technique in practical biomonitoring is assessed, to offer a method that can offer less biased interpretation of monitoring results.

### 1.2 Project goal:

To assess the suitability of serial elution technique in the practical biomonitoring of Zn, Ca, Mg and K deposition, using concentrations in *Hylocomium splendens*.

### 1.3. Project objectives:

- 1) To test the validity of using Zn concentrations, obtained by serial elution, to estimate cellular concentrations.

- 2) To determine the impact of desiccation on cellular concentrations.
- 3) To determine if the uptake of Zn, Ca, Mg and K to new additional growth depends on the pre-growth pools.
- 4) To test the application of a serial elution method in the assessment of A. cellular Zn concentrations around a metal smelter, and B. of cellular Ca, Mg, K and Zn concentrations around a lime production factory.
- 5) To determine the impact of seasonal factors on cellular concentrations.

#### 1.4. Scientific importance:

For the first time, the serial monitoring technique has been applied in practical biomonitoring projects. The lack of clear definition of particles in a specific elution is identified, and a method is recommended to estimate the role of soluble particles in the various fractions. It is demonstrated that the use of moss killed by boiling to provide only cell wall exchange sites for use in uptake studies, is biased, likely due to the presence of sites on denatured intracellular material, that are less easily subject to ion association / displacement. Seasonal patterns in cellular concentrations of Ca, Mg, K and Zn at two polluted and two unpolluted sites are identified and discussed in relation to practical monitoring. The uptake of Ca, Mg, and K are shown to depend on the pre-growth available pools, during growth in the laboratory, demonstrating the potential role of recycling between segments in the field.

#### 1.5. Practical importance:

Serial elution technique has been shown to provide results which can be interpreted without the major biases inherent in the use of total concentrations.

Recommendations are given on the age of segments to be sampled, and the season



in which sampling occurs, in practical projects that use serial elution technique. Problems in the use of the method are identified, such as the possible impact of soluble particles, that exist on moss surfaces, to cellular concentrations; suggestions are also made for assessing the potential significance of this problem.

## 1.6 Approbation and published papers

To initially determine the areas of concern regarding high heavy metal deposition, Latvia was mapped using total concentrations as part of a European survey (published as 2 Nordic Council reports). Also, the distribution of pollution around Mazeikiai, Liepāja, Olaine, Skrunda and Jūrmala has been investigated (one published as co-author in *The Science of the Total Environment*, three in *The Proceedings of the Latvian Academy of Science*, and one as a Latvian Ministry of the Environment report). The problem in the use of total concentrations in moss was identified by differences in total concentrations between forest types in Latvia (presented in Kuoppio, Finland at the International Biomonitoring Conference and published in "Bioindicators of Environmental Health"). A test of the serial elution method for biomonitoring work has been presented as a paper accepted in *The Science of the Total Environment* (co-author). Another two papers on the serial elution technique have been submitted for publication. The serial elution technique has been discussed (Ēdole 1994, Talinn 1996) with the leading scientists involved with biomonitoring in Scandinavia, and the work has been given recognition.

## 2. LITERATURE REVIEW

### 2.1. Monitoring of metal concentrations using total concentrations

The use of moss to survey atmospheric deposition of metals, assumes the premise that pollutant concentrations in an organism can portray the integrated deposition loads over some period of time. Plants can accumulate metals, and a one-time sampling can hence provide the same information as continuous measurements of concentrations in rain or air. The metal concentrations in a plant are affected by (Folkeson 1979):

1. the physical and chemical nature of the available metal,
2. retention of the metal,
3. total metal load,
4. climate,
5. environmental exposure,
6. growth rate,
7. chemical properties (e.g. exchange capacity and amounts of ligands) and
8. Habitat.

A plant is a good monitor of deposited metals if it obtains its chemical supply directly from the atmosphere, the metals are efficiently retained (a relatively high number of exchange sites) and that the plant age (time of accumulation) can be inferred (Rühling et al 1992). Based on these considerations, nine mosses and lichens were tested for their applicability to assessment of deposition (Folkeson 1979). *Hypnum* and *Pohlia* species accumulate more metals from the substrate, but concentrations in lichens are low and they cannot be aged. *Pleurozium schreberi* and *Hylocomium splendens* were regarded as the most suitable monitors of metal deposition. Experimental trials of sorption and retention of metals by *Hylocomium splendens* during immersion and leaching in columns, in pure or mixed solutions (Rühling and Tyler 1970, Gjengedal and Steinnes 1990) have been used to substantiate the moss method for most of the metals of interest. The sorption and retention generally follows the order Cu, Pb>Ni>Co>Zn (Rühling and Tyler 1970).

Direct uptake from below to feather mosses has been assumed to be low, an idea based from the extensive work of Tamm (1953), who showed a high correlation between feather moss location on the forest floor and nutrient sources from above (direct precipitation or canopy leaching).

The factors which most affect concentrations in moss, according to Steinnes et al (1994), are wet and dry deposition of pollutants, natural cycling (e.g. sea salt and biogenic emissions), root uptake by vascular plants and subsequent leaching, and mineral particle as wind-blown dust. Higher metal concentrations in moss than those expected by deposition of other pollutants are commonly explained by these other factors. A concentration of 20-30 mg/kg Zn in moss in northern Scandinavia, too high to be explained by atmospheric inputs, was suggested to be due to uptake from below via higher plants (Rühling et al 1992). Poor correlations between wet deposition and concentrations in moss, have been attributed to dry deposition and canopy leaching (Ross 1990). Steinnes et al (1994) partitioned elements into those explained by long-range transport (V, As, Se, Cd, Sb, Pb), point sources of pollution (Cr, Ni, Cu), marine factor (Br, I), vegetation factor (Zn) and soil factor (Al, Sc, Fe, Co). However, the lack of an adequate representation of deposition of pollutants from local sources, using total concentrations, cannot be used to objectively prove that the cause of the pattern is another source, unless the effects of the various sources can be partitioned. This emphasises the limitations of total concentrations.

The effect of inputs to moss via other vegetation can be largely eliminated by the use of *Sphagnum* species in ombrotrophic raised bogs, which receive, at least by definition, all primary chemical inputs from the atmosphere (Glooschenko et al 1986, Percy and Borlan 1985, Nikodemus 1991). However, elements in *Sphagnum* can be redistributed vertically, to and from the peat, and horizontally via water flows (Malmer et al 1992). A major drawback of the use of *Sphagnum* as a monitor is its absence in many regions. Problems of locating suitable sampling

locations for moss can be by-passed by hanging moss bags (an inert mesh that holds moss) for a given period of time in a transect or grid around a pollution source (Temple et al 1981, Mäkinen 1987a).

Since the total concentrations of metals in moss are usually much higher around major pollutant emitters than the variation in concentrations due to other sources, the moss method has been used successfully regionally to identify major sources of pollutants and background levels (Rühling and Tyler 1973, Pakarinen 1981, Rinne and Mäkinen 1988, Rühling et al 1992, Steinnes et al 1994, Rühling et al 1994). When repeated collections have been made, retrospective comparisons are possible (Rühling et al 1992). Also, the distribution of metals around point sources can be mapped (Folkeson 1981, Mäkinen 1987b).

In Latvia, in the early 1990's when very limited data existed on the regional distribution of metals and the sources of the metals, a national survey using *Hylocomium splendens* as a biomonitor showed the "hot spots" where further work should be undertaken (Brūmelis 1992) As part of a European mapping project, the total concentrations in moss were compared to those in other countries (Rühling et al 1992). However, the measured total concentrations in moss in the Latvian survey were also related to the forest soil types, where an increased component of spruce in the canopy and thinner organic horizon resulted in higher concentrations of some elements (Brūmelis and Nikodemus 1995). This result, although at first seeming minor in relation to the anthropogenic loads near pollution sources, indicated the need for a method that could assess the natural contribution to total concentrations.

In local surveys in Latvia, the spread of metals around pollution sources have also been determined, restricting sampling in strictly defined forest types. Sloka and Jaundubulta have been determined to be the areas of concern in Jūrmala (Nikodemus et al 1993-using pine bark as a monitor, Brūmelis, unpublished - *Hylocomium splendens*). The transboundary spread of pollutants into Latvia from the Mažeikiai oil refinery, Lithuania, was mapped, and levels were determined to

be much lower than local pollution in Rīga (Nikodemus and Brūmelis 1993). The major emission source of metals in the pharmacological industry centre of Olaine was identified as the thermal-electrical power facility (Brūmelis and Nikodemus 1993). Also, the concentrations in moss around the Skrunda Radar System, Latvia, were mapped in an attempt to relate an observed decrease in tree growth to a local pollutant emission source; which was not found (Balodis et al 1996). These local monitoring surveys have provided important information about the sources of pollution, that was previously lacking. Nevertheless, the total concentrations bear little relevance to ecological interpretation.

An emphasis has been placed in biomonitoring on the cell wall exchangeable sites as the major location for cellular inputs of deposition. However, metals can also occur in the free intercellular space, intracellularly in the cytoplasm of organelles, and as adhered particles on the exterior of moss. The use of total concentrations cannot distinguish between these locations. According to Rühling and Tyler (1973), Na, K, Mg and Ca are to be avoided in monitoring using moss, since retention is low, equilibrium occurs between inputs and losses, and accumulation can be by active transport driven by physiological demand. However, equilibrium must always occur between metal pools in moss and the environment, and between the cellular locations. Also, the lack of a physiological demand for an element, for example Cd, cannot be equated with no intracellular input, since the carrier molecules in the cell membrane are rather non-specific (Wells and Brown 1990).

Of the commonly surveyed metals in monitoring projects, Pb and Cu have the highest absorption and retention for cell wall exchangeable sites. Cu and Pb are class B metal ions, with high affinity for N- and S- centres (for example,  $\text{NH}_3$ ,  $\text{NH}_2^+$ ,  $\text{S}^{2-}$ ,  $\text{S}^-$ ), to which they can become relatively irreversible bound (Niebor and Richardson 1980). Class A elements, for example K, Na, Mg and Ca, have higher affinity to O- centres (e.g.  $\text{COO}^-$ ,  $\text{OH}^-$ ,  $\text{PO}_4^{2-}$ ) than elements with class B character. Borderline elements (Zn, Ni, Fe) have both class A and B character. It seems

reasonable that knowledge of binding affinity and nature, and the cellular location of metals in moss, will aid interpretation of moss survey results.

Clearly, the toxicity of elements cannot be discussed solely by total concentrations, since a proportion of a given element will likely be unavailable to intracellular uptake. Very little is known of the sources and fates of particles deposited on moss, including their retention on the moss surface and solubility. A particle, attached to moss, that can only be dissolved by extreme methods, such as concentrated  $\text{HNO}_3$  extraction, may have minimal immediate effect on inter-, cell wall (extra-) and intracellular uptake, especially if it can be easily washed off by a rain event, but its ecological importance to moss during a season or years is unknown. Methods have been proposed to assess the contribution of particles to total concentrations, and to correct total concentrations for naturally occurring particles. Scanning electron microscopy, in combination with X-ray emission spectrography (Johnsen 1981), can provide information on the densities of particles on plant surfaces and concentrations of elements in various particle types. The contribution of soil derived particles can be estimated by enrichment factors, the ratio of element X over Al in moss compared with the ratio of element X over Al in soil, to correct total concentrations for this natural factor (Bargagli et al 1995). For the organic horizon of soils, loss on ignition data have been used to estimate the mass of particles, and to subtract the concentrations due to particles from total concentrations based on concentrations in the parent C horizon (Steinnes and Njåstad 1993). If the background ash contents in moss, without particles, are known, as well as the types of particles and their metal concentrations, then this method may have potential in moss surveys.

## 2.2. Serial elution to determine cellular concentrations of metals.

Using the serial elution technique (Brown and Wells 1988), one can determine the concentrations of metals in various cellular fractions (intercellular, cell wall exchangeable, intracellular and residual/particles). Potentially, the method offers an improvement to the use of total concentrations in practical biomonitoring. The

intercellular fraction, determined by elution with deionised water, estimates the concentrations of ions released into the free space between cells, and the water soluble material on the moss exterior.

The cell wall, including outer cell membrane, associated metals, are exchangeable on negatively charged anionic sites or chelated to side groups of polymers. Various elutants have been used ( $\text{NiCl}_2$ ,  $\text{Pb}(\text{NO}_3)_2$ , EDTA) to displace ions from cell wall sites, depending on the affinity of the metal in question to the existing sites. Cell wall carbohydrate polymers likely dominate the binding of Class A metals, but aminoacid side groups of proteins with N- and S- ligands, firmly bind Class B metals (Brown and Wells 1988). A divalent ion is more efficient as a displacing agent than a monovalent ion, and a higher Class B character will displace Class A ions. In bryophytes, a 20 mM concentration of  $\text{Ni}^{2+}$  will displace the exchangeable K, Mg, Ca, Na, Cd and Zn, similarly Pb will displace Cu, and low pH EDTA will remove Pb (Branquino and Brown 1994).

Metals which cross the cell membrane into the cell interior form the intracellular fraction. These may be soluble in the cytoplasm, or bound on, or within, organelles. This fraction is measured by disruption of the cell membrane in combination with a displacing agent. Many uptake studies have shown that 1M  $\text{HNO}_3$  is suitable for the removal of intracellular ions (Brown and Wells 1990, Wells and Brown 1989, 1990, 1995, Wells et al 1995). However, this fraction (and also the previous two fractions) may also elute some of the attached particles. A boiling 20 mM  $\text{Ni}^{2+}$  step before the 1M  $\text{HNO}_3$  elution has been recommended (Branquino and Brown 1995) to measure intracellular concentrations, but this may physically remove attached particles.

The insoluble particle fraction is argued to be the residual fraction, extracted by boiling in concentrated acid ( $\text{HNO}_3$ ), and defined as the insoluble material not extracted by previous fractions. If low amounts of ions are released from particles by the previous fractions, then the residual fraction will be a suitable estimate of the total particle fraction.

Clearly, the understanding of the physiological effect of a metal requires knowledge of the quantities and locations of the metal, as well as the uptake kinetics and mechanisms. Binding of a metal to the cell wall (extracellular uptake) occurs at anion sites by displacement and depends on the character of the binding site, the quantity of the displacer in the intercellular solution and the affinities of the displacing and bound ions to the specific sites. Intracellular uptake occurs by dissociation-association to carrier molecules located in the cell membrane. Interactions between ions affect intracellular uptake by competitive and non-competitive inhibition for the carrier molecule binding sites (Brown and Wells 1988).

Considering the above, uptake of metals to moss can only be described as an equilibrium process, and mosses can not be assumed to be passive absorbers of cations that provide an integrated assessment of deposition (Brown and Bates 1990). In the case of ions with high Class B character (Cu, Pb), that efficiently bind and are retained on the cell wall, total concentrations will provide a better estimate of cell wall exchangeable deposited ions, but not of particles. Bates (1992) presents a model for the uptake of minerals to moss, involving wet and dry deposition inputs, losses, inputs from the substrate and internal recycling from young to old tissues. Input of metals to moss from the substrate has been assumed to be of little importance in monitoring work, but has been shown to occur in the field (Bates and Farmer 1990). Vertical movement of ions in moss plants has been demonstrated experimentally (Brown and Brown 1990, Sidhu and Brown 1996).

Measurement of the various cellular pools of metals can be expected to be an improvement to the use of total concentrations in pollution studies, and would allow an objective ecological assessment of results. In monitoring studies, seasonal effects have been overlooked, and it is also necessary to consider the impact of meteorological conditions on the equilibrium processes of uptake.



### 3. THE APPLICATION OF ZINC CELLULAR CONCENTRATIONS IN MOSS TO MONITOR DEPOSITION: EXPERIMENTAL UPTAKE TRIALS.

The ability of the serial elution technique to portray cellular concentrations of Zn in *Hylocomium splendens*, was assessed in experimental uptake trials. Zn was applied to moss samples by immersion in solutions in the laboratory for given periods of time. The uptake to cellular locations was investigated using the serial elution technique, to determine if the uptake kinetics resembled expected patterns. Uptake to cell wall sites occurs by saturation kinetics, while intracellular uptake is lower and linear with time (at least in the first 1/2 h of immersion in solution), dependent on dissociation/association to cell membrane carrier molecules (Brown and Wells 1988). A non-linear intracellular uptake can indicate incomplete displacement of cell wall cations. Uptake to the residual particle fraction from solution would indicate incomplete displacement of intracellular zinc. K, Mg and Ca concentrations are monitored during uptake trials, to indicate cell membrane damage. Since K is actively transported into the cell, and the greatest proportion is located intracellularly in healthy moss (Brown and Wells 1988), the appearance of higher inter- and extracellular K concentrations will indicate membrane damage.

#### 3.1. Methods

Fresh *Hylocomium splendens* was collected from grassy slopes of the Mendip Hills, Somerset, UK, and stored moist before treatments. The collected *H. splendens* showed no growth increments, typical of moss found in these conditions, but atypical of that found in most of its range. However, the use of healthy moss was the only limitation required in choice of sampling location. Green moss segments (2 cm in length) were bubbled in 1 l of 0.005 and 0.01 mM zinc solutions. At intervals of 0, 10, 60 and 90 min, 3 replicates of 3 moss segments were removed and rinsed by leaching with 2 l of deionised water. Immediately, the moss was sequentially eluted as follows (Brown and Wells 1988).

1. Intercellular ions.

- a. Shaking with 15 ml deionised water for 40 min.
- b. Shaking with 15 ml deionised water for 30 min.

2. Extracellular ions (cell wall plus outer cell membrane sites).

- a. Shaking with 15 ml 20 mM  $\text{NiCl}_2$  (or  $\text{Pb}(\text{NO}_3)_2$ ) for 40 min.
- b. Shaking with 15 ml 20 mM  $\text{NiCl}_2$  (or  $\text{Pb}(\text{NO}_3)_2$ ) for 30 min.

3. Cell wall plus cytoplasm weight.

Drying at  $80^\circ\text{C}$  overnight and weighing.

4. Intracellular ions

Shaking with 1M  $\text{HNO}_3$  for 1h.

Rinsing in 15 ml deionized water followed by blotting.

5. Cell wall weight.

Drying at  $80^\circ\text{C}$  overnight and weighing.

6. Residual ions (relatively insoluble particles).

Boiling in 5 ml concentrated  $\text{HNO}_3$  to dryness and redissolving of ions to make 10 ml deionised water.

Various displacing agents (step 2) were tested, including  $\text{NiCl}_2$ ,  $\text{Ni}(\text{NO}_3)_2$ ,  $\text{Pb}(\text{NO}_3)_2$  and  $\text{NiCl}_2$  plus  $\text{Pb}(\text{NO}_3)_2$ , for their ability to remove zinc from cell wall exchange sites. A boiling  $\text{NiCl}_2$  treatment after cold Ni elution has been recommended to determine intracellular ion concentrations, since the boiling would damage the membrane, and the  $\text{Ni}^{2+}$  would displace the intracellular ions. In preliminary trials, the boiling step, after the drying at  $80^\circ\text{C}$ , caused the moss to become too brittle for subsequent transfer. Hence, in a parallel elution to the cold  $\text{Ni}^{2+}$  elution series, a boiling  $\text{Ni}^{2+}$  treatment (1 h) was applied immediately to moss

(intercellular plus extracellular plus intracellular concentrations), with the idea that subtraction of the cold  $\text{Ni}^{2+}$  intercellular and extracellular concentrations would provide a more objective estimate of the intracellular concentrations.

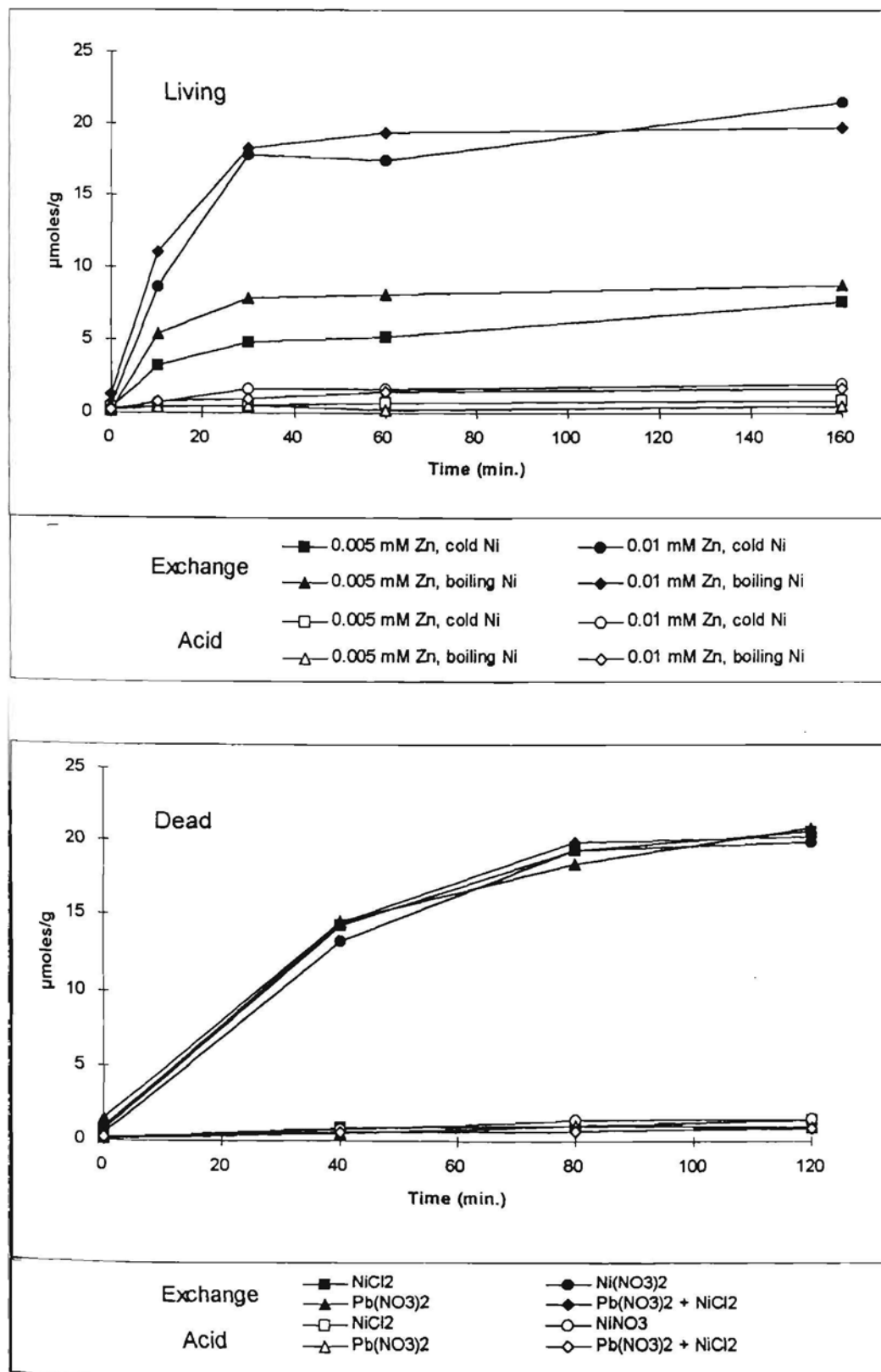
To assess the efficiency of displacement of ions bound to extracellular cell wall sites, in the absence of intracellular sites, moss was previously killed by boiling for 2 h in deionised water.

Concentrations of Zn, Ca, Mg and K were determined by atomic absorption spectrophotometry (Varian Techtron AA-6, Varian Techtron AA-775 and Varian Techtron AA 1100).

### 3.2. Results and Discussion.

The uptake of Zn (0.01 mM and 0.005 mM) to extracellular sites (cold  $\text{Ni}^{2+}$  treatment) showed typical saturation kinetics - a rapid initial increase, followed by a gradual levelling off with saturation of sites (Figure 3.1) Intercellular plus extracellular plus intracellular uptake, estimated by the boiling treatment, was slightly higher. Since the intracellular concentrations were low, and the variability between replicates was rather high, the subtraction of the cold  $\text{Ni}^{2+}$  intercellular plus extracellular values from the boiling  $\text{Ni}^{2+}$  estimates did not provide an adequate estimate of the intracellular concentrations. However, these calculated intracellular values were in the range of direct intracellular concentration estimates made by elution in 1M  $\text{HNO}_3$ , suggesting a minimal effect of soluble particles in the sampled moss.

Figure 3.1. Zn uptake/time curves of healthy and killed moss immersed in bubbled 0.005mM and 0.01 mM Zn solutions. For healthy moss, cold NiCl<sub>2</sub> and Boiling NiCl<sub>2</sub> treatments were used. Extracellular uptake on exchangeable sites in killed moss was determined by elution in 20mM of NiCl<sub>2</sub>, Ni(NO<sub>3</sub>)<sub>2</sub>, Pb(NO<sub>3</sub>)<sub>2</sub>, or NiCl<sub>2</sub> plus Pb(NO<sub>3</sub>)<sub>2</sub>. 1M HNO<sub>3</sub> was used for acid elution.



Intracellular uptake (1M HNO<sub>3</sub> treatment) appeared linear with time for 30 min (Figure 3.1), with a hint of subsequent saturation kinetics, suggesting incomplete removal of Zn by the Ni<sup>2+</sup> displacing agent. However, the use of a displacing agent with greater Class B character (Pb<sup>2+</sup>) did not improve Zn removal from the extracellular sites on killed moss, which would likely have occurred with inefficiency of the Ni<sup>2+</sup> displacing agent. The intracellular uptake to killed moss contradicts this idea, and implies that the acid elution removes also Zn not displaced from extracellular sites by Ni<sup>2+</sup> or Pb<sup>2+</sup> elution. Moss killed by boiling has lost cell membrane integrity and an unknown amount of cytoplasmic content has leaked out. However, the cells cannot be assumed to be empty, since likely many of the cellular macromolecules are still located within the cell, but have changed in structure, for example denatured proteins. Thus, the interior of the killed cells, may contain many exchangeable sites, which may not be as accessible to the used displacing agents, compared with the cell wall.

The adequacy of the cold Ni<sup>2+</sup> displacement of ions from killed cells was tested by increasing elution time. Boiled (1 h) moss segments (youngest mature segment) were bubbled in 0.1 mM Zn for 1 h to saturate binding sites and rinsed as above. The moss (three replicates of 2 segments) were then eluted in 20 mM Ni<sup>2+</sup> for varying lengths of time, followed by elution in 1 M HNO<sub>3</sub>. The elution times used for the first of two Ni<sup>2+</sup> elutions were 30, 40, 50, 60 and 90 min; the second elution time was 30 min. Fresh mature segments (one- and 3-year old) were likewise analysed for comparison. As expected, the extracellular concentrations of Ca, and especially Mg, were increased in boiled cells, at the expense of the intracellular concentrations, due to rupture of the cell membrane, however a considerable component of Ca and Mg was still evident in the 1M HNO<sub>3</sub> fraction. The "intracellular" concentrations of Ca and Mg in killed cells decreased with increasing time of Ni<sup>2+</sup> elution of killed cells, while the extracellular concentrations increased (Table 3.1). Leakage of intracellular K and Mg from healthy moss was observed with the longer elution times, no doubt due to cell membrane damage, and most of

the K was removed by the boiling and bubbling with Zn. However, the losses of "intracellular" ions in killed moss with increasing elution time cannot be interpreted as membrane damage.

The results indicate that boiled moss cannot be interpreted as pure cell wall and its use for testing the efficiency of extracellular uptake can give misleading results. The decreasing Ca and Mg "intracellular" concentrations with an increase in  $\text{Ni}^{2+}$  elution time, suggest the presence of intracellular material in boiled cells that lack a cell membrane, which provides exchange sites from which ions are displaced less efficiently in comparison with cell wall sites. This may be due to a diffusion gradient within the cell. Shaking killed moss in boiling  $\text{Ni}^{2+}$  will ensure a surplus of the displacing agent to the cell surface, but not necessarily to the sites in the killed cells. Also, intracellular sites may be protected from displacement by hydrophobic molecules formed from boiled cellular lipids, or the intracellular sites may have higher Class B character. The Zn concentrations in the extracellular and intracellular locations in killed moss were only slightly lower than those of healthy moss, suggesting that a similar proportion of Zn binding sites is not easily displaced by  $\text{Ni}^{2+}$ , regardless of whether the cell membrane is intact or damaged. Perhaps this reflects a chelated intracellular Zn fraction which is minimally affected by boiling. Zn uptake to these sites in boiled cells would explain the increasing concentrations in the 1M  $\text{HNO}_3$  elution of boiled moss. Thus, the incomplete displacement from extracellular sites in boiled moss, may be due to an artifact. Killing cells with 1M  $\text{HNO}_3$  may be an alternative to use in testing the efficiency of displacement from cell wall sites, but this treatment will also alter the nature and amounts of the cell wall sites.

Table 3.1. Concentrations of Zn, Ca, Mg and K ( $\mu\text{moles/g}$ ) in *Hylocomium splendens* after 1 h immersion in bubbled 0.1 mM Zn solution, and subsequently serially eluted in 20 mM  $\text{NiCl}_2$  and 1M  $\text{HNO}_3$  solutions. Treatments used were one- and three-year-old healthy segments, and killed (boiled) one-year-old segments. Elution times are given for the first of two elutions. The time of the second elution was 30 min. nd - not detectable.

metal	Elution time (min)	Metal concentrations ( $\mu\text{moles/g}$ )					
		one-year old		three-year old		killed	
		$\text{NiCl}_2$	$\text{HNO}_3$	$\text{NiCl}_2$	$\text{HNO}_3$	$\text{NiCl}_2$	$\text{HNO}_3$
Zn	30	51.3	8.5	48.9	5.1	45.1	7.0
	40	47.9	8.6	46.4	5.1	44.4	6.0
	50	46.1	8.1	48.9	5.1	45.3	7.2
	60	48.9	7.1	46.3	5.1	40.0	6.8
	90	46.9	7.9	45.4	4.2	45.9	5.9
Ca	30	60.48	17.4	97.6	24.5	78.3	13.6
	40	65.1	17.5	89.1	21.3	82.1	11.5
	50	60.0	15.5	101.5	17.8	83.5	12.4
	60	60.7	17.8	93.1	15.5	86.7	12.8
	90	61.9	19.9	92.9	13.7	88.7	10.0
Mg	30	17.1	49.6	17.0	12.3	34.7	7.4
	40	16.9	49.0	16.6	15.2	38.6	6.1
	50	16.4	46.1	18.0	10.1	40.0	5.7
	60	16.3	43.9	16.3	15.8	40.6	6.0
	90	16.8	38.8	17.0	15.6	42.9	4.0
K	30	nd	224.3	nd	120.4	nd	13.9
	40	nd	227.5	nd	120.1	nd	11.8
	50	nd	204.3	nd	93.4	nd	12.7
	60	nd	205.8	nd	157.6	nd	13.1
	90	nd	178.2	nd	129.3	nd	10.2

The above results also create doubt to the validity of a boiling elution with a displacing agent, previously recommended to use after elution of cell wall ions (Branquino and Brown 1994), to adequately determine intracellular concentrations. Therefore, in further work, the 1M HNO<sub>3</sub> elution was used to represent intracellular concentrations, plus an unknown amount of dissolved particles.

In all experimental uptake trials, in conditions of high zinc total concentrations, the intracellular zinc concentrations, measured by 1M HNO<sub>3</sub> elution, were always less than 8% of the extracellular concentration. In all field collected material, the total zinc concentrations are much lower, and intracellular concentrations are in the range of about 50% of the extracellular concentrations. Therefore, the error of inadequate displacement displacement of Zn by Ni<sup>2+</sup> elution can be assumed to be relatively small. Hence, the sequential elution technique using the cold Ni<sup>2+</sup> treatment appears to be adequate for the determination of inter-, extra- and intracellular concentrations, provided that the error of displacement from particles by 1M HNO<sub>3</sub> is small relative to the true intracellular concentrations.



## 4. Effects of desiccation of moss on cellular concentrations

In practical biomonitoring work, usually samples are required to be collected over a wide area, in as short a period of time as possible. Fresh samples, thus collected, obviously cannot all be subjected, immediately upon return to the laboratory, to the serial elution technique, due to the time requirements of the method. Frequent trips to the sampling area(s) for fresh samples would make this approach cost-prohibitive. Therefore, air drying of samples would be of benefit. However, it is then necessary to know the effects of desiccation, and subsequent revival by rehydration, on the cellular concentrations. Samples, collected from a dry pine forest and a drained pine swamp, were serially eluted fresh, and after 2 weeks and 2 months air drying with subsequent rehydration.

### 4.1. Methods

*Hylocomium splendens* was collected on 25 July and 29 August, 1994, from an unpolluted dry pine forest with a spruce understorey, and an adjacent drained pine swamp in Tinuži, Latvia. The July sampling period was after 20 days of dry weather, but the August sampling followed 20 rainy days. The cellular concentrations of K, Mg and Ca were determined (Chapter 3) upon 24 h rehydration in the laboratory immediately, and after 2 weeks or 2 months (July only) air dry storage. Moss plants were divided into annual segments (1991, 1992, 1993+1994 bud in July, 1991-1994 in August with bud expansion) before elution of 5 replicates of 2 segments.

### 4.2. Results and Discussion

Figures 4.1 and 4.2 show the effect of air dry storage on the cellular concentrations, and relative concentrations (%) of K, Mg and Ca in *Hylocomium splendens*. Before storage, over 80% of the K was located intracellularly for the July collection, and excepting the oldest segment, also the August collection. The relative cell wall and

intercellular concentrations of K increased, at the expense of intracellular K, in older segments and with longer storage times. This redistribution was less apparent for Mg cellular concentrations. Ca cellular concentrations after 2 months storage were redistributed in the reverse direction; intracellular concentrations increased with a decrease in cell wall Ca. The total Ca concentrations also decreased. This indicates that some of the extracellular Ca had been displaced, perhaps by displacement with the leaked Mg, and has entered to a intracellular location or has been lost to the moist filter paper during rehydration.

Moss species are adapted to various patterns of changing moisture regimes, ranging from, mostly moist, sometimes moist/sometimes dry, to mostly dry. These adaptations are reflected in the redistribution of cellular concentrations of moss species, in laboratory inflicted drying and rehydration timetables (Brown and Buck 1979). Since feather moss grows in environments that are intermittently seasonally dry or moist, and that also diurnally vary in air humidity, it could be expected that these mosses can quickly re-establish metabolically optimum intracellular concentrations upon rewetting after desiccation. Relating this to the use of mosses in monitoring surveys, it is important that the measured cellular concentrations after rehydration of stored material, can estimate the pre-collection concentrations. The monovalent K cation was rapidly redistributed upon desiccation, but two weeks of storage of the moss collected in July had much less effect on cellular redistribution than the August collection (Figures 4.1 and 4.2). Desiccation induced membrane damage (not necessarily irreversible) was suggested by K intracellular concentrations that approached 50% after two months storage. For longer storage times, longer rehydration times will likely result in patterns that approach the pre-collection cellular concentrations, as long as the membrane has not been damaged irreversibly. The divalent Mg and Ca cations were much less affected by storage.

Figure 4.1. Cellular concentrations ( $\mu\text{moles/g}$ ), obtained by serial elution, of moss collected in a dry forest and a swamp, and stored air dry for 2 weeks or 2 months, or immediately analyzed. particles - residual insoluble; cell - intracellular; wall - cell wall exchangeable; inter - intercellular.

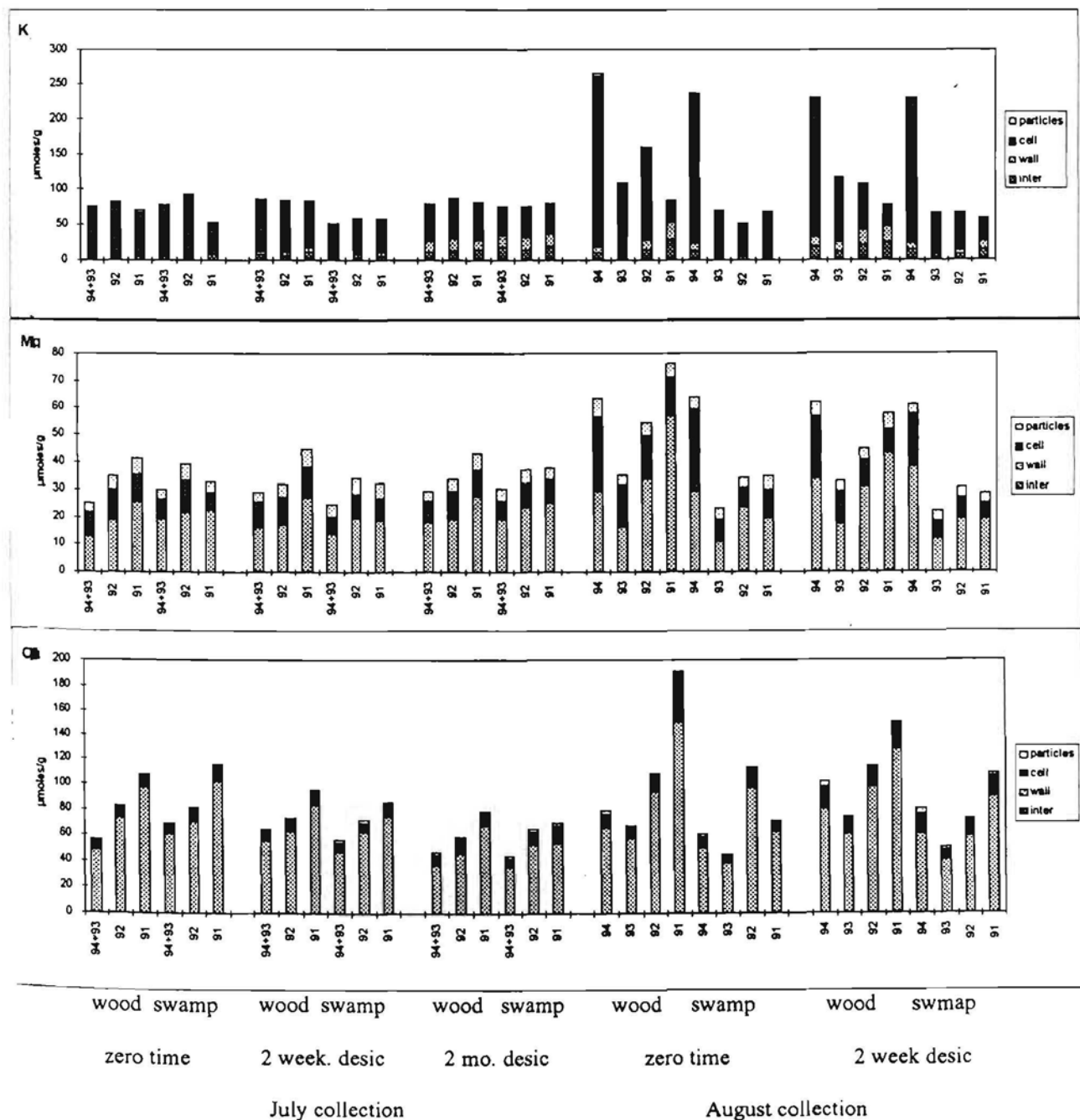
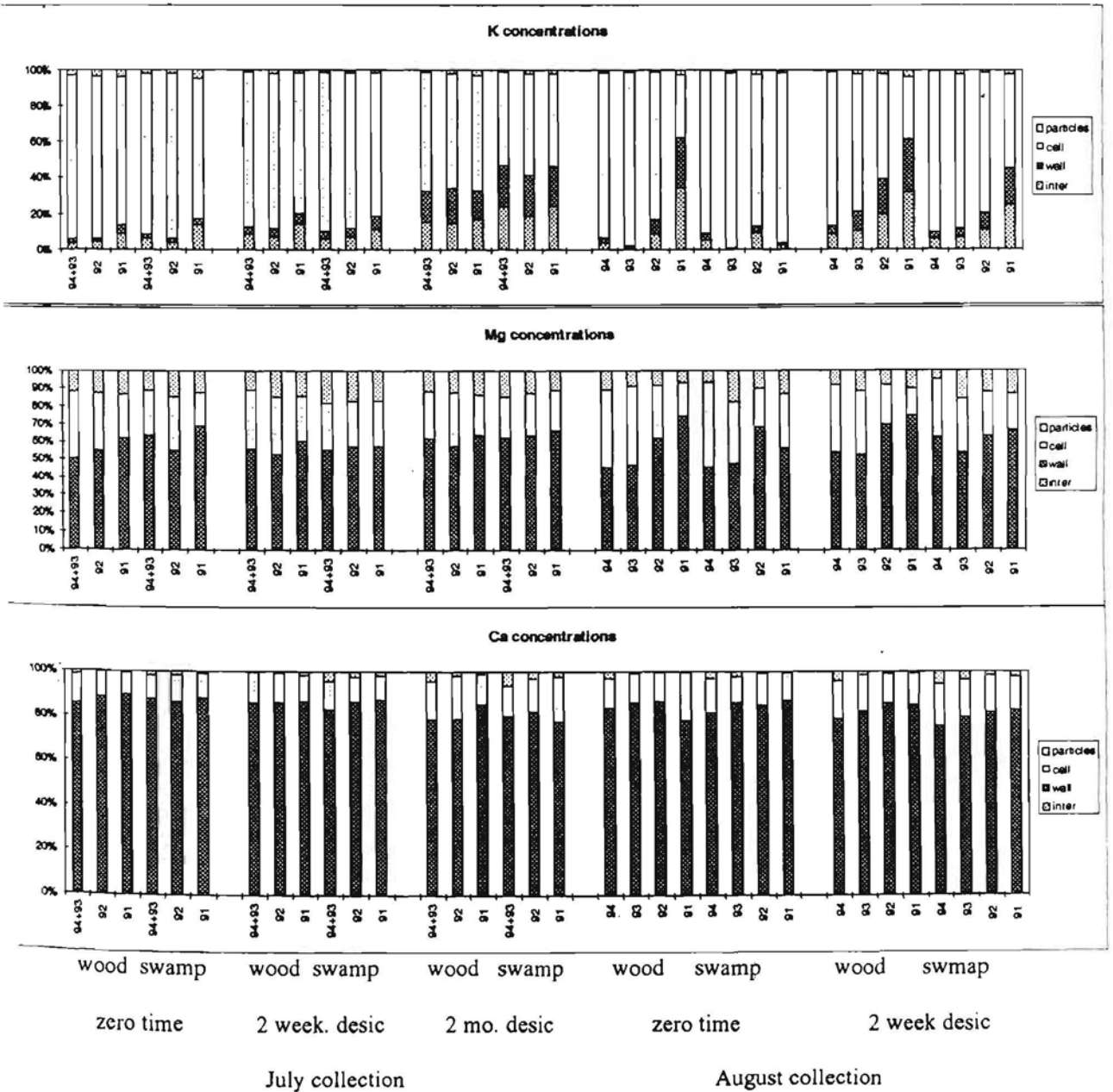


Figure 4.2. Relative cellular concentrations (%), obtained by serial elution, of moss collected in a dry forest and a swamp, and stored air dry for 2 weeks or 2 months, or immediately analyzed. particles - residual insoluble; cell - intracellular; wall - cell wall exchangeable; inter - intercellular.



In August (wet period), rapid production of biomass is occurring, and the high K concentrations in the youngest segments are consistent with the idea of mineral recycling between moss segments (Bates 1992). At this time, the oldest segments in the dry forest moss are visibly brown with loss of some material, and the lower K and Mg concentrations in these segments likely indicate ageing. In the swamp forest, at least 5 segments of moss were green to green/yellow, i.e. relatively healthy, hence the relative K intracellular concentrations were more similar for the youngest 4 segments. Nevertheless, the older segments were still more susceptible to desiccation after two weeks of storage.

Of the elements investigated, the relative Ca extracellular concentrations were most stable with desiccation and above 80%, but total Ca concentrations were increased in August, in comparison with July, especially in the older segments. This indicates increased uptake of Ca in autumn, perhaps due to canopy leaching, and downward leaching or more available cell wall sites in older segments. The higher extracellular exchangeable Ca concentrations in older segments in the dry pine forest may be due to exposure of intracellular sites after membrane damage (see Chapter 3), or to increased deposition by canopy leaching. Increased cell wall concentrations in older moss tissue have been also previously attributed to maturation of the cell wall, bacterial and fungal growth, and also particles (Brown and Bates 1990).

While 2 months storage resulted in considerable leakage of K from intracellular locations, Mg and Ca was redistributed minimally. This suggests that the cell membrane is not irreversibly damaged, but further study is required to determine the optimum rehydration times for various meteorological conditions and ecosystem moisture regimes. It may be assumed that cations with greater Class B character (the ions of interest in pollution surveys) are even less easily displaced, and, provided that the cell membrane is intact, that the serial elution technique can estimate the cellular concentrations of these elements after storage. At certain times

of the year, at some locations, collected samples may be more susceptible to desiccation induced cellular redistribution of elements caused by storage before elution.

## 5. UPWARD MOVEMENT OF METALS TO NEW GROWING TISSUE IN THE MOSS *HYLOCOMIUM SPLENDENS*

The feather mosses are, throughout most of their ranges, associated with coniferous canopies (Tamm 1953, Weetman and Timmer 1967, Āboliņa 1974). It is widely supposed that these mosses are reliant on deposited nutrients for growth. Since feather mosses also lack a developed conduction system for transport of water and nutrients (Callaghan et al 1978), it is also believed that minerals are not transported between segments. These claims, along with the observed high sorption and retention efficiencies of some metals applied in solution (Rühling and Tyler 1970, Gjengedal and Steinnes 1990) and significant correlations between metal concentrations in wet deposition and moss (Ross 1990, Berg et al 1995), are the prerequisites for an ideal biomonitor of atmospheric deposition.

However, it has been shown that metals, both mineral nutrients and heavy metals, can move between segments of moss, from young to old segments (Brown and Brown 1990), from old to young (Sidhu and Brown 1996, Wells and Brown 1996) or from soil solution to plant (Bates and Farmer 1990). This movement, and losses due to leaching, are dependent on meteorological conditions and seasonal growth (Skre and Oechel 1979, Brown and Brown 1990, Bates and Farmer 1990, Bates 1990). Even metals with high retention in moss (Rühling and Tyler 1970), such as Cu and Pb, have been observed to move into additional growth of *Rhytidiadelphus squarrosus* in the laboratory, when supplied from below (Brown and Brown 1990, Brown and Sidhu 1992).

The extent of movement of Ca, Mg, K and Zn into additional growth, and the dependence of this movement on the previous cellular pools in the older segments was investigated. The existing pre-experimental pools of metals were varied by using moss from different sites, which varied in deposition and growth, and by excision of branches.

## 5.1. Methods.

In Latvia, moss segments initiate in late autumn, or, less often in spring, as buds, which rapidly produce first and second order branches in late July through autumn. The 1-year-old segment in late autumn, initiated as a bud in the previous autumn or current spring, appears mature. In Scandinavia, development occurs during two seasons (Økland 1995), but this process appears to be more rapid in Latvia, at least in the sites studied around Rīga.

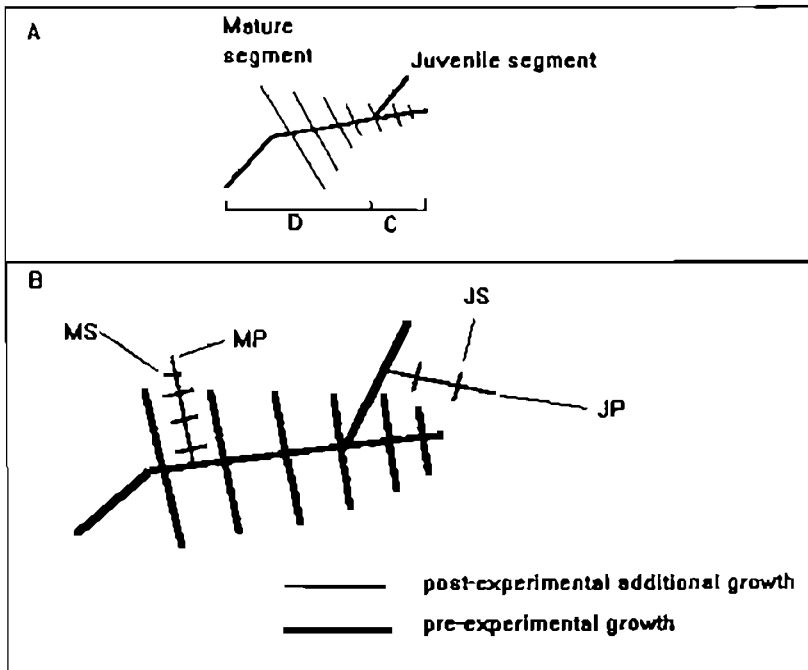
*Hylocomium splendens* moss was collected in six pine forest stands (with mixed spruce) around a metal smelter in Liepāja in June 1993. Moss was air dried, transported to Bristol and restored to physiological activity by spraying with deionised water and storing wet for 24 h in covered plastic trays lined with filter paper. The youngest mature segment, with the new 1- to 2- cm long bud, were used in the growth experiments. The stems of eight moss individuals per site were placed vertically through a 5-mm mesh glued on a 1.5-cm-high cylinders. Humidity was maintained by placing cylinders in plastic boxes with lids, filled with 40 ml of deionised water, enough to immerse the bases of the moss stem. The moss was grown under a 24-h  $110 \mu\text{mol m}^{-2}\text{s}^{-1}$  light regime at  $15^\circ\text{C}$  for 30 days.

Six treatments were applied to moss samples from the most contaminated site: A, the new unexpanded segment was removed; B1, moss with short (1 cm) unexpanded segments; B2, moss with long (2 cm) unexpanded segments; C, branches located above the point of initiation of the unexpanded segment were removed; D. branches located below the unexpanded segment were removed; and E, all branches were removed (Fig.5.1). For treatments C-E, the unexpanded segment lengths were about 1.5 cm. Three replicate segments per treatment were hooked onto each of three Velcro strips and placed vertically into plastic boxes with 40 ml deionised and allowed to grow, as above. Only visually healthy moss was chosen, but no effort was made to obtain standard sizes of expanded segments, to better portray field variability.



After 30 days of growth, the new additional branches, were clearly visible as a lighter shade of green. The new additional branches produced in the laboratory were measured and excised from the old previous growth. Three replicates of old and additional growth from three (or two) segments were subjected to sequential elution (cold Ni treatment - see Chapter 3) and analysed for Ca, Mg, K and Zn. Pre-growth concentrations of elements were estimated by analysis of replicate material for each treatment without placement in the growth room.

Figure 5.1A. Schematic representation of excised mature and juvenile segments used for laboratory growth experiments. The branches labelled D and C indicate locations of excised branches, excised below the juvenile increment and above the juvenile increment, respectively. B. Locations of additional branches during laboratory growth. MP and MS are primary and secondary branches, respectively, that have developed from the mature segment. JP and JS are primary and secondary branches, respectively, of the juvenile increment.



## 5.2. Results

The observed locations of new primary and secondary branches, that develop from juvenile and mature segments, are depicted in Figure 5.1. Additional growth was observed to occur from both mature and juvenile segments (site 1), but mostly from the juvenile segment at sites 4 and 6, and from the mature segment at sites 2, 3 and 5. Tertiary branch development was observed only at site 4 (Figure 5.2).

In the various treatments of moss collected at a polluted site (site 1), the additional growth varied in relation to the location of excised branches (Figure 5.3). The excision of mature branches above (Figure 5.3,-Treatment C) or below (Treatment D) the juvenile segment promoted the initiation and subsequent growth of primary and secondary branches from the juvenile segment. When all mature branches were removed (Figure 5.3,Treatment E), less additional growth occurred from the mature segment and lengths of the additional branches were shorter. More branches from the juvenile segment developed when the mature branches located below it had been removed (Figure 5.3-Treatment D). Fewer new additional primary and secondary branches developed from the mature segment, following excision of mature branches (2 times less), in comparison with treatments without branch excision. However, the removal of branches from mature segments resulted in greater development of branches in the juvenile segment.

Figure 5.2. The mean number of additional branches and mean total additional branch length (mm) per *Hylocomium splendens* segment, collected from 6 sites and grown for 30 days in the laboratory. Additional branches are classified as primary (prim.) and secondary (sec.), initiated on mature or juvenile parent segments.

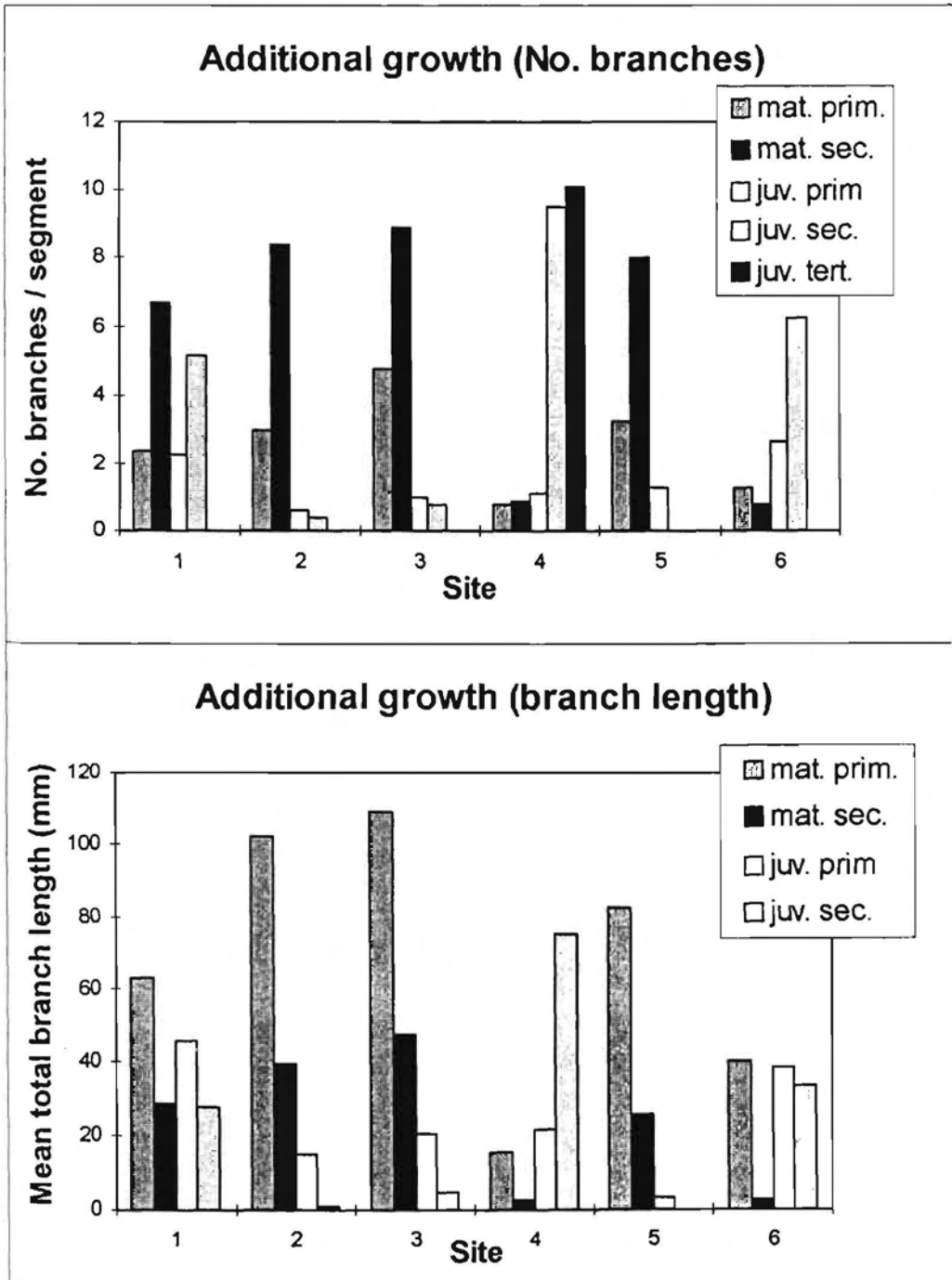
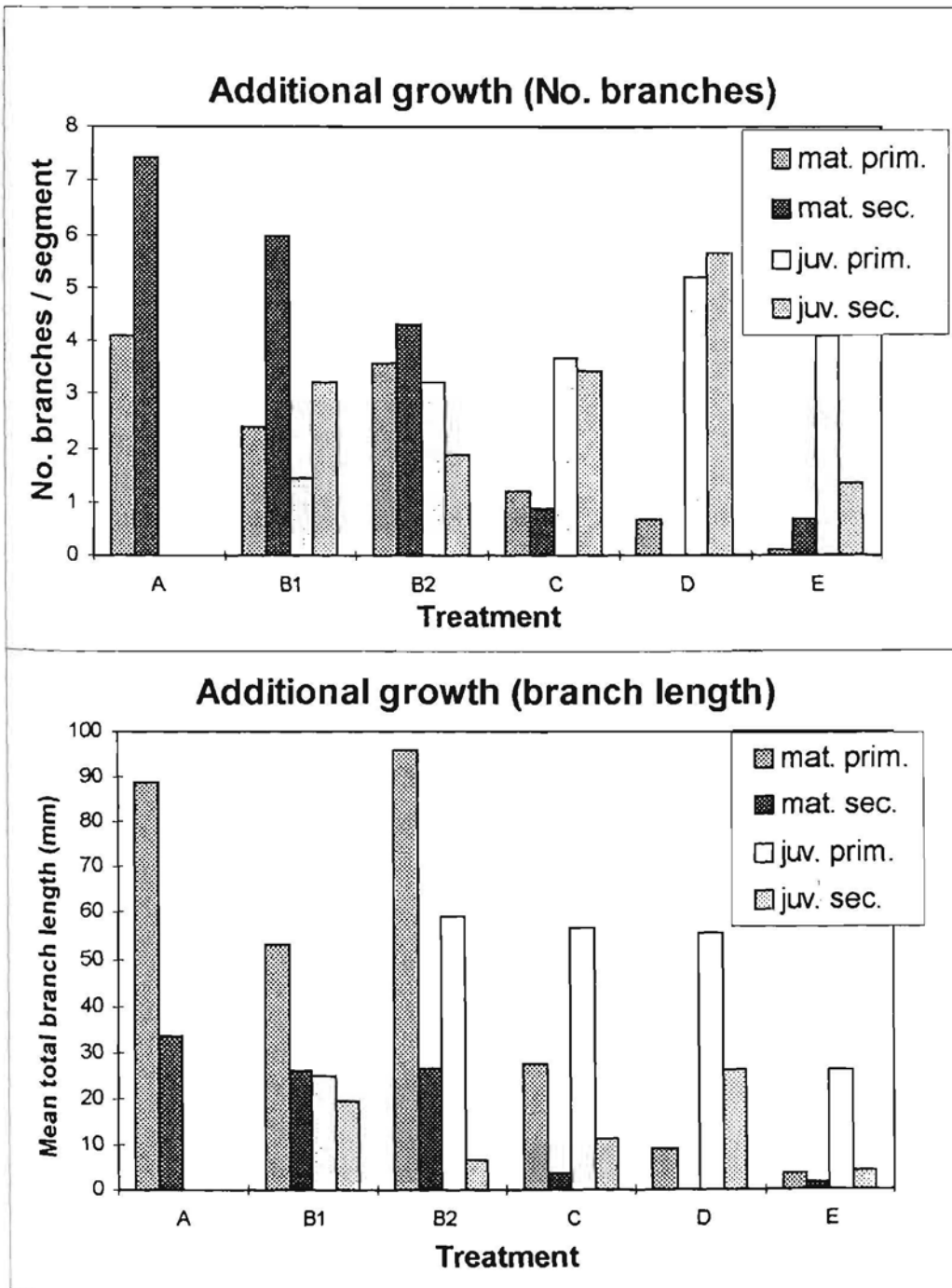


Figure 5.3. The mean number of additional branches and mean total additional branch length (mm) per *Hylocomium splendens* segment, grown for 30 days in the laboratory. The treatments applied to segments were: A. The new unexpanded segment was removed, B1. moss was chosen with short (1 cm) unexpanded segments, B2. moss was chosen with long (2cm) unexpanded segments, C. branches located above the point of initiation of the unexpanded segment were removed, D. branches located below the unexpanded segment were removed, and E. all branches were removed. Additional branches are classified as primary (prim.) and secondary (sec.), initiated on mature (mat.) or juvenile (juv.) parent segments.



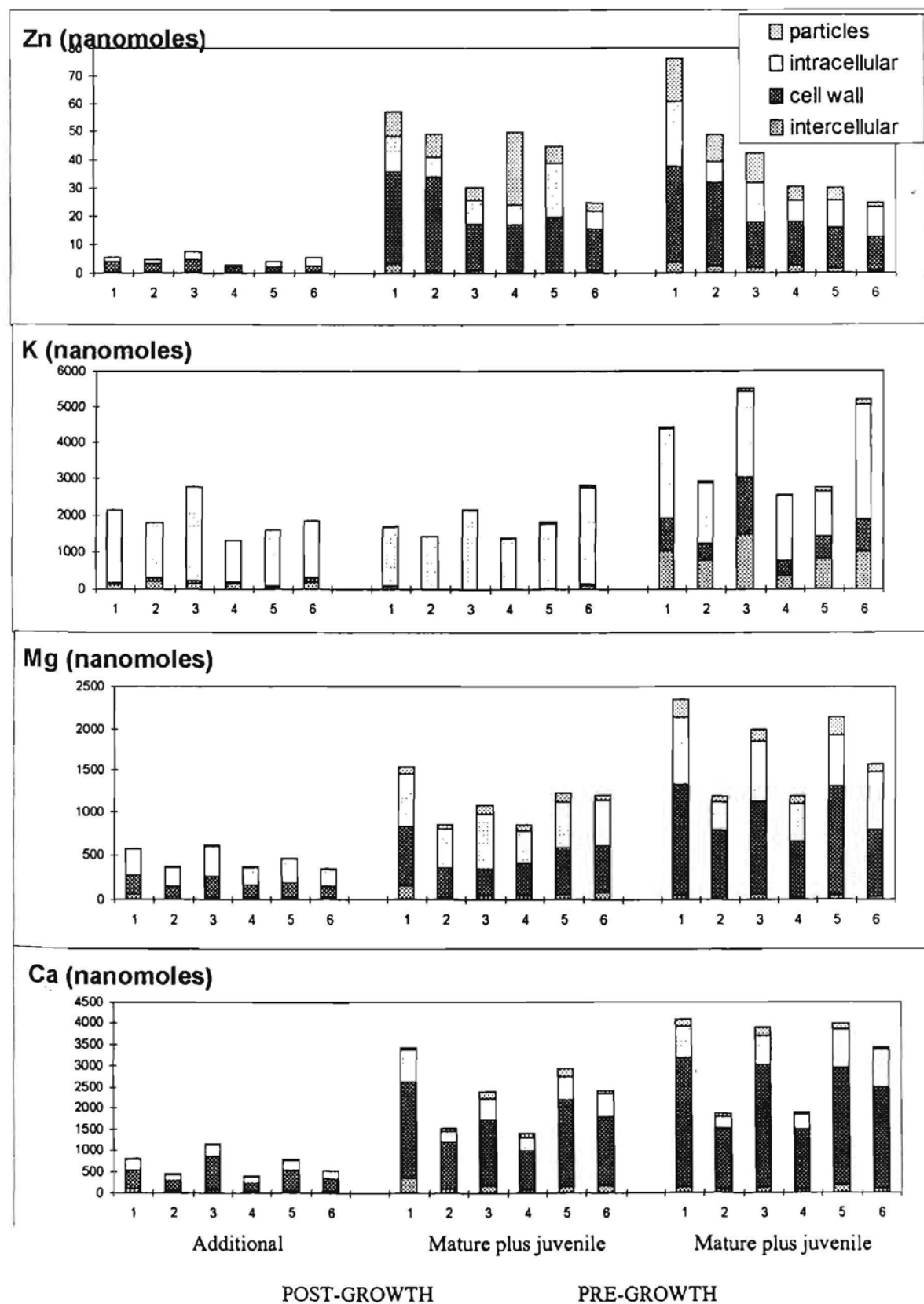
## Metal contents in additional growth

The correlation coefficients between pre-experimental cellular contents or concentrations of Zn, K, Mg and Ca in *Hylocomium splendens* collected at six sites, with the post-experimental values in additional growth or the current tissue, are shown in Table 5.1. The cellular contents depend on both the existing concentrations and segment weights; thus, they represent the total pools of metals potentially available for uptake to additional growth. The pre-growth pools (intercellular plus extracellular plus intracellular contents) of K, Mg and Ca in the juvenile plus mature segments were significantly correlated with the post-growth amounts in additional growth, and for Mg and Ca, also in the post-growth juvenile plus mature segments (Table 5.1, Figure 5.4). The contribution to the pre-growth pool from the juvenile segment was low for K (less than 30%), and even less for Mg, Ca and Zn (Figure 5.4). However, the concentrations of Ca, Mg and K were higher in the pre-growth juvenile segment, compared with the mature segment. The concentrations of K in additional growth were correlated with the concentrations in both mature and juvenile pre-growth tissue (Table 5.1). The uptake of Zn (concentrations and contents) to additional growth was not related to pre-growth levels, but small levels of uptake did occur (Figure 5.4). The Zn pre- and post- growth contents or concentrations in mature tissue were correlated, and losses were not apparent.

Table 5.1. Correlation coefficients between the amounts and concentrations of Zn, K, Mg and Ca in pre-growth moss segments, versus those in post-growth additional growth and post-growth mature and juvenile segments. Pre-growth contents were calculated as the total pools in mature plus juvenile increments, but pre-growth concentrations were determined for each segment separately for sites. Post-growth concentrations are determined as juvenile plus mature tissue and for additional growth. ns - not significant.

		Pre-growth versus post-growth additional			Pre-growth versus post growth mature and juvenile segments		
		Amounts	Concentrations		Amounts	Concentrations	
Sites			mature	juvenile		mature	juvenile
	Zn	ns	ns	ns	0.65	0.71	0.76
	K	0.68	0.63	0.67	ns	ns	ns
	Mg	0.68	ns	ns	0.71	ns	ns
	Ca	0.82	ns	ns	0.91	ns	ns
Treatments	Zn	0.76	ns		0.79	ns	
	K	0.95	ns		ns	0.92	
	Mg	0.76	ns		0.93	ns	
	Ca	0.82	ns		0.91	ns	

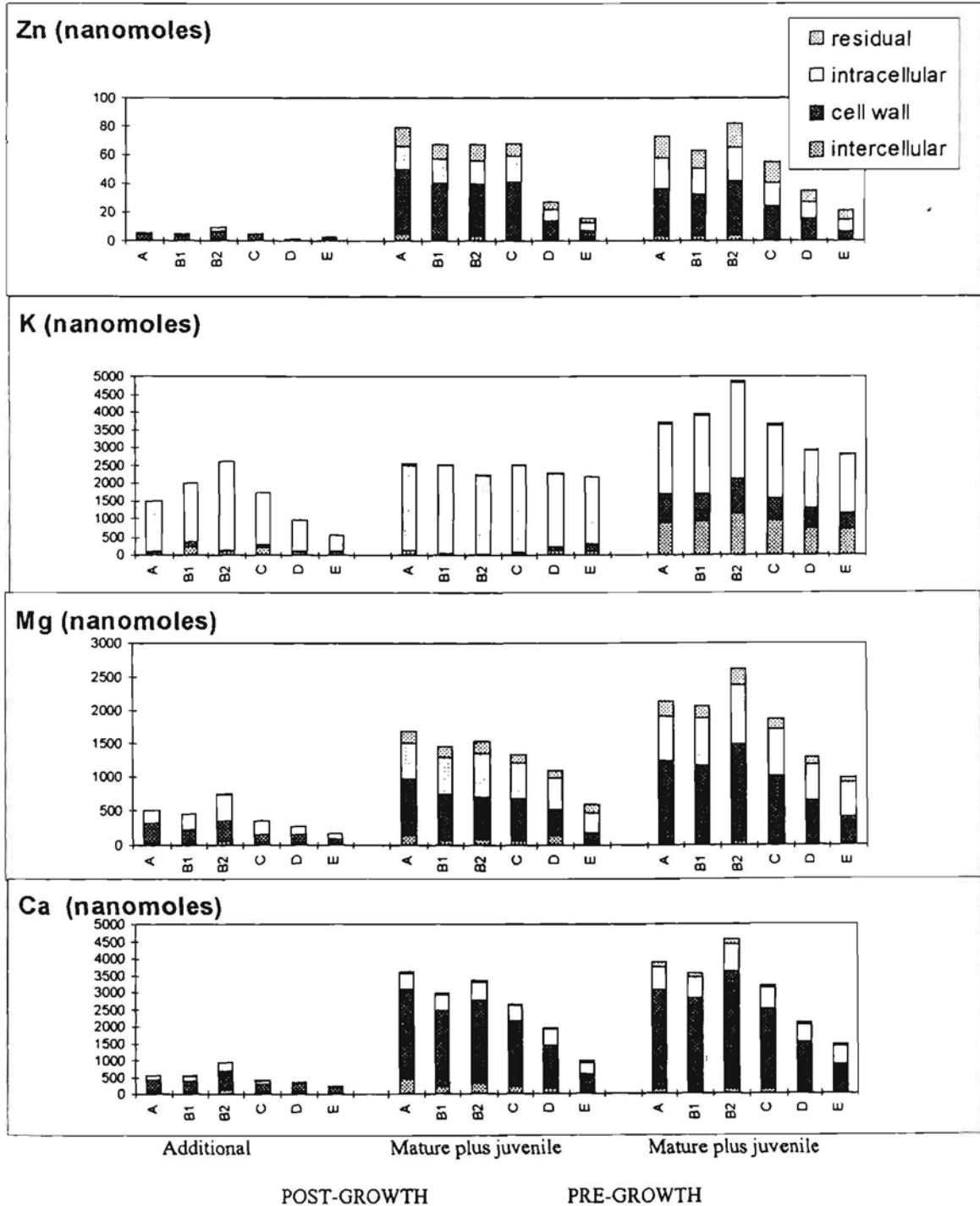
Figure 5.4. Zn, K, Mg and Ca contents (nanomoles) in *Hylocomium splendens* segments collected at 6 sites and grown for 30 days in the laboratory. Pre-growth contents in the mature plus juvenile segments and the post-growth mature plus juvenile and additional tissue are given.





The estimated pre-growth contents of Zn, Mg, Ca and K were correlated to those in additional growth in the excision treatments used (Table 5.1, Figure 5.5), but this relation was not evident for the concentrations. Excision of branches above the juvenile segment (Figure 5.5, Treatment C) decreased the pool of these metals, and even more was removed with the excision of the lower branches (Treatment D). A longer juvenile segment (Figure 5.5-Treatment B2) resulted in higher contents in additional growth. Although the juvenile segment is a minor component in terms of total weight, its excision from the mature segment lowered the K contents in additional growth to the same level as when all branches on the mature segment located below the point of attachment of the juvenile segment were excised. Thus, when the existing pools of metals were lowered by the excision treatments, the additional growth had lower contents. The contents of K in all treatments were similar in the post-growth mature tissue, regardless of whether juvenile segments, some or even all branches were excised. The pre-growth pools of potentially available Zn, Mg and Ca were also correlated with the post-growth pools in the juvenile plus mature segments.

Figure 5.5 Zn, K, Mg and Ca contents (nanomoles) in *Hylocomium splendens* grown for 30 days in the laboratory. The treatments applied to segments were: A. The new unexpanded segment was removed, B1. moss was chosen with short (1 cm) unexpanded segments, B2. moss was chosen with long (2cm) unexpanded segments, C. branches located above the point of initiation of the unexpanded segment were removed, D. branches located below the unexpanded segment were removed, and E. all branches were removed. Pre-growth contents in the mature plus juvenile segments and the post-growth mature plus juvenile, and additional segments are given.



## Discussion

It is known that metals in moss can move upward to new additional growth from current tissue, and also when supplied with an additional metal source from below (Brown and Bates 1990, Brown and Brown 1990, Brown and Buck 1978, Bates and Farmer 1990, Bates 1992, Brown and Sidhu 1992, Sidhu and Brown 1996, Skre and Oechel 1979) . The upward movement from current tissue is shown here to depend on the initial pools of potentially available metals, both in trials using moss collected at different sites (Figure 4), and by manually lowering contents by branch excision (Figure 5). The differences in branching patterns between sites did not seem to be related to the uptake of cations to additional growth.

K movement into additional growth was most favoured, and by the end of the experiment, the pre-growth pool of K was no longer correlated to post-growth juvenile plus mature segment contents. In the excision trials, post-growth K contents in juvenile plus mature segments were similar, even when all branches had been removed, suggesting that a major portion of the K pool is located in the moss stem, which is less available for uptake, compared with the K pool in branches.

The determination of uptake in terms of pools provided more information than the use of concentrations, since in this study, high heterogeneity of moss segment total weight existed between sites and between the excision treatments. A segment with a low total biomass, but with a high concentration of an element, can potentially supply to additional growth the same amount of the element as a segment with high biomass but with low concentration. In situations where the sampled moss weights are homogenous, cellular contents will likely relate to the concentrations.

Potassium was located in rather high amounts in the intercellular or cell-wall exchangeable locations at the beginning of the experiment, probably due to desiccation damage during storage and transport. However, during growth in the laboratory, the K was redistributed to a mostly intracellular location, indicating a non-stressed pattern. Relative to the pre-growth contents, K was most readily transferred upwards, followed by Mg, Ca and Zn. An increasing proportion of total metal was recovered in new tissue, compared to the pre-growth contents, according to the sequence  $Zn < Ca < Mg < K$ , which negatively correlates with affinity of metals for cell wall exchange sites (Brown and Wells 1988, Wells and Brown 1990). When an ion is displaced from the cell wall, unless it passes through the cell membrane, it enters the intercellular solution that is subject to upward movement with evapotranspiration or leaching with rainfall. Therefore, the more easily displaced ions are more capable of bi-directional vertical movement. Testing of the extent of movement (upward with evapotranspiration and downward with leaching) in field conditions is problematic, considering the lack of control over meteorological events and inputs (Brown & Brown, 1990), but seasonal patterns of cellular concentrations determined using the sequential elution technique may help to increase understanding. The residual component was not transferred upward into additional growth, indicating the likely particulate nature of this fraction.

In monitoring surveys, if total concentrations in moss are due mainly to exchangeable ions on the cell wall, and assuming 100% sorption and retention, but no vertical movement of metals, then the concentrations in a particular aged segment should be equal to age multiplied by the concentration in the first segment. In the available literature (Rühling & Tyler, 1970; Bengtson et al, 1982; Mäkinen, 1987b), this is never the case. This implies that the metals have been redistributed between segments or lost by leaching.

Brown and Bates (1990) and Bates (1992) outlined a dynamic equilibrium model of mineral nutrition, based on inputs from both deposition and substratum, losses from dead and damaged tissue and recycling from old to young tissue. The extent of upward movement of metals in natural conditions is, however, not known. Pb has higher affinity to the cell wall, and therefore is less mobile than Zn and Cd in the laboratory and in field conditions (Brown and Brown, 1990). The observed upward movement of metals in *Hylocomium splendens* is likely to be apoplastic, a wicking effect, driven by an imposed vertical water pressure gradient: a water source from below and evapotranspiration from above. This same type of gradient likely occurs in forests in dry periods after rainfall, where the upper A<sub>0</sub> horizon will remain moist for some time. Growth and uptake of mineral nutrients by excised *Rhytidiadelphus squarrosus* segments in the laboratory, when offered only a deionised water source from below, was dependent on the time interval of remoistening with deionised water (Wells and Brown 1996). More frequent watering resulted in less uptake, but more growth, which can be explained capillary downward flow, but upward movement to additional growth during conditions of decreased moisture is likely apoplastic via loss to the intercellular fraction from old tissue, and subsequent extracellular and intracellular recapture in the new growth.

The extensive work of Tamm (1953) suggests efficient scavenging of metals, supplied by precipitation or throughfall, by *Hylocomium splendens*. However, the concentrations of elements in moss have been sometimes observed to be too high to be explained solely by atmospheric deposition (Skre and Oechel, 1979), including Zn (Gjengedal and Steinnes 1990). Laboratory and field studies have shown that mosses can obtain metals from below, and that metals can be redistributed by internal recycling. New additional growth of *Rhytidiadelphus squarrosus* has been shown to accumulate Zn, Cu and Pb from existing tissue soaked in metal solutions for 10 min, although the uptake of Pb and Cu was low in short-term (3 week) experiments (Brown and Brown, 1990; Sidhu and Brown,

1996). *Pleurozium schreberi*, when supplied with Ca applied underneath the moss carpet, can utilise this supply for uptake (Bates and Farmer, 1990). Less mobile metals (Cu and Pb) show higher retention because of their greater affinity for cell wall exchange sites, and thus are less subject to vertical movement (Brown and Brown 1990).

The excision of branches below and above the juvenile segment, and the removal of all branches, still allowed subsequent growth. The removal of branches above the point of insertion of new growth resulted in lower amounts of elements in the new growth, due to diminished available resource pools. This reduction in metal supply was even more obvious when existing branches below the newly developing segment were eliminated. This may imply that movement toward the apices of more or less horizontally arranged segments is likely greater than movement towards the base. However, the dependence of the total amounts of metals in additional growth on the pre-growth available pools, suggests, rather, that the location of the existing pool of metals in branches is of little importance to uptake, at least in this experiment.

The preferred expansion of the juvenile segment, rather than further growth of the mature segment, after removal of mature branches may imply that the typical burst of expansion in the autumn is not directly due to a surplus supply of nutrients from rain and canopy leaching. Rainfall events after desiccation of moss can be particularly damaging in terms of nutrient loss (Brown & Buck, 1979; Brown and Bates, 1990). The typical expansion of *H. splendens* juvenile segments in late summer/autumn may be a response to losses due to leaching, and subsequent uptake from recycling and substratum. However, virtually nothing is known on factors which initiate branching and branch expansion on the already expanded versus juvenile segments.

## 6. THE MONITORING OF ZN DEPOSITION AROUND A METAL SMELTER IN LATVIA USING SERIAL ELUTION TECHNIQUE.

It has been claimed that serial elution technique to determine metal cellular concentrations can be used to provide results that can be interpreted without the speculation inherent in the use of total concentrations (Brown and Brown 1990). While serial elution has been shown to be useful in laboratory and field physiological studies of metal uptake (Bates and Farmer 1990, Brown and Bates 1990 ), it has not yet been applied in a monitoring survey. Before recommendation of the serial elution technique for practical monitoring projects, the application of the method must be tested. Therefore, the deposition of Zn along a transect from the metal smelter in Liepāja was surveyed, using cellular concentrations in *Hylocomium splendens*, estimated by serial elution technique. Moss collected in this area is known to have high concentrations of Zn, in comparison with the rest of Europe (Rühling et al 1992).

### 6.1 Methods

In late June 1993, three *Hylocomium splendens* samples were collected in each of nine dominant pine (*Pinus sylvestris*) forests in the east-south-eastern direction, at an angle to the prevailing wind direction (north-east and east). Previous surveys of total concentrations in moss (integrated samples of three annual segments) had shown high concentrations of Zn (>100µg/g), Pb (>30µg/g) and Cu(>10µg/g) in the area (Brūmelis and Nikodemus 1994). This area has been identified as a "hot spot" in Latvia, with regard to the high Zn concentrations, but nothing is known of the impact of these high total concentrations on organisms. Therefore, the area was chosen for study, to attempt to obtain information, using the serial elution technique, that could be better related to effects.

The moss samples were air dried, transported to Bristol, UK, and rejuvenated by spraying with deionised water and storing for 24 h by in trays lined with filter paper and fitted with lids. Immediately before elution, moss was separated into 4 annual segments (1990-1993). The youngest segment was a 1-2 cm-long bud that had developed in the previous autumn or current spring. Three replicates of three mature annual segments, and three replicates of ten developing buds, were serially eluted as described in Chapter 3.

## 6.2 Results

In the 1990-1992 *H. splendens* segments, total Zn concentrations decreased exponentially with distance from the emission source; the decrease was less obvious in the youngest, 1993 segments (Figure 6.1). The highest Zn concentrations occurred in the oldest segment. The highest increases of concentrations toward the source were observed in the extracellular exchangeable and intracellular fractions. In the young unexpanded segment, no clear trends with distance were apparent, except perhaps at the two closest sites for extracellular and intracellular concentrations (Figure 6.2). In the three fully expanded segments (1990-1992), extracellular exchangeable and intracellular Zn concentrations rapidly decreased between 4 and 6 km from the source, followed by a more gradual decrease to 28 km. The insoluble fraction showed a gradual decline in concentrations to 8.5 km from the source, which was less apparent in the younger segments. The water soluble intercellular zinc concentrations were small, compared to the other fractions, but concentrations were higher close to the source.

The proportions of intracellular Zn at the sites are highest (35% - 65% of the total Zn) in the young 1993 segment (Figure 6.2), but did not appear to be related to distance from the Zn source. The cell wall dry weights and cytoplasm to cell wall weight ratios were also not related to distance from the source (Table 6.1), but the



cytoplasm weight was higher, in relation to cell wall weight, in the younger segment.

The cellular Ca and Mg concentrations in moss at the sites did not appear to be related to the distance from the source of the Zn contamination (Figures 6.3 and 6.4). The relation of the concentrations to overstorey vegetation (forest type) was also investigated, but no effect was found. While total concentrations of Ca and Mg varied between sites, the proportions of intra- versus extracellular exchangeable Ca and Mg concentrations were similar. As expected, the greater portion of Ca was located on cell wall sites, and a higher intracellular proportion in the 1993 segment is likely explained by immature cell walls. Mg was distributed more evenly between intra- and extracellular locations, with higher intracellular concentrations in the 1993 segment (bud).

Table 6.1. Mean cell wall dry weights of mature annual segments and the ratio's of cytoplasm/cell wall weights of *Hylocomium splendens*, collected along a transect from a metal smelter in Liepāja, Latvia. Distances to the pollution source are given.

Distance (km)	Cell wall dry weight (mg)			Ratio cytoplasm/cell wall weight			
	1992	1991	1990	1993	1992	1991	1990
4	16.3	14.9	14.8	0.24	0.14	0.16	0.13
5	15.7	14.4	11.5	0.33	0.14	0.11	0.12
6.5	21.8	18.3	14.8	0.26	0.21	0.16	0.11
8.5	18.9	14.4	11.7	0.22	0.16	0.13	0.11
15	18.9	20.0	19.6	0.19	0.13	0.10	0.11
16.5	24.3	28.9	18.8	0.19	0.19	0.14	0.14
22	15.8	18.6	17.6	0.24	0.18	0.14	0.13
25	20.1	16.4	16.4	0.23	0.16	0.13	0.12
28	15.4	11.1	10.4	0.23	0.17	0.16	0.12

Figure 6.1. Total Zn concentrations ( $\mu\text{g.g}$ ) in *Hylocomium splendens* collected along a transect from a metal smelter in Liepāja, Latvia. Concentrations are given for four annual segments, beginning with the young unexpanded bud (1993 segment).

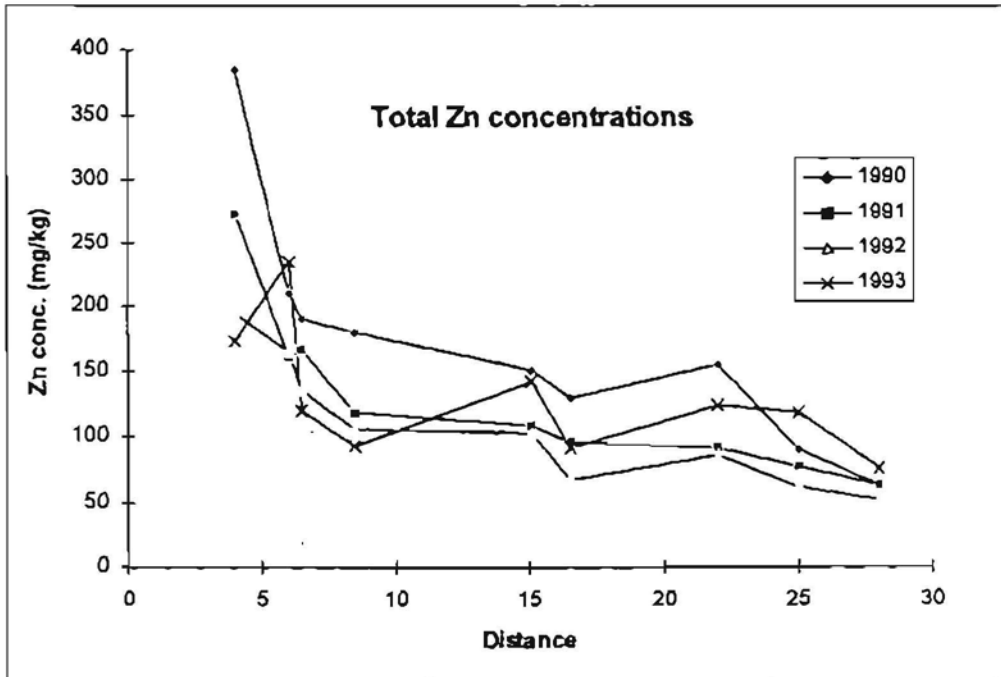


Figure 6.2. Cellular Zn concentrations ( $\mu\text{g/g}$ ) in *Hylocomium splendens* collected along a transect from a metal smelter in Liepāja, Latvia. Concentrations are given for four annual segments, beginning with the young unexpanded bud (1993 segment).

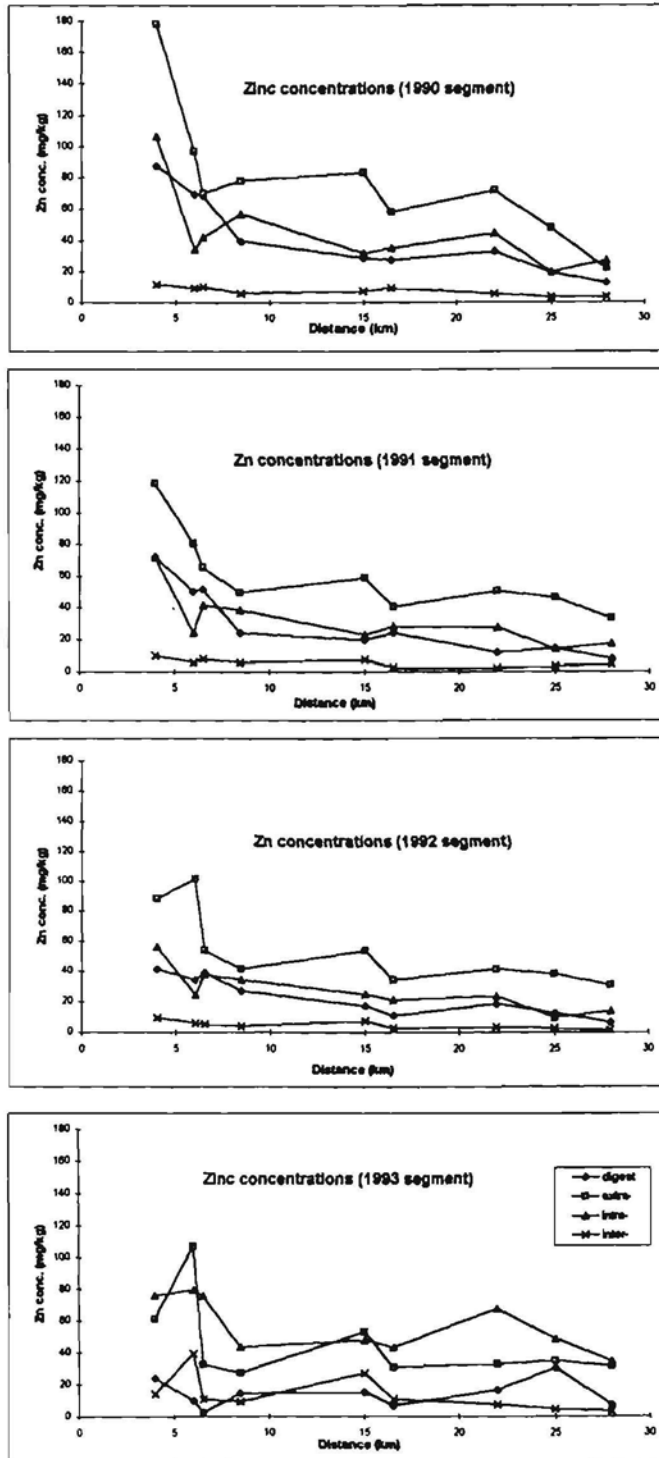


Figure 6.3. Cellular Ca concentrations ( $\mu\text{g/g}$ ) in *Hylocomium splendens* collected along a transect from a metal smelter in Liepāja, Latvia. Concentrations are given for four annual segments, beginning with the young unexpanded bud (1993 segment).

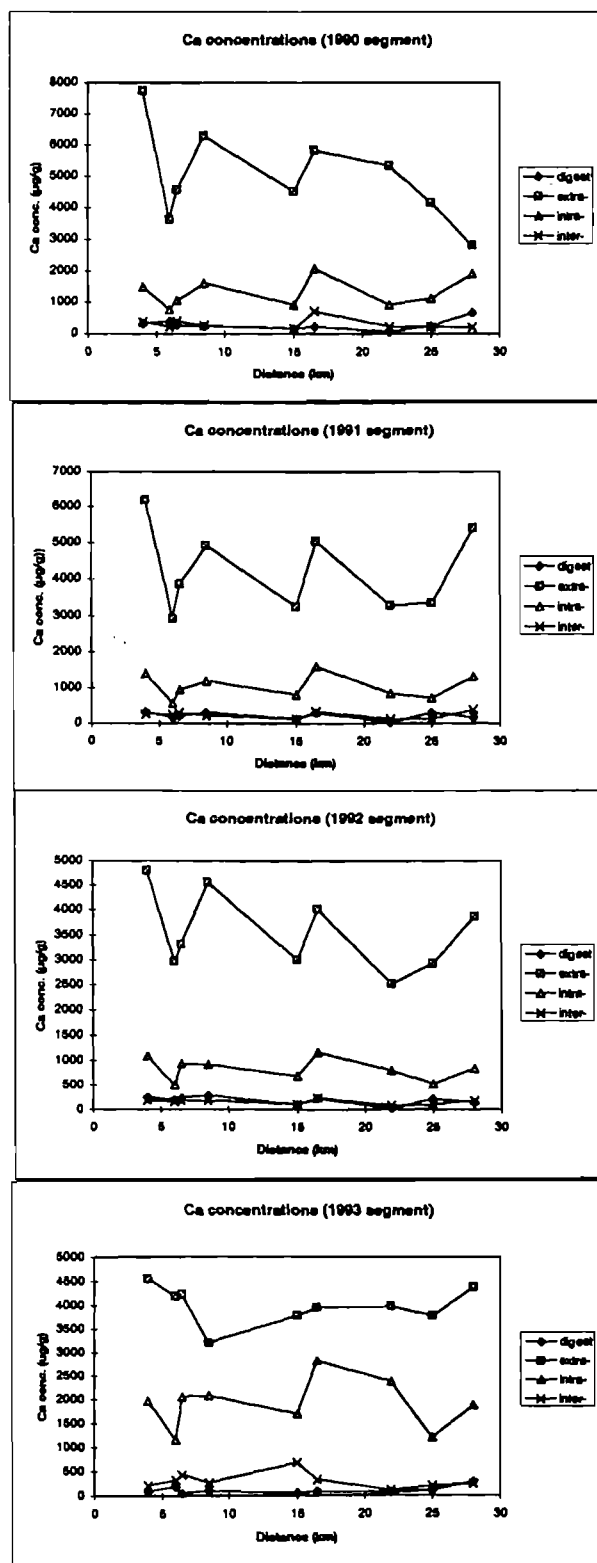
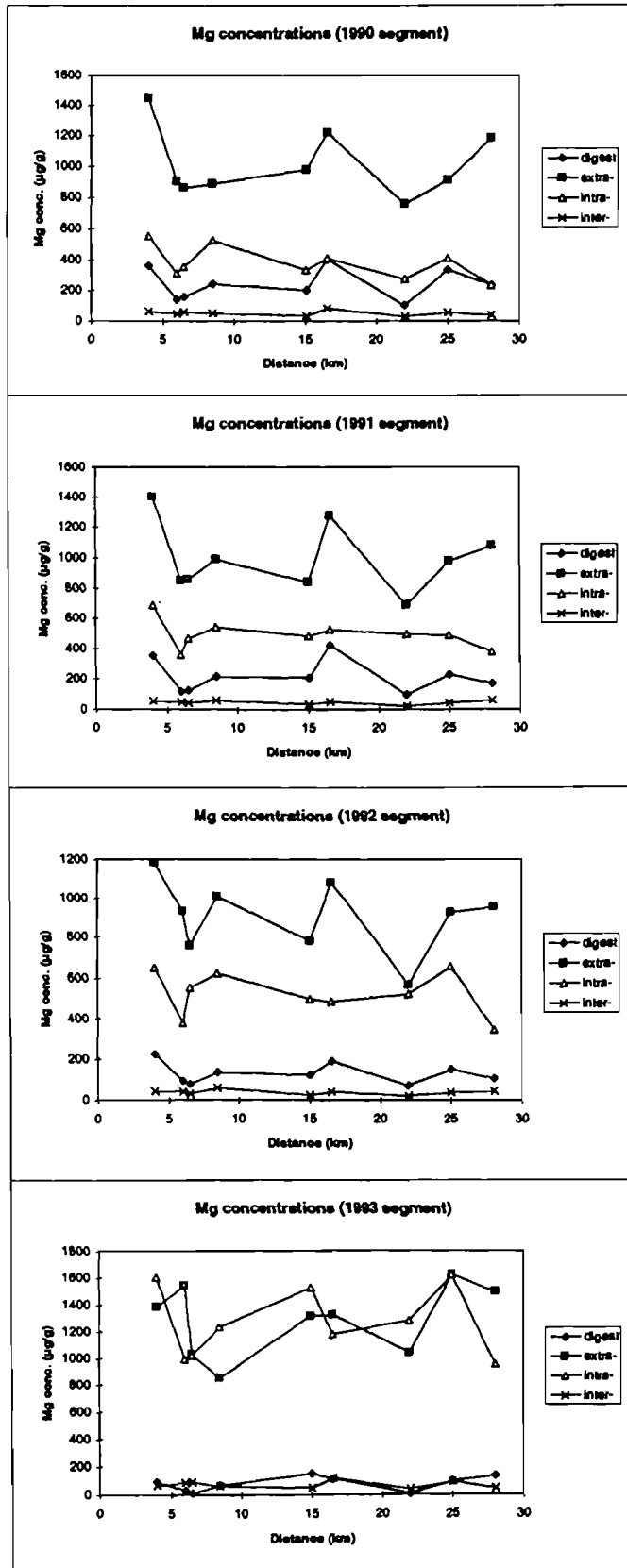


Figure 6.4. Cellular Mg concentrations ( $\mu\text{g/g}$ ) in *Hylocomium splendens* collected along a transect from a metal smelter in Liepāja, Latvia. Concentrations are given for four annual segments, beginning with the young unexpanded bud (1993 segment).



## Discussion

Mathematical models of particle deposition from point sources predict an exponential decrease with distance (Gutkovs and Viržbickis 1993). The predicted exponential decrease is supported by study of deposition, as measured by concentrations of elements in a bioindicator organism (Folkeson 1981, Mäkinen 1987b, Heliövaara and Väisänen 1989). Around a pollution source, the elemental composition of organisms can thus be linked to the fallout from the smoke stack plume. The extracellular and intracellular Zn concentrations in *Hylocomium splendens* segments follow the expected pattern (Figure 5.2), especially the older segments, which have obviously received higher total amounts of deposited material from the atmosphere. However, the past history of losses from the segments is unknown, and the older segments can potentially obtain inputs from younger segments by leaching. Evidently, most of the soluble Zn aerosols are deposited close the emission stack, but the Zn containing insoluble fraction showed a less rapid decrease with distance. If the relatively insoluble Zn fraction is due to heavier particles, that may be expected to be deposited closer to the source, then this zone of deposition may correspond to the 0-4 km zone where moss was not found. The data may also indicate low efficiency of trapping and retention of particles by the moss, leading to losses of the increased loads of particles near the source. However, the relative stability of proportions of Zn in the various fractions between sites suggests a homogenous nature of deposition.

Mathematical models of plume fallout cannot incorporate the interactions between particles; for example, the aggregation of small particles into larger particles. Also, the interactions between aerial dispersed particles and meteorological factors is likely dynamic, and simple models are likely to be ineffective in portraying deposition patterns. Little is known of the role of particles in total concentrations, the extent to which they release metals, efficiency of their entrapment and rates of their loss in the environment. However, this work indicates that the insoluble

particle fraction is a major component (up to one third) of total concentrations in moss. A significant portion of the particles may be soil derived, especially close to the smelter, due to more erosion and dispersal of wind blown dust in the immediate urban environment. The significance of this factor may be determined by the proportions of metals due to soil contamination by soil enrichment ratio's relative to elements naturally high in soil (Bargagli et al 1995), or by electron microscopy, in combination with X-ray emission spectrography (Johnsen 1981, Mäkinen 1987a), to estimate the amounts of elements in individual soil particles, with classification of particles by source.

Ca and Mg deposition does not appear to be dependent on distance to the Zn pollutant source, except perhaps at the closest site (Figures 5.3 and 5.4), but this may be masked by other factors, such as leaching, competition between ions and high background inputs. Close to major cities, with the combustion of fossil fuel, Ca and Mg concentrations in deposition are expected to be higher (Nikodemus et al 1993). Competition of Ca and Mg with other deposited ions at extracellular exchange sites is likely greater close to the source, but the existing molar concentrations of Zn alone were too low, in comparison to Ca and Mg, for this to be a major factor near Liepāja. The Ca, Mg and also Zn, concentrations along the transect are undoubtedly affected by natural factors, such as tree canopy, soil conditions, past desiccation stress, and meteorological conditions, and also anthropogenic inputs. Canopy throughfall is the major source of Ca, Mg and Zn deposition in natural conditions, and the chemistry of throughfall depends on canopy vegetation (Bergkvist et al 1989, Helmisaari and Mälkönen 1989). The Mg, Ca and Zn concentrations along the transect did not appear to be related to the canopy type (not shown). Likely, the classification by forest types used to define canopy vegetation here, is a poor reflection of the throughfall chemistry, along with the above other factors that can affect concentrations.

The growth rate of moss did not appear to be affected by the pollution source, and the variability in cell wall dry weight along the transect (Table 1) from the metal



refinery, is not paralleled by changes in concentrations of Zn, or also Ca and Mg, except perhaps the high Ca and Mg concentrations in the site located 16.5 km from the source, which also had the highest moss dry weight productivity. Thus, the higher Zn concentrations near the source, and the local maxima of Ca and Mg concentrations, cannot be explained by lower productivity.

In a study on the effects of a brass metal foundry in Sweden, total Zn and Cu concentrations of 160 and 700 mg/kg in *Hylocomium splendens*, respectively, were correlated with a 54% reduction in growth (Bengtson et al, 1982), and an obvious reduction in cover occurred with total concentrations of 80 µg/g Cu and 240 µg/g Zn (Folkeson and Andersson-Bringmark, 1988). However, the correlation of total concentrations to impact has little ecological interpretative value, since knowledge of metal availability and the effects of existing metal combinations are lacking. Thus, regional studies of pollution, that are based on multifactor analysis of total concentrations versus effects on organisms, for example tree growth, will fail to prove direct effects, but may be of indicative value. For example, a high V concentration will indicate the combustion of oil, and the associated other pollutants (SO<sub>2</sub>).

The physiological effects on moss species are best related to intracellular metal concentrations (Brown and Sidhu 1993; Sidhu and Brown, 1996, Wells and Brown 1995). Thus, a high intracellular concentration can be a direct indication of toxicity. The highest intracellular concentrations in *Hylocomium splendens* around the Liepāja smelter (about 120 µg/g) have been observed experimentally to cause a slight reduction in growth when applied alone, depending on whether Zn was applied by pulse incubation or to cut bases (Sidhu and Brown 1996). However, when Zn was applied in combination with other metals, the effects were either antagonistic (low concentrations) or synergistic (high concentrations). A valid assessment of toxic effects of a metal, based on intracellular concentrations, requires knowledge of the existing concentrations of other metals. Therefore, when surveys based on total concentrations in moss have identified an area of concern,

the next logical step in environmental assessment would be to determine the intracellular concentrations, together with experimental study of toxic effects of the combined action of the metals in the range of existing concentrations in the field.

The higher proportion of intracellular Zn in the young 1993 segments (Figure 5.2) may be explained by immature cell walls in the new segment. Intracellular concentrations based on cytoplasm weight have been recommended, since this removes the bias of cell wall maturation with age (Wells and Brown 1987). Some of the collected *H. splendens* samples were relatively brittle, causing loss of material during elution, particularly after drying. The source of the lost material, damaged tissue or associated debris, is not known. This introduced an error in the estimation of cytoplasmic weight, and therefore the concentrations are expressed based on cell wall, which were minimally affected by the losses.

The serial elution method, used here in a monitoring study, has been able to extract more information than the traditional total concentrations in moss method. Disadvantages of the method are, many solutions to analyse, a necessity for relatively fresh moss and too low concentrations for some elements. Soluble particles on the surface of moss can increase concentrations in all fractions. The soluble particle component in the intercellular fraction can be regarded as existing in the free intercellular space, and does not bias interpretation. However, the presence of soluble particles can potentially result in the overestimation of extra- and intracellular components, especially after dry periods of deposition. The potential error due to soluble particles may be minimal in this survey, since samples were collected during a rain event. After a period of dry deposition, with subsequent rainfall, an unknown amount of metal as soluble particles will be retained in moss by extra- or intracellular uptake. Therefore, knowledge of the solubility of particles, and the seasonal patterns of deposition and cellular concentration, would aid interpretation. Nevertheless, the ability to differentiate between metals in the various cellular components and to detect insoluble particles allows one to better

understand the environmental significance of results, and, in the future, to better budget the inputs and outputs to moss carpets.

## 7. THE EFFECT OF A LIME PRODUCTION PLANT, SAULKALNE, LATVIA, ON CONCENTRATIONS OF CA, MG, K AND ZN IN *HYLOCOMIUM SPLENDENS* AND IN THE UPPER ORGANIC HORIZON.

The metals nutrients, Ca, Mg and K are often avoided in monitoring surveys of deposition. The natural inputs of these elements are typically high, which may muffle the anthropogenic loads deposited on moss. Budgets of inputs and outputs to the forest floor in *Pinus sylvestris* forests, show that the major source of K, Mg, Ca (Helmisaari and Mälkönen 1989) and Zn (Bergkvist et al 1989) is canopy throughfall. The use of the serial elution technique was tested in a survey of Ca, Mg, K and Zn concentrations in *Hylocomium splendens* around a lime production factory, to determine if additional information can be obtained, above that provided by total concentrations. Since the emissions around the factory were suspected to be soluble aerosols (lime dust), some particle types were also serially eluted to determine their potential contribution to the fractions.

The organic A<sub>o</sub> horizon, which represents decomposed moss among other sources, initially receives all losses of particles and soluble ions from the above moss carpet. If the metals are retained somewhat efficiently, the A<sub>o</sub> horizon may be useful for assessing the losses that occur from moss, perhaps long-term deposition patterns, and also the potentially available mineral nutrient pool available to moss by upward movement. To investigate the practical use of the A<sub>o</sub> horizon in monitoring, the decomposed material under the moss carpet was also sampled and eluted.

### 7.1. Methods

On 17 October, 1994, *Hylocomium splendens* moss and the underlying A<sub>o</sub> horizon were sampled in 5 sites along a transect from the Saulkalne Lime Factory. Site 1 (1 km from the source) and site 2 (1.5 km) were urban forests grown on abandoned agricultural land, evident from a plough layer. Sites 3 (3.8 km) and 4 (4.4 km) were

dry pine forests with a sparse spruce understorey, and site 5 (4 km) was a drained pine swamp.

Three replicates of two segments (1992-1994), from each site, were serially eluted, after transport to the laboratory, and moist storage for 24 h (Chapter 3). The 1994 mature segment had, for the most part, developed buds that would expand in the subsequent year. The 1992 segments in Sites 1 and 3 were visibly deteriorated with much loss of leaves and branches.

Ao horizon samples (four replicates) were collected under the moss carpet, transported to the laboratory and air dried. 0.5 g samples were eluted (not serially) in 15 ml solutions as follows:

1. 70 minutes shaking in distilled water, filtered, retain solution,
2. 70 minutes shaking in 20 mM NiCl<sub>2</sub>, filtered, retain solution,
3. 60 minutes shaking in 1 M HNO<sub>3</sub>, filtered, retain solution,
4. boiling in concentrated HNO<sub>3</sub>.

Filtrak 89, GDR, filters were used.

Since each retained solution from elution of the Ao material represented a cumulative concentration of the previous fractions, concentrations for each serial elution for comparison with moss were estimated by subtraction from the concentration in the previous elution.

To attempt to discover the impact of various particles on the concentrations in moss and humus, 0.5 g clay and 0.5 g sand, collected from an urban environment in a small city, Ikšķile, and 0.005 g commercial lime produced at Saulkalne, were also eluted in 15 ml solutions as for humus, but centrifuged at 5000 rpm for 5 min after each elution instead of filtering.

## 7.2. Results and Discussion

### 7.2.1. Elution of particles

The elution of clay and sand particles with concentrated HNO<sub>3</sub> resulted in lower Ca concentrations than those eluted with 1M HNO<sub>3</sub>, hence the negative values in Table

7.1. Tests using various concentrations of  $\text{HNO}_3$  showed that maximum displacement of Ca occurred with 25% or 50%  $\text{HNO}_3$ , and least with concentrated  $\text{HNO}_3$ . The loss of Ca may be explained by complexation in the mineral soil lattice structure, or the increased release of anions with acidity, which react with Ca to form insoluble compounds. These results clearly demonstrate that concentrated  $\text{HNO}_3$  will highly underestimate the Ca existing as sand or clay particles attached on the moss surface. Also, the use of  $\text{HNO}_3$  to estimate intracellular concentrations should be re-examined in conditions of varying anion concentrations and/or mineral soil particles. Nevertheless, the extensive previous experience with the use of the serial elution technique in the laboratory has shown the suitability of 1M  $\text{HNO}_3$  for estimating Ca intracellular concentrations. In further discussion, the Ca concentrations in the concentrated  $\text{HNO}_3$  elution are excluded. This problem of loss of a cation by elution with concentrated  $\text{HNO}_3$  was not observed for the other elements studied.

A portion of the Ca and Mg in lime was immediately soluble in distilled water and  $\text{Ni}^{2+}$  solution, but the bulk of Ca and Mg in lime was soluble only in 1M  $\text{HNO}_3$  (Table 7.1). Undoubtedly, in field conditions, where leaching during rain events is more intense, a greater proportion will be soluble in the rain water solution. Certainly, the practical use of clay in construction requires soaking for at least 1 day to produce a suitable slurry for use, and likely the 70 min elutions cannot portray the potential availability of a metal. The K and Zn in lime is less soluble, and a larger proportion is eluted only in concentrated  $\text{HNO}_3$ .

The high proportion of Mg, and especially K, in the concentrated  $\text{HNO}_3$  elution of clay (Table 7.1), suggest that increases of both elements in this fraction during elution of moss or humus, can suggest the presence of greater amounts of clay particles attached to the moss surface. The same is true, to a lesser extent, for sand particles.

Table 7.1. Ca, Mg, K and Zn concentrations ( $\mu\text{moles/g}$ ) in eluted clay, sand and lime, estimated as serial elutions by subtraction from the previous elution. Negative values in concentrated  $\text{HNO}_3$  elutions represent losses due to interaction to form insoluble substances.

Particle	elution	Metal concentrations ( $\mu\text{moles/g}$ )			
		Ca	Mg	K	Zn
Clay	Distilled water	0.2	1.0	1.7	.01
	20 mM $\text{NiCl}_2$	37.9	11.0	0.9	.02
	1M $\text{HNO}_3$	1.4	17.8	2.8	.15
	conc. $\text{HNO}_3$	-25.0	76.2	40.4	.41
Sand	Distilled water	1.0	.7	2.2	.01
	20 mM $\text{NiCl}_2$	28.4	4.8	3.1	.02
	1M $\text{HNO}_3$	14.0	25.3	4.7	.11
	conc. $\text{HNO}_3$	-24.3	25.1	6.1	.16
Lime	Distilled water	93.1	152.8	6.0	nd
	20 mM $\text{NiCl}_2$	272.6	76.3	3.0	nd
	1M $\text{HNO}_3$	5359	2785.8	17.3	0.17
	conc. $\text{HNO}_3$	8.3	15.4	20.1	0.08

### 7.2.2. Cellular concentrations of Ca, Mg, K and Zn in *Hylocomium splendens*

Inter-, extra- and intracellular Ca concentrations increased with segment age in all sites (Table 7.2), excepting intracellular concentrations at the site closest to the pollution source (site 1). The larger proportion of Ca is located on cell wall exchange sites. The intracellular Ca concentrations in the youngest segments (1993 and 1994) were also increased at the closest site. Moss from the site closest to lime factory had the highest inter- and extracellular concentrations, suggesting the presence of easily dissolvable Ca aerosols. In light of the concentrations of eluted lime, it is somewhat surprising, that the intracellular concentration of the oldest moss segment (1992) collected near the source, is in the range of those at other sites, since the bulk of Ca in lime was eluted only in 1M HNO<sub>3</sub>. Possible explanations are: the laboratory elution does not represent the more intense leaching in field conditions, the particles are readily leached, a more soluble Ca aerosol is emitted than commercial lime dust, or an artifact due to reaction with anions in an acidic environment as discussed above. The first two seem to be most likely, but knowledge of the real emitted particles, rather than the suspected, would be required. This could be investigated by entrapment of particles on an inert source, and identification by comparison with known particles using microscopy.

The Mg concentrations in moss are more evenly proportioned between the intra- and extracellular locations, and the proportion of intracellularly located Mg is highest in the youngest segments. Intracellular Mg concentrations, based on cell wall weights, are related to the ratio of cytoplasm to cell wall weights, and cell membrane integrity. The oldest segments in Site 3 were brown with disintegration of frond structure, strongly suggesting membrane damage, which can explain their low intracellular Mg concentrations. The oldest segment in Site 1 was similarly damaged, in appearance, but the intracellular Mg concentrations were similar to those of the youngest segment. Since the low intracellular K concentrations in the oldest segments in Sites 1 and 3, do indicate membrane damage at both sites (Table 7.3), Mg particles likely cause the higher intracellular concentrations in Site 1.



Table 7.2. Cellular Ca and Mg concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens* collected along a transect from the Saulkalne Lime Production factory. Distance to source is given. nd - not detectable.

		Cellular concentrations ( $\mu\text{moles/g}$ )						
			Site					
	Cellular location	Segment	1	2	3	4	5	
Ca	intercellular	1994	4.3	1.9	1.8	nd	1.0	
		1993	4.8	1.9	1.6	.9	1.1	
		1992	9.6	2.6	4.1	1.8	3.4	
	extracellular	1994	80.9	60.3	49.2	65.6	41.1	
		1993	123.2	58.2	50.7	94.9	41.8	
		1992	186.7	86.4	104.0	121.7	70.0	
	intracellular	1994	25.3	8.2	8.8	15.1	11.6	
		1993	26.5	8.2	8.8	15.1	11.6	
		1992	21.6	19.2	16.1	24.3	12.6	
Mg	intercellular	1994	4.1	nd	nd	nd	nd	
		1993	4.4	nd	nd	nd	nd	
		1992	6.3	nd	.7	.4	.8	
	extracellular	1994	49.5	35.6	33.5	32.7	21.4	
		1993	50.5	21.6	16.2	26.6	9.8	
		1992	65.4	25.7	30.1	44.8	18.9	
	intracellular	1994	27.4	30.1	26.1	21.4	19.5	
		1993	14.5	20.7	14.7	16.4	19.1	
		1992	30.1	22.8	8.9	18.7	12.8	
	insoluble	1994	1.9	1.6	3.0	2.3	.8	
		1993	4.5	5.0	3.8	3.2	1.5	
		1992	7.5	7.4	3.3	4.5	2.7	
	distance from source (km)			1.0	1.4	3.4	4.4	4.0

The patterns of inter- and extracellular Mg concentrations between sites are similar to Ca concentrations, which substantiates the idea of deposition of lime dust near the source. Intracellular Mg concentrations were increased in Site 1 only in the oldest 1992 segment. The Mg concentrations in the insoluble fraction were also highest in Site 1, especially in the oldest segments. Since at this site, the oldest segments were closest to, and often part of, the thin humus layer above mineral soil, sand and clay particles may explain the insoluble Mg concentrations.

As expected, most of the K was found intracellularly, and the relative intracellular concentrations decrease with segment age and health (Table 7.3), following the pattern described above for Mg. Among sites, the greatest proportion of intracellular K, for all segments, is found in the swamp site, where the moss appears the healthiest (greenest). However, the K concentrations at the swamp site were the lowest, explained by less inputs from canopy leaching or recycling from below.

With membrane damage, K is lost from the predominant intracellular location, and enters the intercellular space or becomes bound to the cell wall. At site 4, a dry pine forest, the intercellular and cell wall K concentrations increased with segment age, suggesting an increase of damage with age; this was not evident at the adjacent Site 5 (pine swamp). At Sites 1 and 3, where the oldest segments were in the last year of showing some evidence of structure, cell wall K concentrations did not increase with segment age, perhaps due to increased competition with divalent cations, such as Ca and Mg, near the lime factory. Higher insoluble K concentrations at the closest site suggest also the presence of clay particles, as observed for Mg.

Table 7.3. Cellular K and Zn concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens* collected along a transect from the Saulkalne Lime Production factory. Distance to pollution source is given. nd - not detectable.

		Cellular concentrations ( $\mu\text{moles/g}$ )						
			Site					
	Cellular location	Segment	1	2	3	4	5	
K	intercellular	1994	5.2	4.5	4.6	7.0	5.4	
		1993	8.1	3.1	6.6	6.4	1.8	
		1992	12.7	4.6	7.6	15.4	6.0	
	extracellular	1994	4.3	6.4	4.6	8.1	3.2	
		1993	3.9	4.8	3.8	7.8	2.5	
		1992	2.9	4.7	2.4	14.5	2.9	
	intracellular	1994	205.8	194.5	156.5	182.0	128.8	
		1993	147.8	120.0	94.5	106.1	86.2	
		1992	64.2	177.1	48.3	113.7	62.2	
	insoluble	1994	1.2	1.6	1.5	1.8	.8	
		1993	3.2	1.9	1.6	1.0	1.1	
		1992	4.5	2.3	2.2	1.7	1.3	
	Zn	intercellular	1994	nd	nd	nd	nd	nd
			1993	nd	nd	nd	nd	nd
			1992	nd	nd	nd	nd	nd
extracellular		1994	0.86	0.72	0.68	0.44	0.52	
		1993	0.77	0.54	0.57	0.71	0.53	
		1992	0.80	0.79	0.75	0.91	0.69	
intracellular		1994	0.38	0.25	0.32	0.45	0.26	
		1993	0.34	0.06	0.23	0.28	0.24	
		1992	0.38	0.15	0.39	0.47	0.29	
insoluble		1994	nd	nd	0.08	nd	nd	
		1993	0.06	0.06	0.06	nd	nd	
		1992	0.11	nd	0.12	nd	0.07	
distance from source (km)			1.0	1.4	3.4	4.4	4.0	

Cell wall and intracellular Zn concentrations did not much vary between sites (Table 7.3), except that concentrations were lower in the pine swamp (Site 5). Excepting Site 1 and 2 (closest to the source), Zn extracellular concentrations increased with segment age, especially in Site 4 (dry pine forest). Possibly, increased competition with Ca and Mg at Site 1 and 2 has led to increased loss of Zn, especially in the older segments. Intracellular Zn concentrations showed no clear relation with distance from the lime plant or with segment age. The insoluble Zn concentrations were low (often below detection limit), and slightly elevated in Site 1 and 3, but never represented more than 10% of total concentrations. Evidently, the Saulkalne Lime Plant is not a source of Zn, but emissions may affect the Zn concentrations in moss by competition. Zn concentrations in lime, sand and clay particles were low, and the dominant input is likely canopy throughfall (Bergkvist et al 1989). Total Zn concentrations in moss are often greater than can be explained by inputs from precipitation, and uptake by recycling through canopy leaching has been suggested (Gjengedal and Steinnes 1990).

### 7.2.3. Ca, Mg, K and Zn concentrations in the upper organic layer $A_0$ .

The distilled water displaced Ca concentrations were lowest, but showed a clear trend in decreasing concentrations with distance from source (Table 7.4). The 1M  $HNO_3$  concentrations were over 50 times higher at the closest 2 sites (Sites 1 and 2), compared with the distilled water displaced concentrations, but they decreased much more rapidly with distance. Ni displaced Ca concentrations in the  $A_0$  horizon showed no clear trend with distance. The bulk of Ca in the  $A_0$  horizon was Ni displaceable in Sites 3 to 5, but the portion 1M  $HNO_3$  extractable Ca dominated closer to the lime factory. Little is known of the efficiency that Ni will release Ca from exchange sites on decomposing plant material. It is, however, clear that the major proportion of the higher Ca amounts deposited close to the source, and that accumulates in the upper organic horizon, is only displaced by 1M  $HNO_3$ . This accumulation of relatively insoluble Ca, is suggestive of lime dust, for which the bulk of Ca was displaced only by 1M  $HNO_3$ . This reservoir of Ca in the  $A_0$  horizon may provide a pool of Ca for upward movement to moss plants.

A loss of Ca occurred in the concentrated HNO<sub>3</sub> extracts (not shown), for the reasons discussed above.

The Mg concentrations in the distilled water and Ni fractions decreased gradually with distance from the source, being over 20 times higher in the Ni fraction than in the distilled water fraction (Table 7.4). However the Mg concentrations in the 1M and concentrated HNO<sub>3</sub> fractions were much higher only in Site 1, with no clear trend with distance further from the source. This may indicate the accumulation of Mg containing particles close to the lime factory, which are not easily dissolved, but further dispersal of more soluble Mg deposition.

The K concentrations in distilled water and Ni fractions show no clear relation to distance from source (Table 7.4). This may suggest lack of K emissions, but K is a monovalent cation that is easily displaced and leached. A significant portion of K in the 1M HNO<sub>3</sub> fraction may represent intracellular K of bacteria and fungi. Therefore, it is interesting that the highest 1M HNO<sub>3</sub> displaceable K in the A<sub>0</sub> horizon was found at the furthest less polluted sites.

The Ni displaceable Zn concentrations clearly increase with distance from the lime production factory, strongly suggesting leaching caused by competition with the pollutant ions (Ca and Mg) near the source, supporting the argument that extracellular Zn in moss was lost by leaching (see above). Between the less polluted dry forest and swamp sites (Sites 4 and 5), Zn concentrations are highest in the swamp forest, which had the lowest Ca and Mg concentrations. 1M HNO<sub>3</sub> displaced Zn concentrations showed no clear trend with distance, but Zn displaced by concentrated HNO<sub>3</sub> is much higher close to the factory, perhaps due to clay, sand or other particles.

Table 7.4. Ca, Mg, K and Zn concentrations ( $\mu\text{moles/g}$ ) in the organic soil layer, collected under moss turf., along a transect for the Saulkalne Lime Production Factory. The concentrations were measured in elutions of the organic material, estimated as serial elutions by subtraction from the previous elution.

		Concentrations ( $\mu\text{moles/g}$ ) in soil organic layer				
		Site				
Elution		1	2	3	4	5
Ca	Distilled water	2.1	1.3	.6	.5	.5
	20 mM $\text{NiCl}_2$	117	100	106	126	97
	1M $\text{HNO}_3$	186	89	.7	2.7	8.9
Mg	Distilled water	2.7	1.8	.5	.7	.7
	20 mM $\text{NiCl}_2$	66	44	18	27	24
	1M $\text{HNO}_3$	58	1.4	3.2	6.8	17.5
	conc. $\text{HNO}_3$	50	4.7	7.0	9.0	3.0
K	Distilled water	4.7	4.8	2.8	5.2	3.7
	20 mM $\text{NiCl}_2$	4.0	1.7	1.9	2.2	3.2
	1M $\text{HNO}_3$	2.7	.4	2.0	2.1	4.8
	conc. $\text{HNO}_3$	14.2	10.7	5.1	10.5	2.5
Zn	Distilled water	nd	nd	nd	nd	nd
	20 mM $\text{NiCl}_2$	.14	.24	.44	.47	.68
	1M $\text{HNO}_3$	.60	.40	.43	.31	.53
	conc. $\text{HNO}_3$	.74	.26	.14	.18	.01
Distance to source		1.0	1.5	3.8	4.4	4

### 7. 3. General Discussion

The patterns of Ca and Mg concentrations in the Ni and 1M HNO<sub>3</sub> fractions differ between moss and the A<sub>o</sub> horizon, with much higher proportions of these elements being in the 1M HNO<sub>3</sub> fraction in the A<sub>o</sub> horizon. Extracellular exchangeable concentrations of Mg and Ca in moss were elevated only in the site closest to the lime factory (Site 1). Ni displaced Mg concentrations in the A<sub>o</sub> horizon were increased in both Sites 1 and 2, but the respective Ca concentrations did not appear to be related to distance from the source. This suggests increased loss of exchangeable Ca in the A<sub>o</sub> horizon by leaching, or, the formation of rather insoluble Ca compounds (i.e. such as the reaction of lime and sand to form plaster).

In the A<sub>o</sub> horizon, the Mg concentrations in the 1M HNO<sub>3</sub> fraction were increased (highly) only in Site 1, but the respective Ca concentrations were increased in both Sites 1 and 2, suggesting that the portion of the Ca aerosols that are less soluble, are dispersed further than the Mg load, or that the Ca deposition accumulates more readily in the A<sub>o</sub> horizon as rather insoluble compounds. The Ca and Mg intracellular concentrations in moss were only slightly elevated near the source. However, the Ca and Mg concentrations in the 1M HNO<sub>3</sub> elution of the organic horizon were over 20 and 10 times higher, respectively, in Site 1, compared with sites 3-5. Two models of deposition and retention can be proposed to explain these results:

- 1) Moss, compared to the soil organic horizon, is inefficient at trapping and retaining the deposition at Saulkalne, which is suggested to consist mostly of relatively insoluble (dissolves in 1M HNO<sub>3</sub>) lime dust particles.

- 2) deposition occurs as soluble Ca and Mg aerosols on the surface of moss, which is readily leached to the A<sub>o</sub> horizon, where it forms rather insoluble substances.

Investigation of seasonal patterns can be used to test the two models. In dry periods, which favour accumulation of dust, if the first model is true, accumulation of Ca and Mg in the 1M HNO<sub>3</sub> fraction is expected, which is rapidly lost with rain.

Closer to the lime factory, the exchangeable Zn concentrations in the  $A_0$  fraction were decreased, likely due to competition with Ca and Mg particles, but the insoluble Zn (also K and Mg) concentrations in the concentrated  $HNO_3$  fraction are likely due to soil particles. Although exchangeable Ca concentrations in the  $A_0$  horizon were not elevated near the source, the very high Ca concentrations in the 1M  $HNO_3$  fraction may be a long-term source of exchangeable Ca. Total Zn concentrations will indicate high Zn concentrations, at least in the  $A_0$  horizon, around the lime factory, but this will not be a reflection of the deposition patterns and the interactions with other cations, which can be obtained using elution technique.

In a study around a fertiliser plant and strip mine in Finland (Tynnyrinen et al 1992), correlations between K, C, Mg, P and F concentrations in lichens, between these elements and distance to the factory, and between the concentrations in lichens and airborne dust, were used to identify the particle source. With obtained data at Saulkalne, the concentrations seem to imply more than one source, and varying rates of accumulation and loss in moss and the  $A_0$  horizon. The Mg/K/Zn ratios in the 1M  $HNO_3$  fraction of eluted decomposed material, at the closest site indicate much lower relative K levels than expected from clay or sand particles (Table 7.5), but are closer to commercial lime, suggesting accumulation of lime dust. On the other hand, the above ratio in the concentrated  $HNO_3$  fraction is more suggestive of sand or clay. Rather, both explanations are likely true, which was also suggested by the moss cellular concentrations. At the closest site, high Ca and Mg concentrations (but not K) in the 1M  $HNO_3$  fraction of moss and the  $A_0$  horizon suggest lime dust, but high Mg and K concentrations in concentrated acid extraction suggest clay or sand.



Table 7.5. Ratio's of estimated Mg / K / Zn concentrations in serial elutions of clay sand and lime particles, as well as organic soil horizon samples collected along a transect from the Saulkalne Lime Production Factory.

Elution		Mg/K/Zn ratio
Clay	1M HNO <sub>3</sub>	121 / 19 / 1
	Conc. HNO <sub>3</sub>	186 / 98 / 1
Sand	1M HNO <sub>3</sub>	223 / 41 / 1
	Conc. HNO <sub>3</sub>	153 / 37 / 1
Lime	1M HNO <sub>3</sub>	16035 / 100 / 1
	Conc. HNO <sub>3</sub>	177 / 231 / 1
Soil organic layer		
Site 1	1M HNO <sub>3</sub>	96 / 4 / 1
	Conc. HNO <sub>3</sub>	66 / 19 / 1
Site 2	1M HNO <sub>3</sub>	3 / 1 / 1
	Conc. HNO <sub>3</sub>	17 / 40 / 1
Site 3	1M HNO <sub>3</sub>	7 / 5 / 1
	Conc. HNO <sub>3</sub>	51 / 37 / 1
Site 4	1M HNO <sub>3</sub>	22 / 16 / 1
	Conc. HNO <sub>3</sub>	50 / 58 / 1
Site 5	1M HNO <sub>3</sub>	33 / 9 / 1
	Conc. HNO <sub>3</sub>	280 / 236 / 1

It must be assumed that particles are dynamic, and without knowledge of their weathering rates and interactions with organic matter, little information can be gained from the use of elemental ratios to determine source. Also, if the ratios are used, it would be preferable to calculate the insoluble metal concentrations due to accumulated particles based on particle weight, and not organic matter weight. Ash content cannot be used as an estimate of particle weight, since mineralization will also increase ash content. For example, in the control dry forest and swamp, where contamination by mineral soil is unlikely due to a thick A<sub>0</sub> horizon, the ash contents are about 5% and 20%, respectively, which can be explained by differences in mineralization rates.

The metal binding affinity to soil organic matter is related to the forest type and pH (Krosshavn et al 1993), and the differences may be due to the vegetation source of the organic matter, decomposition processes, and mixing with the underlying soil particles. Considering the suggested even higher impact of natural factors on total concentrations in the A<sub>0</sub> horizon, in comparison to moss, it is surprising that the total concentrations in the A<sub>0</sub> horizon of forest soils will be mapped as part of an international survey (Rühling, in progress). The obtained results here, indicate that a serial elution method may be more informative, regarding deposition of metals and their form in the A<sub>0</sub> horizon. However, a clear understanding of the chemistry of the A<sub>0</sub> horizon, and the impact of the above natural factors, in relation to concentrations in the fractions of the serial elution procedure, is yet required.

## 8. SEASONAL CHANGES IN CELLULAR CONCENTRATIONS OF K, MG, CA AND ZN IN *HYLOCOMIUM SPLENDENS*

Cellular concentrations are rapidly redistributed with environmental changes in moisture, temperature and inputs (see Chapter 4). During dry periods, inputs of metals to moss will occur as dry deposition, and upward movement by internal recycling is favoured. Intercellular concentrations will likely be increased with rainfall, after a dry period, due to dissolved dry deposition and lost intracellular ions from desiccation. These dissolved ions may be leached downwards with water flow, or be retained in extra- and intracellular locations. Therefore, it is necessary to determine the seasonal changes in cellular concentrations, before the serial elution technique can be applied in practical monitoring of deposition. Markert and Weckert (1989) has described major changes in the seasonal concentrations of cations in *Polytrichum formosum*. However, studies have shown that the total concentrations in 3 year integrated segments of *Hylocomium splendens* are stable seasonally (A. Mäkinen, E. Steinnes, Å. Rühling, personal communication).

### 8.1. Methods

*Hylocomium splendens* was collected from two polluted and two unpolluted pine forest stands (following the Latvian Forest Type Classification - “mētrajs”, except swamp which is “slapjais mētrajs”); site descriptions follow:

#### Polluted sites -

Saulkalne. Located 1 km from the Saulkalne lime manufacturing plant and 100 m from the Saulkalne Rail Station. Small patches of *H. splendens* carpet occur among graminoids and calcifile pleurocarpous

moss species. The forest is monoculture mid-aged pine with no understorey trees or shrubs. Soil is podsolic with a plough layer.

Rīga. Located 1.2 km east-north-east of the Dārziņi Rail Station in an urban young pine plantation. *Vaccinium vitis-idaea*, *V. myrtillus* and graminoids dominate in the herbaceous layer. Tall broad leaf shrubs are abundant, but not in the vicinity of the collected material. About ten 30 cm high hummocks of *H. splendens* are found. Soil is podsolic with a plough layer.

Unpolluted sites -

Dry forest. Located in Tinūži about 4 km north of Ikšķile. Ground cover is dominated by *Pleurozium schreberi* and *H. splendens*; *Vaccinium vitis-idaea* and *V. myrtillus*. Soil is podsolic.

Drained swamp. Collected at the forested edge of a raised bog, about 400 m south of the dry forest site. *Sphagnum* spp occur in hollows and *Hylocomium splendens* and *Pleurozium schreberi* on hummocks. *Vaccinium* spp., *Rubus chamaemorus* and *Ledum palustre* are common.

Beginning with snow melt in 1994, samples were collected monthly from the four sites, (during thaws in the winter of 1994/1995), for about 1 year. Precipitation and mean daily temperature data was obtained from the Rīga Hydrometeorological Station. During the 1994 growing season, two predominantly dry spells occurred (Figure 8.1), April 23 - May 13 (30-50 days) and June 24-August 11 (90-140 days). The winter of 1995 was mild with many thaws (Figure 8.1), but the winter of the previous year (not shown) was cold with more stable snow cover.

Figure 8.1a. Daily precipitation (mm) at the Rīga Hydrometeorological Station.

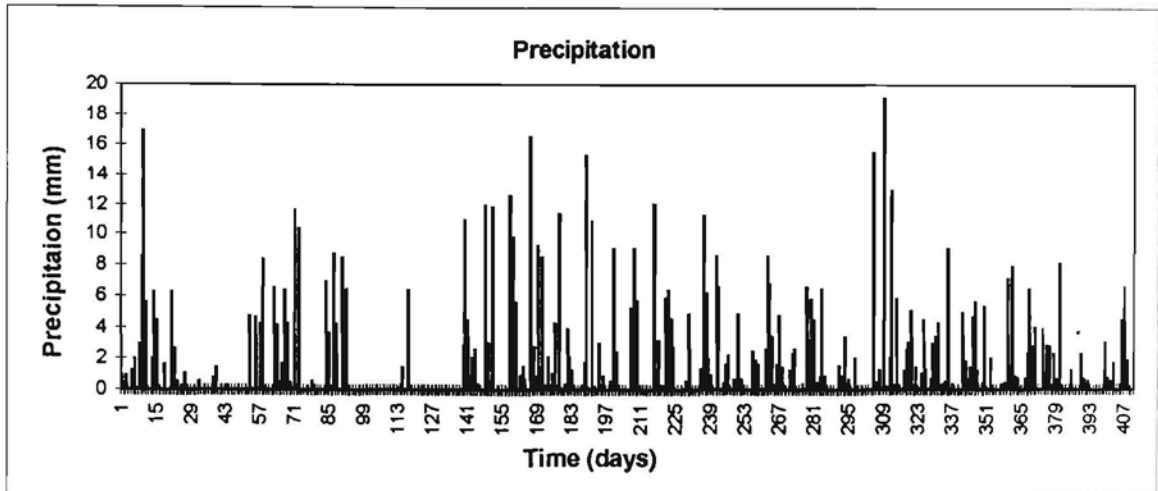
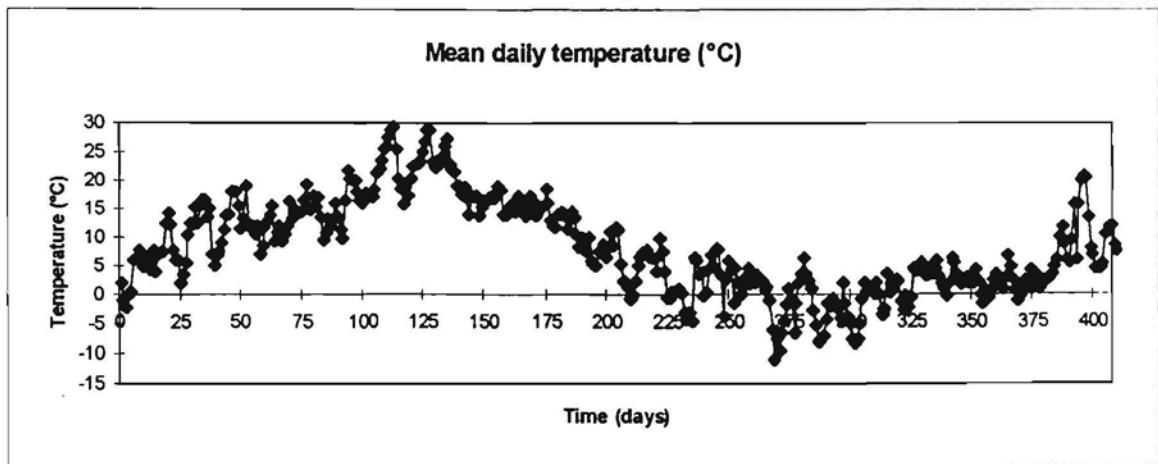


Figure 8.1b. Mean daily temperature (°C) at the Rīga Hydrometeorological Station.



Day No.	0	50	100	150	200	250	300	350	400	
Year	1994						1995			
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28	

Collected *Hylocomium splendens* samples were transported immediately to the laboratory, and stored humid for 24 h (sprayed with distilled water if dry) in trays lined with moist filter paper and with lids. Samples were separated into annual segments (1991-1994) and immediately serially eluted (Chapter 4). The 1994 bud was analysed together with the 1993 segment, until the autumn expansion began to occur.

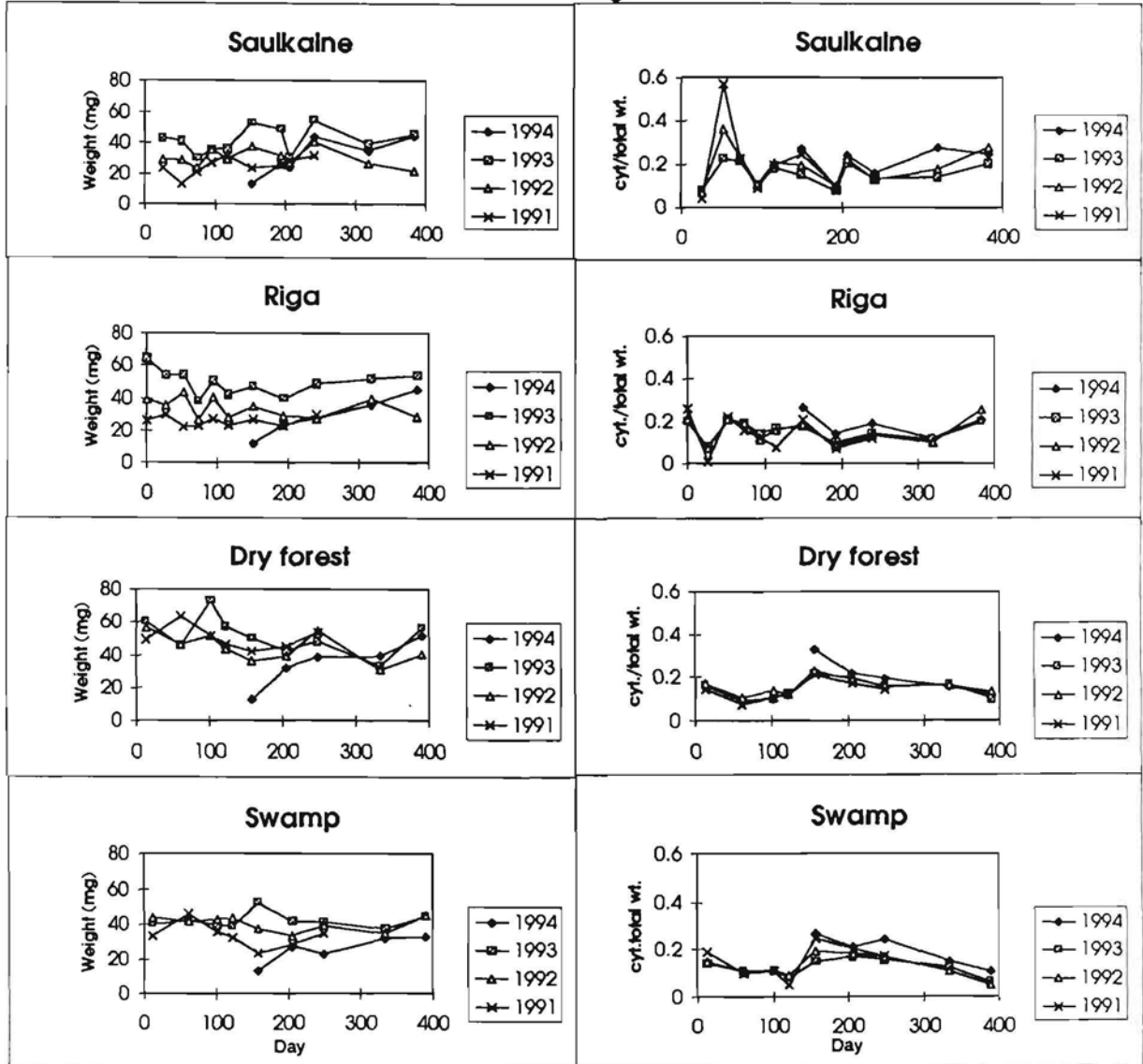
## 8.2. Results and Discussion

### 8.2.1. Segment weights and percent cytoplasm weight

The seasonal changes in segment weight (cell wall plus cytoplasm) and the percent cytoplasm weight of *Hylocomium splendens*, at two polluted (Rīga and Saulkalne) and two unpolluted sites (dry pine forest and pine swamp), are shown in Figure 8.2. The lowest segment weights and lowest rates of weight increase of the youngest 1994 segment, occurred at the swamp site. The youngest segment rapidly began to increase weight in late July through autumn, with a slight increase in weight in the subsequent year (days 350 - 400; Figure 8.2). Thus, in the temperate forests of Latvia, the expansion of *Hylocomium splendens* seems to be quicker than in the boreal forest (Busby et al 1978), which may explain why new buds are produced here mostly in the autumn, and not in the subsequent spring, as in Norway (Økland 1995).

Figure 8.2a

Figure 8.2b.



Day No.	0	50	100	150	200	250	300	350	400
Year	1994				1995				
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28

Figure 8.2a. Seasonal mean dry weights of two segments. Weight represents cell wall weight plus cytoplasm weight.

Figure 8.2b. Seasonal mean cytoplasm to total cell wall plus cytoplasm weights.

In the next summer after initiation of expansion in the previous year, the 1994 segments had similar weights to, or slightly less than, those of the previous years segment (1993). Before the expansion of the bud in 1994, which had formed in autumn 1993, or less often in spring 1994, the bud weight was included in the 1993 segment weight. The initial weights of expanding buds in late summer 1994, indicate that the unexpanded bud weights were rather insignificant, relative to the 1993 segment weights. This is also substantiated by the lack of a sudden decrease in weight of the 1993 segments after bud expansion, when weights were determined separately for the 1994 and 1993 segments.

The lack of an apparent increase in the 1991-1993 segment weights suggests that most development from bud to mature segment occurs during late summer to autumn of one growing season. The weights of the 1992 segments at the polluted sites (Rīga and Saulkalne) and the unpolluted dry pine forest, gradually decreased during the studied period, reaching the weight of 1991 segment in the previous year. This decrease in weight was associated with visible browning of tissue and the beginning of disintegration of frond structure (obvious losses of leaves and branches).

At the two polluted sites, the weight differences between 1991-1993 segments were greater than for the unpolluted sites, with the older segments having less weight, indicating hastened ageing and/or damage at the polluted sites. By the autumn of 1994, the 1991 segments at the polluted sites had clearly lost most branches, and they were extremely fragile; hence they were no longer sampled. At the same time, the 1991 segments in the dry forest had lost many branches and were brown, but the corresponding swamp segments were only slightly yellowed.



The percent intracellular weight of moss in the control sites increased in all segments with expansion of the young segment in late July, but again decreased with the onset of winter. Thus the moist and cooler conditions in August were associated with increased physiological activity of the moss, compared with the intermittent wet and dry spring to summer. The percent intracellular weights were erratic at the polluted sites, and may be affected by loss of damaged material during elution, and increased amounts of debris associated with the moss in the urban environment. In particular, a peak of percent cytoplasm is observed in early spring at the Saulkalne site during a very dry and warm period after a rapid and rainy thaw. These conditions may have damaged the moss more in this polluted site, compared to the other sites, since the humus layer is thinner with likely less moisture retention, the open shrub and herb free environment creates a greater desiccation stress, and, being close to a well used path, trampling may be more common. Since debris was also observed during elutions of material collected at this time, likely the spuriously high percent cytoplasm values are caused by damaged moss fragments released into the elutions.

### 8.2.2. K concentrations

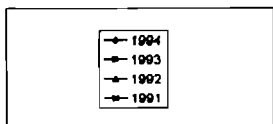
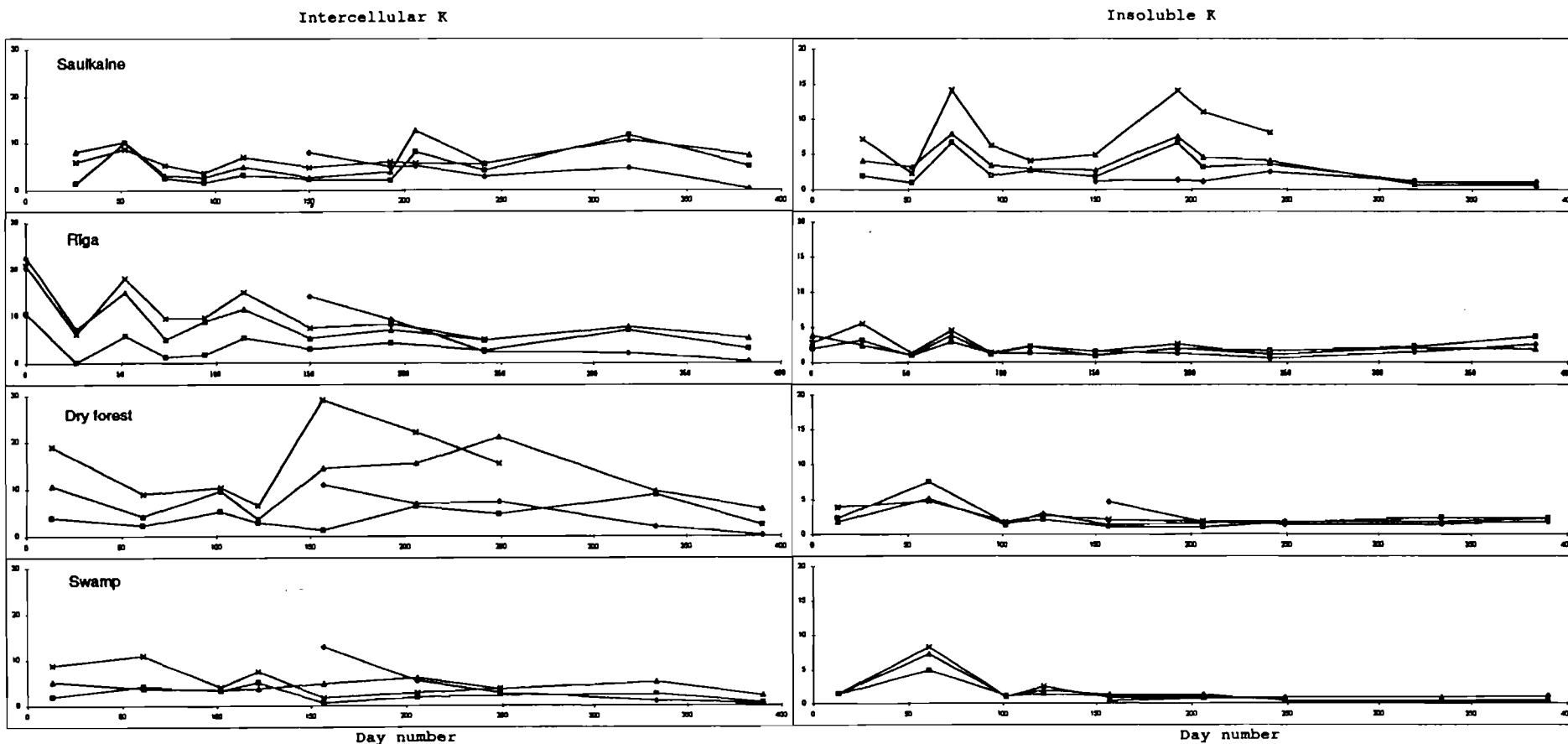
The cellular distribution of K followed the normal pattern of most K located intracellularly in healthy segments Figure 8.3a-b. The highest intracellular K concentrations occurred in the youngest segments at beginning of expansion, but subsequently gradually decreased to the level of the previous years increment by the spring of the next year. The lowest total K concentrations occurred in the most nutrient poor swamp forest, and the cellular concentrations were similar between segments, suggesting minimal damage or ageing of older segments. There is no evidence that the higher concentrations in the youngest segment had occurred at the expense of older segments by upward movement, since a

parallel decrease of K concentrations were not observed in the 1992-1993 segments.

In the dry forest, extra- and intercellular K concentrations (Figure 8.3a-b) much increased with the onset of rains and cooler temperatures at about 150 days (August 21), especially in the oldest 1991 segments. Since the abruptly higher concentrations are much above those at previous sampling days, and not matched by a corresponding decrease in intracellular concentrations, this increase cannot be explained solely by intracellular K losses, suggesting input from canopy leaching. The lack of such an increase at the polluted sites and swamp may be explained by lower K inputs by canopy leaching or increased competition with divalent cations promoting loss. The higher intercellular concentrations at the Riga site and the dry forest after snow melt in 1994 at the beginning of collections, may be due to intracellular losses during thaw, or accumulation of K in snow during winter

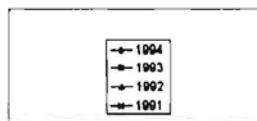
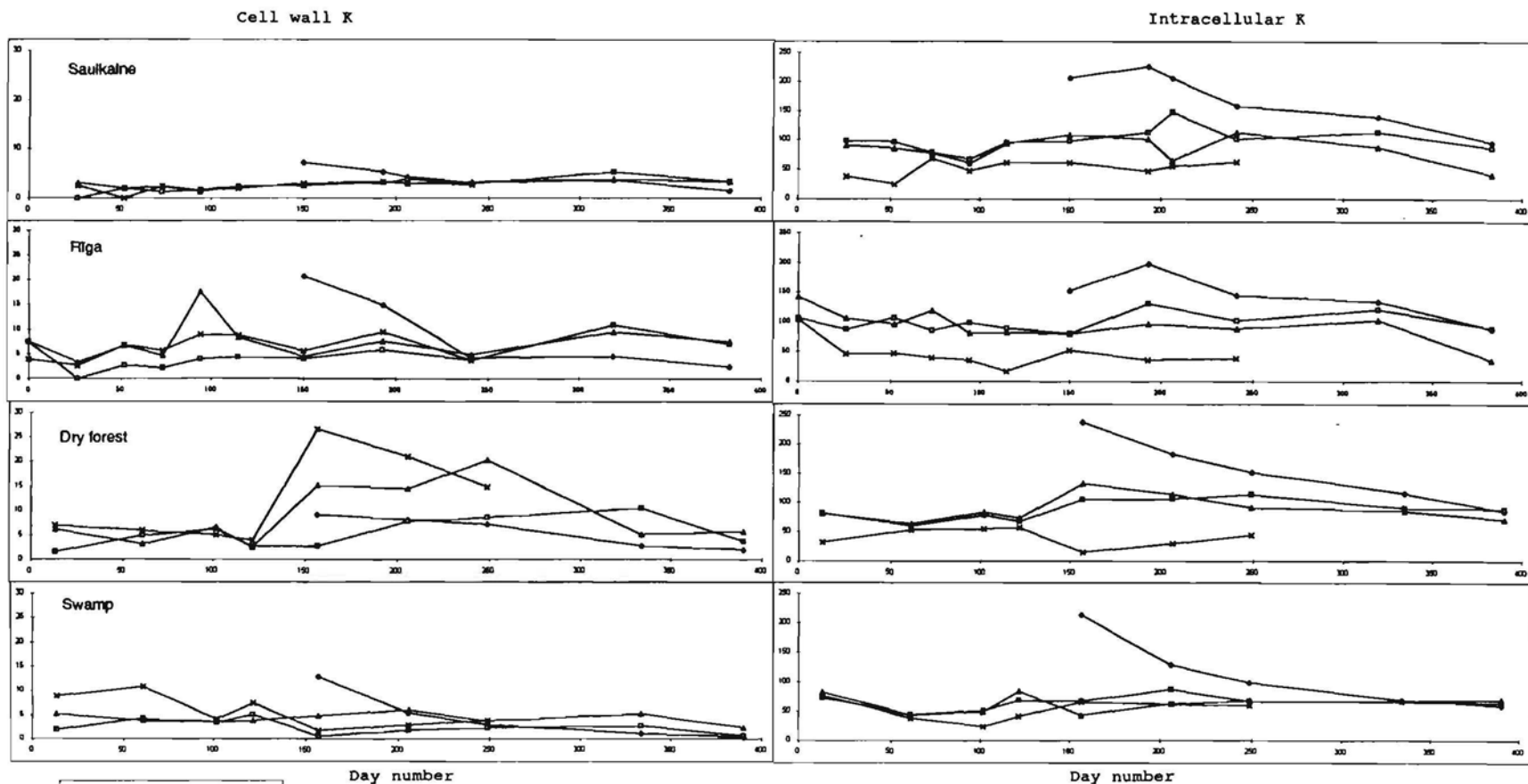
At all sites, but especially at the Saulkalne lime factory, peaks of relatively insoluble K were observed during a rainy period (50-100 days) after a dry spell, and, for only the Saulkalne site, also during the early stages of autumn rains (Figure 8.3a). This may be explained by the washing of particles from the tree canopy. These peaks were highest in the oldest segment, and it is evident these K inputs were later quickly lost by leaching, or were gradually dissolved and incorporated into other cellular locations.

Figure 8.3a. Seasonal cellular K concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*.



Day No	0	50	100	150	200	250	300	350	400
Year	1994					1995			
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28

Figure 8.3b. Seasonal cellular K concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*.



Day No	0	50	100	150	200	250	300	350	400
Year	1994						1995		
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28

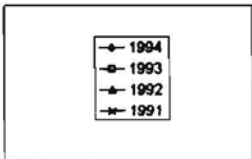
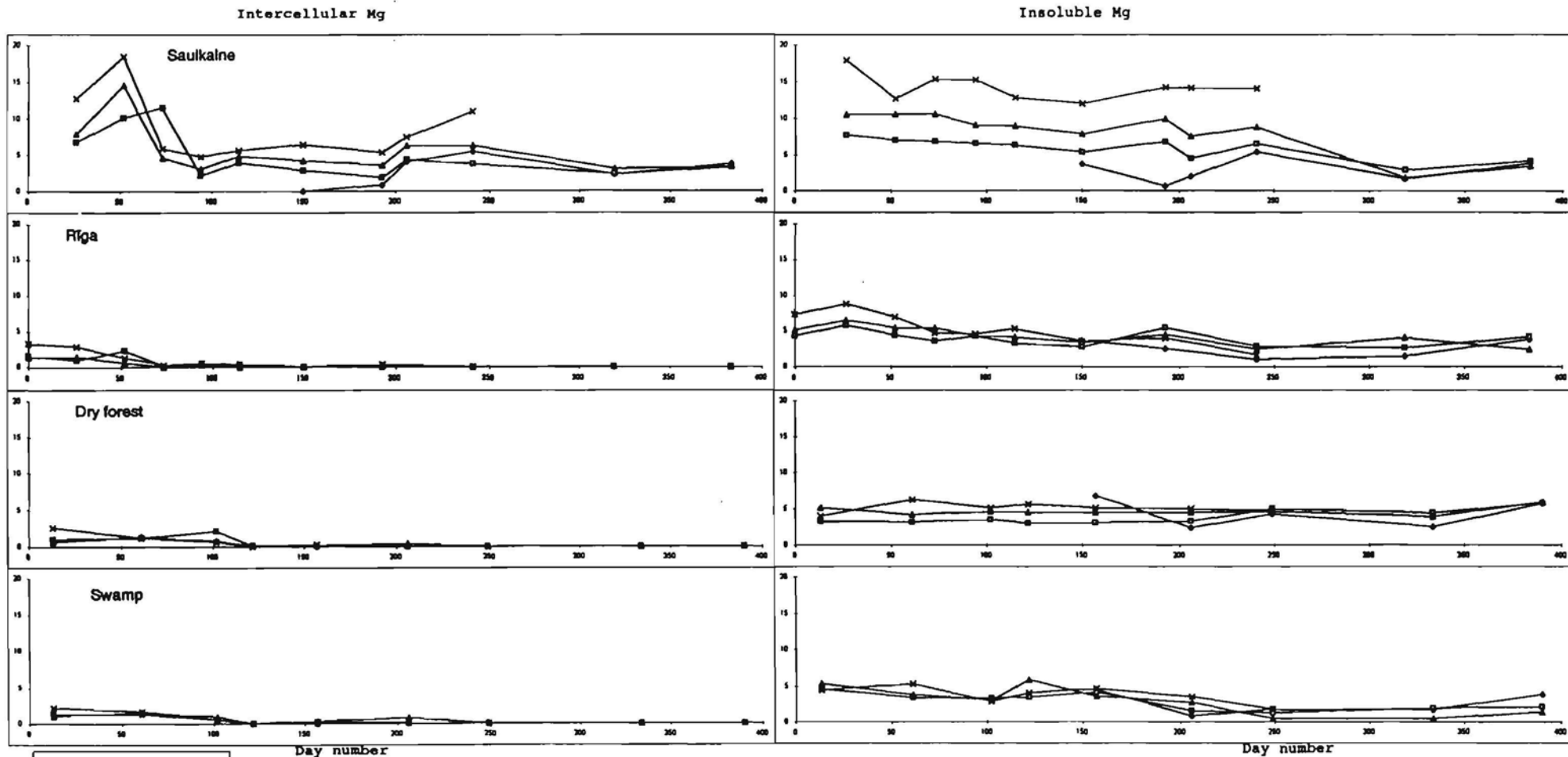
### 8.2.3. Mg concentrations

In all sites, Mg intracellular concentrations, similar to K concentrations, were highest in the expanding segments (Figure 8.4b). Mg concentrations were highest in the site close to the Saulkalne lime factory. Mg is a component of lime dust (Chapter 6), explaining the increases in Mg concentrations in all cellular locations. The peak of intercellular concentrations in spring 1994 (Figure 8.4a) at the Saulkalne site is likely due to damaged material, also evident by very high percent cytoplasm weights. Intercellular Mg in moss from other sites is negligible, but also higher in spring.

Cell wall Mg exchangeable concentrations in moss close to the lime facility were increased in the two dry periods of 1994 (Figure 9.4b). This can be explained by dry deposition, but it is not clear if the Mg was associated with the moss as particles at time of sampling, and became bound on exchange sites during elution with distilled water, or was released from particles by elution with Ni, or if a humid microclimate existed for some period of time allowing the particle to dissolve and absorb on the cell wall. Which ever the case, with the onset of rain, the extracellular concentrations decreased, perhaps due to competing cations in the rainfall or the washing of particles from the moss surface.

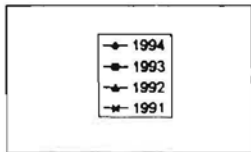
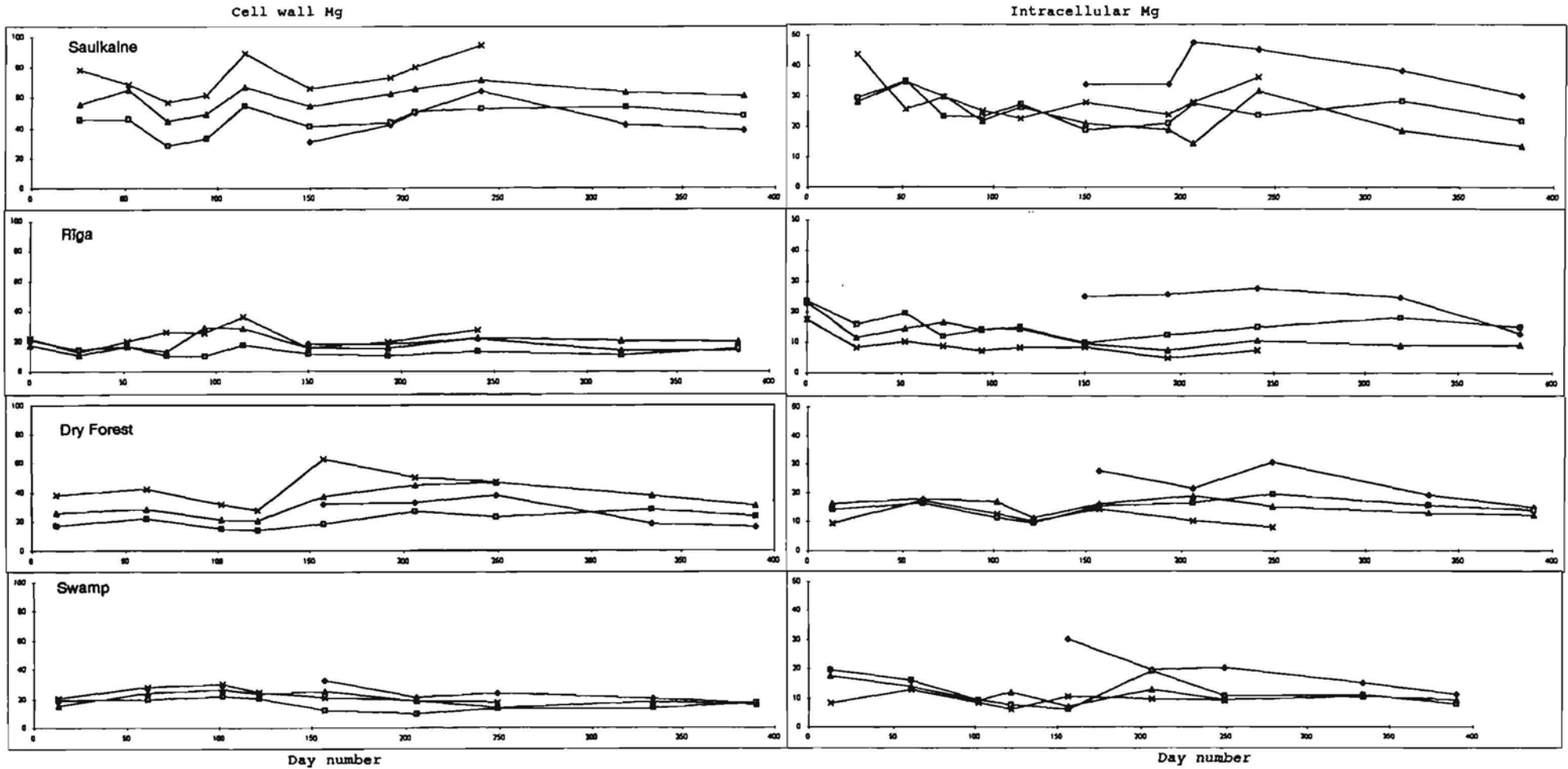
At the Rīga site, a slight extracellular Mg increase was also observed in the July dry period. In the control swamp, extracellular Mg concentrations were low and stable, but in the adjacent forest they increased in autumn with rains, as for K, perhaps due to canopy throughfall or desiccation stress. The lack of peaks during dry periods at the unpolluted sites indicate less dry deposition. Certainly, the control sites are less disturbed, receive less windblown surface material, and they are further from pollution sources.

Figure 8.4a. Seasonal cellular Mg concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*



Day No.	0	50	100	150	200	250	300	350	400
Year	1994						1995		
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28

Figure 8.4b. Seasonal cellular Mg concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*



Day No.	0	50	100	150	200	250	300	350	400
Year	1994						1995		
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28

The insoluble Mg concentrations (particles) at the Saulkalne site were fairly constant, until the first winter (Figure 8.4a.) when they decreased, perhaps due to leaching during the many intermittent thaws (Figure 8.1). The previous winter (not shown) was colder with stable snow cover, thus without complete thaws.

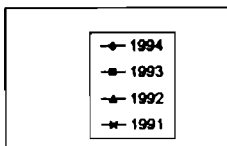
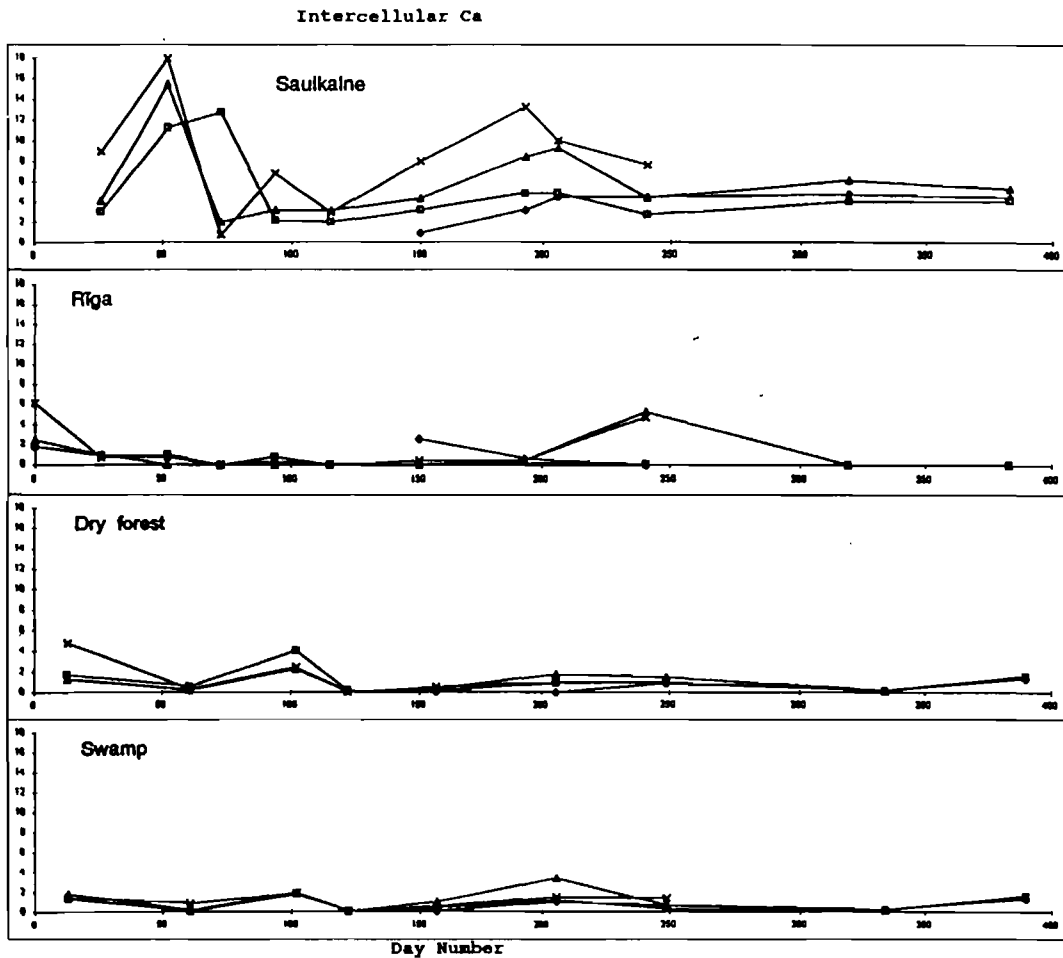
The two peaks in extracellular Mg concentrations peaks in the dry periods in the Saulkalne site, do not occur for insoluble particles, perhaps suggesting differing patterns of deposition and/or retention of soluble versus insoluble substances. The insoluble Mg particle concentrations were increased in older segments, without a general apparent seasonal pattern of increased leaching in the growing season at the four sites. The stable concentrations, excepting winter 1994/5, indicate an equilibrium of inputs and losses.

#### 8.2.4. Ca concentrations

Ca concentrations were over twice as high in the Saulkalne site than in the other sites (Figure 8.5a-b). Intercellular Ca concentrations peaked at the Saulkalne site in spring and late autumn, 1994. In the other sites, peaks occurred in the dry July period in the unpolluted sites. These patterns may be explained by soluble Ca dry or wet deposition, canopy throughfall or desiccation stress.

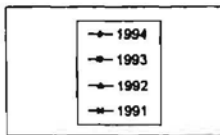
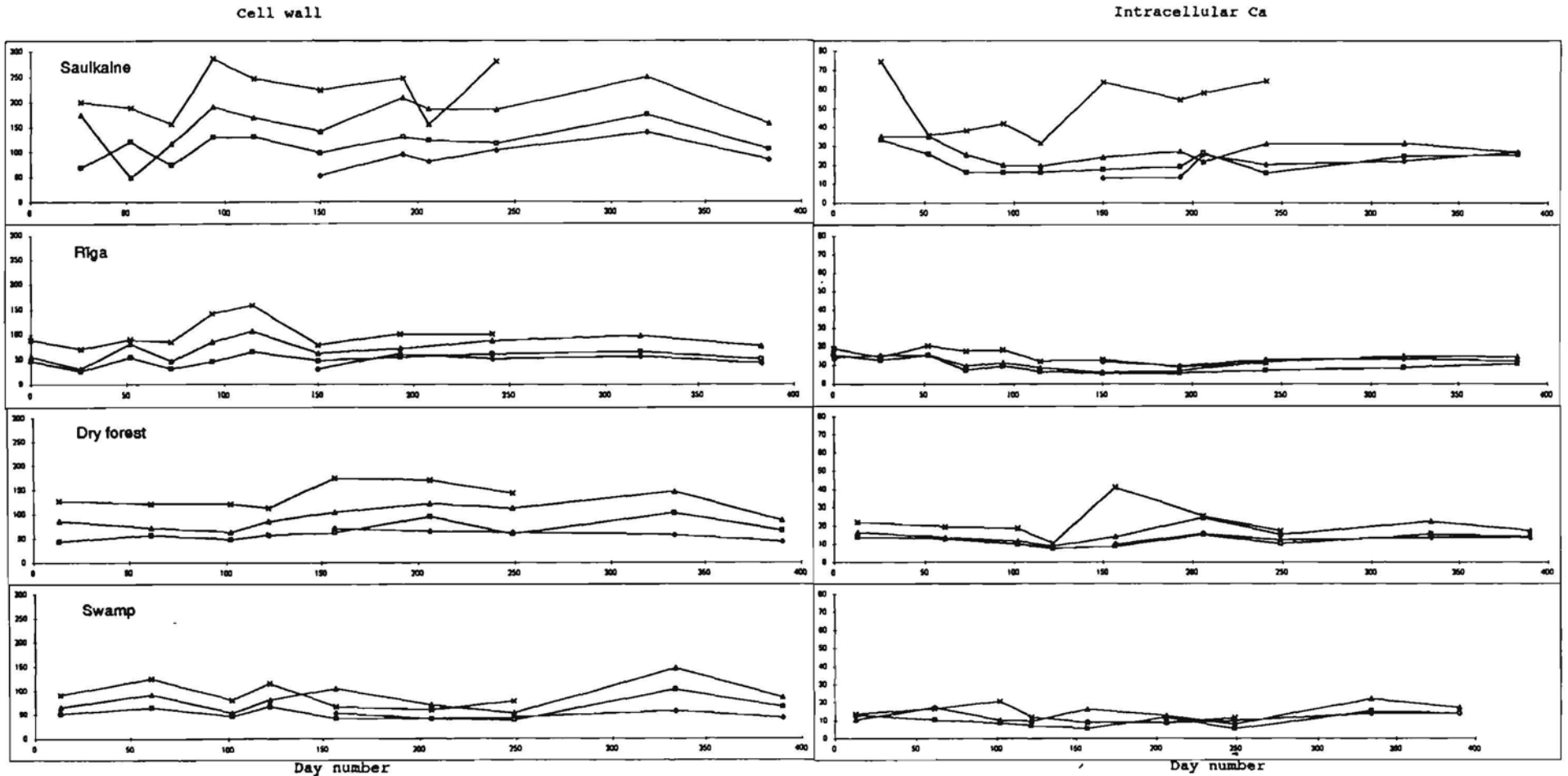


Figure 8.5a. Seasonal cellular Ca concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*



Day No.	0	50	100	150	200	250	300	350	400	
Year	1994					1995				
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28	

Figure 8.5b. Seasonal cellular Ca concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*



Day No.	0	50	100	150	200	250	300	350	400	
Year	1994					1995				
Day	Mar 25	May 13	Jul 2	Aug 21	Oct 10	Nov 29	Jan 18	Mar 9	Apr 28	

Excepting the swamp site, extracellular Ca concentrations generally increased from spring, 1994 to winter 1994/5, in the 1993 segment, with an overlaying maximum during the dry July at the Rīga site. This steady increase may be explained by maturation of the cell wall, i.e. more available sites. The accumulated Ca in July in the Riga site, which was likely due to deposition, was subsequently lost. The erratic behaviour of extracellular Ca concentration in the oldest segment in the Saulkalne site is likely due to its damaged condition, which is quite variable between sampled moss plants.

Excepting the oldest segments in the Saulkalne and dry forest sites, intracellular Ca concentrations were quite seasonally stable in all sites. Thus, there is little evidence of the desiccation induced Ca displacement from the cell wall to intracellular locations, described in Chapter 4. In Chapter 7, two testable models were proposed to explain the deposition and retention of Ca and Mg in moss and the A<sub>0</sub> horizon near Saulkalne: 1) deposition of relatively insoluble particles which are readily washed off and accumulate below, and 2) deposition of soluble substances on moss, which, when leached, accumulate below by the formation of relatively insoluble compounds. The predominance of exchangeable Ca, and to a lesser extent Mg, compared to concentrations in other fractions, at all times of the year, and the lack of intracellular Ca and Mg peaks in the dry periods, suggest that the second model better explains the results. Higher exchangeable Mg concentrations in moss in dry periods in Saulkalne, also substantiates the idea that the dry deposition is readily soluble.

#### 8.2.5. Zn concentrations

Intercellular concentrations were below detection limit and are not presented. In the polluted sites, cell wall exchangeable Zn concentrations

were highest in the snow melt period, at the beginning of data collection in the Rīga site, indicating that the snow had accumulated Zn in the frost period. However, this Zn was rapidly leached. In the lime production area (Saulkalne), and near Rīga, maximums of cell wall Zn concentrations occurred after the spring dry spell, with the onset of rains, but decreased with subsequent rain. This can be explained by a pulse of Zn deposition with canopy throughfall, which is retained on moss, but which is subsequently lost by competition with other cations. Data from the Saulkalne transect (Chapter 7) suggest that competition with Ca and perhaps Mg result in a depletion of Zn from moss and humus near the source, and this is further supported by decreasing Zn concentrations in rainy periods, after an initial increase.

The peaks of seasonal maxima appear to be superimposed on a general trend of increasing Zn intercellular concentrations, seen clearly in the unpolluted sites. This may be due to increased numbers of cell wall sites on older moss tissue, or accumulation with age. The seasonal patterns of intracellular Zn concentrations parallel the cell wall concentrations, suggesting a chemical equilibrium between exchangeable and intracellular locations.

In the polluted sites, insoluble (particle) Zn concentrations were highest in spring, but high seasonal cell wall and intracellular concentrations did not occur with similar trends for insoluble Zn, suggesting that the Zn as particles is from a different source as the soluble Zn, perhaps soil dust. Aside from the high spring Zn levels, including at the swamp controls site, the insoluble Zn concentrations were seasonally similar.

Figure 8.6a. Seasonal cellular Zn concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*

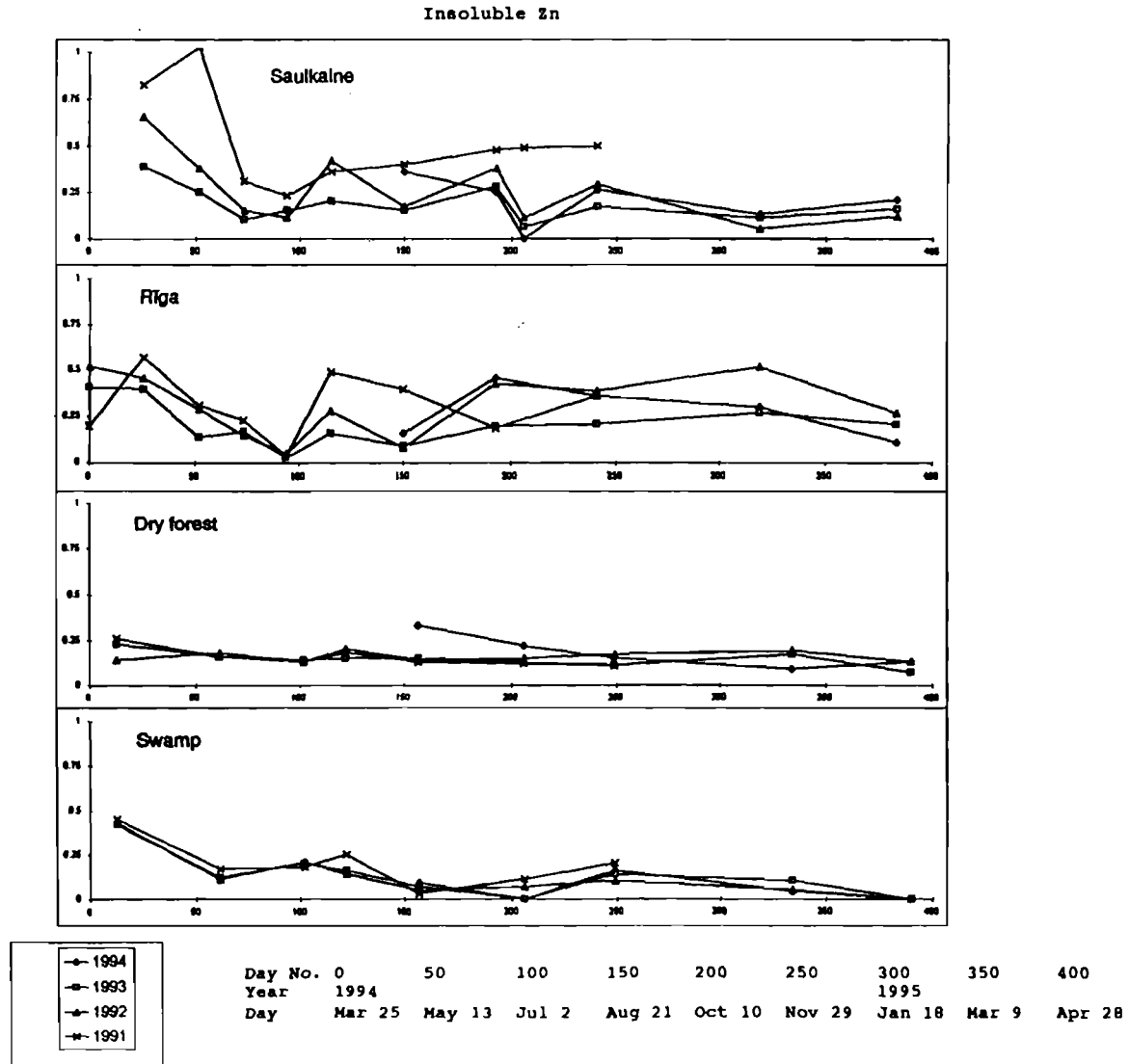
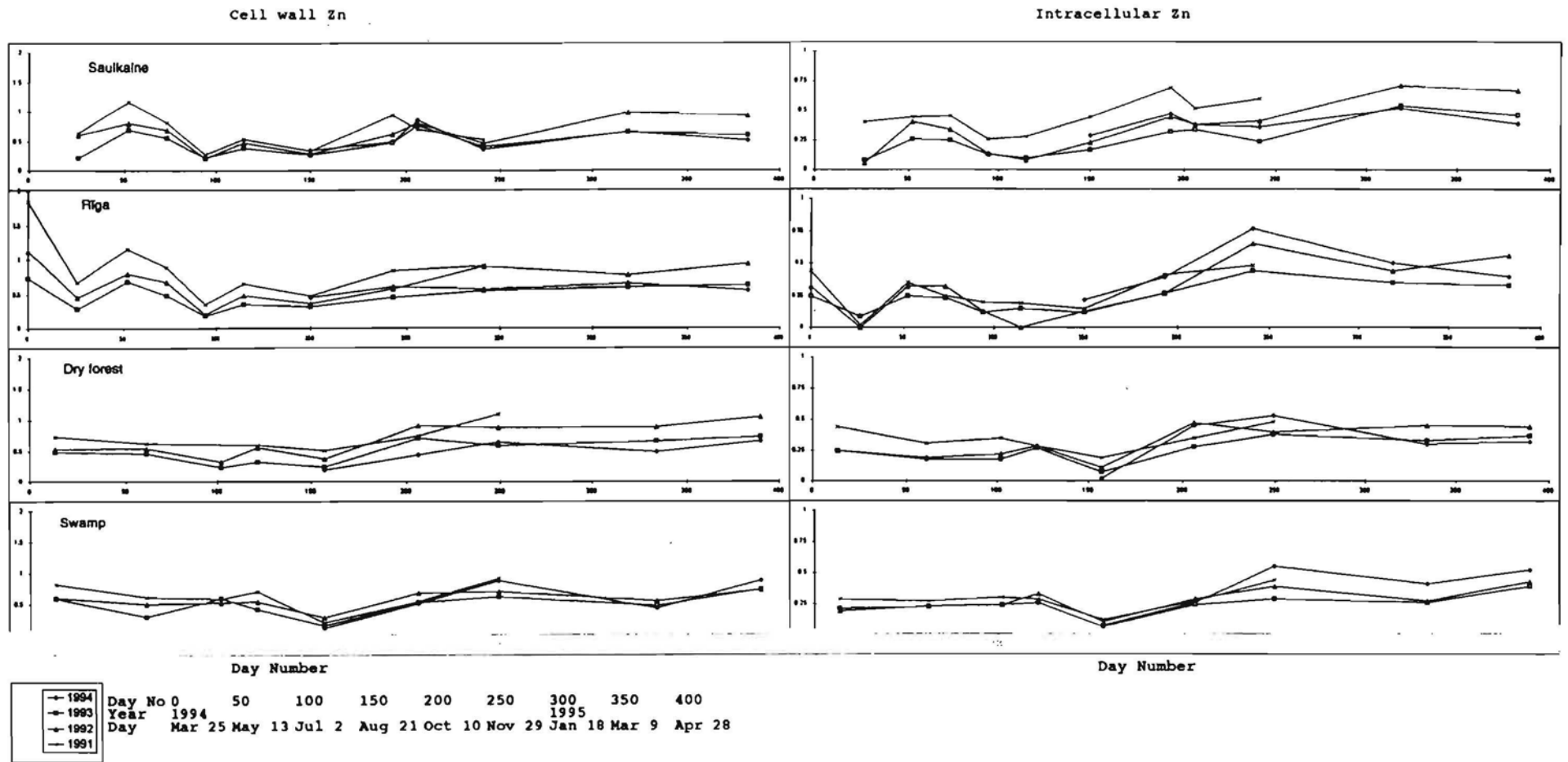


Figure 8.6b. Seasonal cellular Zn concentrations ( $\mu\text{moles/g}$ ) in *Hylocomium splendens*



## General Discussion

The seasonal patterns of cellular concentrations appear to be related to the seasonal dynamics of the nature and quantities of inputs, as well as the growth of moss. The highest K and Mg intracellular concentrations occur in late summer to autumn in the expanding bud, which is no doubt related to differences in the cytoplasm to cell wall weight ratio.

However, while the relative cytoplasm weight increased in all segments in the unpolluted sites with the beginning of moist and cool temperatures, a parallel increase in intracellular concentrations was not observed in the mature segments. This indicates that the intracellular K and Mg concentrations, expressed as per cytoplasm weight, have actually decreased. This suggests a loss of K and Mg from mature segments at the time of bud expansion, and supports the idea proposed in Chapter 5 that the expansion may be associated with nutrient loss. Losses of metals from mature segments in late summer with the onset of favourable conditions, may be associated with upward movement of nutrients into the expanding buds, thus promoting autumn growth.

Busby et al (1978) have observed that bud expansion occurs after termination of growth of the previous segment, and is not associated with cold treatment or photoperiod change, but suggest hormonal control or apical dominance as the regulatory mechanism. A more even seasonal distribution of growth (absence of annual segments) in *Hylocomium splendens* is found in grass swards in the southern UK, where the microclimate is more stable, moist and cool.

Tamm (1953) considered the autumn expansion to be related to optimum abiotic conditions of high humidity, low temperatures and an overhead source of nutrients. However, it seems more likely that the unexpanded

bud with low surface area, at least initially at the early stages of expansion, obtains its mineral nutrient supply from the previous segment. Excepting the dry forest site, there does not appear to be a rapid increase in cellular concentrations of Ca, Mg and K in mature segments in the autumn, when canopy leaching is expected to result in greater deposition. This suggests that in autumn, the inputs of these metals are at least matched by losses. Losses of minerals from mature segments, via the intercellular compartment, can potentially move apoplastically upwards to the expanding buds.

The cell wall and intracellular concentrations of Ca, Mg, K and Zn were most variable within sites between dry versus wet periods, which could be explained by dry deposition (Mg and Ca) or canopy throughfall (Zn), and also interactions between these cations. It is somewhat surprising that, during the dry periods and beginning of wet periods, intracellular losses of K due to desiccation were not evident. Also, for the mature one-year-old 1993 segment, intercellular K concentrations were low annually in all sites; this is not the case for the older 1992 segment. Desiccation damage increased seasonally with segment age at some sites, indicating variability in ageing and/or damage between sites. Therefore, the mature one-year-old 1993 segments would provide a more adequate assessment of deposition, since it is less likely to be damaged.

In the Saulkalne site, most of the deposition of Ca and Mg, is located in cell wall exchangeable or intracellular locations, with little evidence of insoluble lime dust deposition. The higher Ca concentrations in the 1M HNO<sub>3</sub> fraction of the eluted A<sub>0</sub> horizon (Chapter 7), thus implies losses from the moss carpet which is retained below in the form of relatively insoluble compounds.



Concentrations of Pb, Cd, Cu and Zn in *Polytrichum formosum* have been shown to be seasonally variable with much higher concentrations of Pb, Cu and Zn in winter, which was explained by lower biomass productivity and increased respiration (Markert and Weckert 1989). However, the possibility of accumulation of metals in moss from deposition in snow, and subsequent losses in spring due to leaching, should be considered. In *Hylocomium splendens* in the studied sites in Latvia, the seasonal dynamics in mature segments seem more related to the patterns of deposition and losses.

The Ca, Mg, K and Zn concentrations were more stable, along an increasing trend, in the autumn when moisture conditions are optimum for growth (wet and cool) and also rather stable. This suggests that monitoring work should avoid intermittent wet and dry periods in spring to summer, but be conducted in autumn in as short a period as possible.

## 9. CONCLUSIONS

### Testing of the serial elution technique

1. Elution with 20 mM Ni solutions is adequate for displacing cell wall Zn.
2. The use of killed cells by boiling to approximate pure cell walls in uptake trials can lead to misleading results, due to less available sites on intracellular substances.
3. Concentrated HNO<sub>3</sub> elution is not appropriate for determination of insoluble Ca.

### Desiccation effects on concentrations

4. Desiccation will alter the cellular distribution of ions, in the order K>Mg>Ca, suggesting that storage will less affect the cellular concentrations of less easily displaced ions. Desiccation induced damage increases with time of storage.

### Uptake of metals to additional growth

5. Uptake to new additional growth depends on the existing initial pools. In growth experiments, when samples differ in weight, the use of contents rather than concentrations is recommended.

### Application of the serial elution technique

6. In a transect from a Zn pollution source, extra-, and intracellular concentrations decreased exponentially, but insoluble Zn concentrations decreased more gradually. Ca and Mg concentrations were not related to distance from the source. Zn intracellular concentrations were similar to those known to have slight toxic affect when Zn is applied alone, but these results cannot be compared with out more knowledge of effects due to combinations of metals.

8. The cellular concentrations of Ca, Mg, K and Zn in moss around the Saulkalne Lime Production Factory indicate deposition of relatively soluble Ca and Mg (lime

dust), and the presence of insoluble K and Mg (mineral soil contamination). These trends are amplified in the organic horizon beneath moss. Soluble Zn near the source is lost by leaching, likely due to competition with Ca and Mg, but insoluble Zn particles are apparent, which are perhaps mineral soil. Ca and Mg accumulates in the A<sub>0</sub> horizon as relatively insoluble (1M HNO<sub>3</sub> displaced) compounds.

#### Seasonal effects on concentrations

9. Seasonal concentrations of Ca, Mg, K and Zn were most variable in intermittent wet and dry periods. Dry deposition increases the concentrations of Ca and Mg, which is subsequently leached with rainfall. Zn concentrations increase with canopy throughfall. In autumn, concentrations are more stable in mature segments, sometimes along an increasing gradient

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