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Review

The sequential elution technique applied to cryptogams: a literature review

Alicia Pérez-Llamazares, J Ángel Fernández, Alejo Carballeira, Jesús R Aboal

Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Spain

The use of bryophytes and lichens as biomonitors of atmospheric contamination, particularly contamination by heavy metals, is a well-known and widely applied technique. Determination of the total concentrations in organisms is often used in such studies, but has some disadvantages that may be resolved by using the sequential elution technique (SET). This technique involves successive washing steps that enable quantification of the elements in the different cell compartments (intercellular, extracellular, and intracellular), and finally determination of the remaining fraction, i.e. particulate material. The key step in the SET is the correct extraction of the extracellular fraction, for which a suitable extractant must be used for each different element considered. We have found only seven studies that have focused on selecting suitable extractants, which may be metal cations or chelating agents. Ethylenediaminetetraacetic acid is presented as the most appropriate extracellular extractant for Al, Co, Cu, K, Mg, Pb, V, and Zn, for which it has been tested, and for Cd and Fe, for which it has not yet been tested (although it is known to be capable of extracting these elements). The only extractant that is capable of extracting extracellular Hg is dimercaprol.

The technique has been used in laboratory studies and less often in field studies carried out in the surroundings of focal points of contamination. The elements analysed include nutrients (e.g. Ca, K, and Mg) and heavy metals (e.g. Cd, Zn, and Cu), usually associated with sources of emission of contaminants. Several problems have arisen in the application of the SET, some of which must be resolved. These include methodological problems (e.g. variability in the extraction process) and problems inherent in the SET itself, which are more difficult to resolve. Other techniques, such as histochemical techniques and electron microscopy with microanalysis, must therefore be used simultaneously with the SET to determine if the uptake of metal is only extracellular. At present, in light of the disadvantages of the quantification of the extracellular fraction, measurement of the intracellular fraction is presented as the best option because it is not affected by the metal burden in the particles, enables evaluation of environmental risks, better represents the average conditions of contamination, and enables better evaluation of phytotoxicity.

Keywords: Bryophytes, Extracellular, Intracellular, Lichens, Particulate, SET

Introduction

The use of bryophytes and lichens as biomonitors of atmospheric contamination, particularly contamination by heavy metals, is a well-known and widely applied technique (Tyler, 1989). Studies usually focus on the deposition of contaminants, by determining the total concentrations of elements in the study organism. While this may provide some measure of the contamination gradients involved and insights into how local conditions may alter distribution patterns, more valuable information can be obtained by a more refined analysis of plant composition. Total analyses may also greatly misrepresent the actual impact of the metals in many of the organisms concerned. Despite this disadvantage, determination of the total concentration of elements is still in widespread use, and is applied to unwashed samples of organisms (see e.g. Harmens *et al.*, 2008), to evaluate atmospheric deposition, or to washed samples (Markert *et al.*, 1999), in an attempt to evaluate those elements theoretically available to the organism.

The sequential elution technique (SET), in which the elements in the different cellular locations are quantified, is an alternative method that may resolve the above-described disadvantage. The definition of each of the cellular locations obtained in the SET is very precise and facilitates toxicological interpretation of the results. Data on the total concentration of contaminants are more difficult to interpret if they

Correspondence to: Alicia Pérez-Llamazares, Área de Ecología, Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain. Email: alicia.perez@usc.es

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are not obtained as the sum of the different fractions analysed by the SET. This technique provides more information about the bioavailability of contaminants and the associated risks, about the temporal representativeness of the bioconcentrated contaminants, and about the toxic effects of these.

Since it was first used (Brown & Slingsby, 1972), the SET has been applied to cryptograms (mainly bryophytes and lichens) and has only been used with algae in two studies (Costas & López, 2000, 2001). The organisms most commonly used with the SET are bryophytes (73%), followed by lichens (22%), although in other studies both types of organisms have been used together (5%) (Table 1). The genera of bryophytes most commonly used are *Pseudoscleropodium* and *Rhytidiadelphus* (22% of total cases in both), *Fontinalis* (12%), and *Hylocomium* (12%), whereas species of *Pleurozium*, *Brachythecium*, *Funaria*, *Grimmia*, *Hypnum*, and *Rhynchostegium* have been used in <10% of cases (Table 1). The genera of lichens most commonly used have been *Peltigera* (7%), followed by *Cladonia* and *Ramalina* (both 5%) and less frequently, *Hypogymnia* and *Pseudevernia*.

With regard to the structure of this review, we first describe the different cellular locations that can be studied with the SET, along with the form in which the elements may reach these locations, which depends on different factors. We then focus on the technique itself, its development throughout studies in which it has been used, and on the problems that have arisen, some of which are still relevant. This review focuses on bryophytes and lichens as these are the organisms most commonly used as biomonitors with the SET.

Cellular Locations

Mineral elements can be located at a number of cellular sites. The main forms and locations in which metals can be recovered from bryophytes and lichens are explained below, following information derived from using the SET (Brown, 1995): (1) intercellular: elements in solution that bathe the exterior of the cell wall matrix and the cell membrane but that are not bound to the cells; (2) extracellular: elements bound to the cell wall and outer layer of the plasma membrane; (3) intracellular: elements present within the cytoplasm or within cell organelles, as well as those bound to the internal layer of the plasma membrane; and (4) particulate: composed of extracellular particles and/or insoluble particles corresponding to crystalline deposits from inside the cells. The extra- and intracellular locations, as well as the particulate fraction, are described in detail in the following sections, as they correspond totally or partially to bioavailable fractions.

Extracellular location

The cell wall is the structure with the greatest cation exchange capacity in bryophytes and lichens, and therefore where most elements are retained. Binding is carried out in accordance with strict physicochemical rules and is a rapid, passive, and reversible process. Studies with these organisms have established that binding of a cation to extracellular cation exchange sites may cause displacement of other cations. Selective binding depends on the nature of the elements, their concentration, and the nature of the cation exchange sites. In the case of bryophytes, most of the binding appears to occur with the carboxylic groups of uronic acids, although cell wall proteins with oxygen, nitrogen, and sulphide groups may be involved, as well as the lipoproteins present in the plasma membrane. It has also been shown that the efficiency of the cell wall in immobilizing heavy metal ions may be the main mechanism of tolerance in bryophytes (Tyler, 1990), which would explain, e.g. the high tolerance to Cu, Zn, and Mn in species of the genus *Mielichhoferia* (Url, 1956). However, there is not yet any convincing evidence that species with a high cation exchange capacity in their tissues show greater tolerance to heavy metals.

The time that cation uptake lasts shows saturation kinetics due to the establishment of an equilibrium between soluble and bound ions. In the laboratory, this equilibrium may occur within 30 minutes in a 0.1 mmol L^{-1} solution in the moss *Rhytiadelphus squarrosus* (Brown & Wells, 1988), but data published by Mouvet (1987) for more environmentally realistic levels indicate that in nature, the equilibrium may be restored later, i.e. within days rather than minutes, and that the release may also be slower.

The valency of the cations is also important; monovalent cations are less effective at binding than divalent cations and this determines the equilibrium between the concentration of the elements in the environment and the exchange sites. The addition of divalent cations may cause loss of another divalent cation or of two monovalent cations. When equal concentrations of different cations are added, the concentration of bound cations depends on the affinity of the elements for anionic binding sites. Nieboer & Richardson (1980) classified cations on the basis of the nature of the compounds to which they are bound: (1) class A elements (e.g. K, Ca, and Mg), which generally show a high affinity for O-rich ligands (such as carboxylic groups); (2) class B elements (e.g. Hg and Au), which show a preference for ligands rich in S and N; and (3) borderline elements (e.g. Ni, Fe, and Zn), with intermediate preferences. In bryophytes, the binding sites on the cell walls are considered to be dominated by O-rich pectic carbohydrate polymers. However, some data (Brown & Wells, 1988) suggest the existence of anionic sites for class B elements, probably proteins that are present in the cell wall matrix, or on the external face of the plasma membrane. The

Table 1 Use of the sequential elution technique in different species of bryophytes (B) and lichens (L) in articles published to date

Species	Elements studied	Extractant	Experience	Reference
Cladonia rangiformis (L)	K, Pb	$H_{(1)}$	F	Brown & Slingsby (1972)
Bryophytes	Ca, K, Mg, Na	$Sr_{(1)}$	L	Bates & Brown (1974)
Grimmia sp. (B)	Ca, K, Na	$Sr_{(1)}$	L	Bates (1976)
Bryophytes	K, Mg, Sr	$Ni_{(1)}$	F	Brown & Buck (1978a)
Funaria hygrometrica (B)	Ca, K, Mg	$Sr_{(1)}$	L	Brown & Buck (1978b)
Bryophytes	Ca, K, Mg	$Ni_{(1)}$	L	Brown & Buck (1979)
Lichens	Ca, K, Mg	$Ni_{(1)}$	L	Buck & Brown (1979)
Bryophytes	Ca, K, Fe, Mg	$Sr_{(1)}$	L	Bates (1982)
Peltigera sp. (L)	Cd	$Ni_{(1)}$	L	Beckett & Brown (1984a)
Peltigera sp. (L)	Ca, Cd, K, Mg, Zn	$Ni_{(1)}$	L	Beckett & Brown (1984b)
Lichen			R	Brown & Beckett (1984)
Rhytidiadelphus squarrosus (B)	Cd	$Ni_{(1)}$	L	Brown & Beckett (1985)
Rhytidiadelphus squarrosus (B)	K	$Ni_{(1)}$	L	Brown & Whitehead (1986)
Rhytidiadelphus squarrosus (B)	Ca, Cd, K, Mg	$Ni_{(1)}$	L	Wells & Brown (1987)
Pseudoscleropodium purum (B)	Ca, K, Mg	$Sr_{(1)}$	L	Bates (1987)
Bryophytes			R	Brown & Wells (1988)
Pleurozium schreberi (B)	Ca, K, Mg	$Sr_{(1)}$	L	Bates & Farmer (1990)
Rhytidiadelphus squarrosus (B)	Ca, Cd, K, Mg	$Ni_{(1)}$	L	Wells & Brown (1990)
Rhytidiadelphus squarrosus (B)	Cd, K, Zn	$Ni_{(1)}$	L	Brown & Wells (1990a)
Bryophytes			R	Brown & Wells (1990b)
Bryophytes + lichen	Ca, K, Mg	$Sr_{(1)}$	F	Farmer *et al.* (1991)
Lichen			R	Brown & Brown (1991)
Bryophytes			R	Bates (1992)
Bryophytes			R	Brown & Sidhu (1992)
Rhytidiadelphus triquetrus (B)	Al, Ca, K, Mg	$Sr_{(1)}$	L	Bates (1993)
Peltigera membranacea (L)	Ca, K	$Ni_{(1)}$	L	Brown & Avalos (1993)
Brachythecium rutabulum (B) and *Pseudoscleropodium purum* (B)	Ca, K, Mg	$Sr_{(1)}$	L	Bates (1994)
Cladonia portentosa (L)	Ca, K, Mg, Pb	$Ni_{(1)}$ $Cu_{(*)}$ $Ni_{(1)} + Cu_{(*)}$ EDTA Ethylene glycol tetraacetic acid Glutatione Ammonium pyrrolidine dithiocarbamate	L	Branquinho & Brown (1994)
Rhytidiadelphus squarrosus (B)	Ca, Cd, K, Mg	$Ni_{(1)}$	L	Wells *et al.* (1995)
Bryophytes			R	Brown (1995)
Bryophytes	Zn	$Ni_{(1)}$	L	Sidhu & Brown (1996)
Hylocomium splendens (B)	Ca, K, Mg, Zn	$Ni_{(1)}$ $Ni_{(2)}$ $Pb_{(2)}$ $Ni_{(1)} + Pb_{(2)}$	F	Brown & Brümelis (1996)
Hylocomium splendens (B)	Ca, K, Mg, Zn	$Ni_{(1)}$	F L	Brümelis & Brown (1997)
Brachythecium rutabulum (B) and *Pseudoscleropodium purum* (B)	Ca, K, Mg	$Sr_{(1)}$	L	Bates (1997)
Lichen	Ca, Cu, K, Mg	$Ni_{(1)}$ EDTA $Pb_{(2)}$	L	Branquinho *et al.* (1997a)
Lichen	Ca, K, Mg, Pb	$Ni_{(1)}$ EDTA	L	Branquinho *et al.* (1997b)
Hylocomium splendens (B)	Cu, Pb, Zn	$Ni_{(1)}$	F	Brümelis *et al.* (1999)
Ramalina fastigiata (L)	Cu, K, Mg	$Ni_{(1)}$ EDTA	F L	Branquinho *et al.* (1999)
Fontinalis antipyretica (B)	Al, Ca, Cd, Co, Cu, K, Mg, Na, Ni, Pb, Zn	$Ni_{(1)}$ EDTA $Pb_{(2)}$	L	Vázquez *et al.* (1999a)
Bryophytes	Cd, Co, Cu, K, Mg, Ni, Pb, Zn	$Ni_{(1)}$ EDTA	L	Vázquez *et al.* (1999b)
Ramalina canariensis (L)	Ca, K, Na, Mg	$Ni_{(1)}$	F	Figueira *et al.* (1999)
Rhynchostegium riparioides (B)	Cu	EDTA	L	Mouvet & Claveri (1999)
Fontinalis antipyretica (B)	Al, Ca, Co, Cu, K, Mg, Ni	EDTA $Ni_{(1)}$	F L	Vázquez *et al.* (2000)
Hylocomium splendens (B)	Ca, K, Mg	$Ni_{(1)}$	F L	Brümelis *et al.* (2000)

Table 1 Continued.

Species	Elements studied	Extractant	Experience	Reference
Bryophytes and lichen	Cd, Cu, K, Pb	$Sr_{(1)}$	L	Badacsonyi *et al.* (2000)
Lichen	Ca, K, Mg, Mn	$Ni_{(1)}$	L	Hauck *et al.* (2002)
Pleurozium schreberi and *Rhytidiadelphus triquetrus* (B)	Ca, K, Mg, Fe	$Ni_{(1)}$ $Sr_{(1)}$	L	Bharali & Bates (2002)
Pleurozium schreberi and *Rhytidiadelphus triquetrus* (B)	Ca, K, Sr	$Ni_{(1)}$	L	Bharali & Bates (2004)
Pseudoscleropodium purum (B)	Cu, K, Mg, Fe, Zn	$Ni_{(1)}$	F	Fernández *et al.* (2004)
		EDTA		
Hylocomium splendens (B)	Ca, K, Mg	$Ni_{(1)}$	F	Brūmelis *et al.* (2004)
Fontinalis antipyretica and *Fontinalis dalecarlica* (B)	Cd	$Ni_{(1)}$	L	Bleuel *et al.* (2005)
Fontinalis antipyretica (B)	Al, Co, Cu, Ni, Zn	$Ni_{(1)}$ EDTA	F	Fernández *et al.* (2006)
Pseudevernia furfuracea (L) and *Hypnum cupressiforme* (B)	K	$Ni_{(1)}$	F	Tretiach *et al.* (2007)
Hypogymnia physodes (L)	Cd, Cu, Fe, Pb, Zn	EDTA	F	Mikhailova & Sharunova (2008)
Fontinalis antipyretica (B)	Cu	EDTA	L	Ferreira *et al.* (2009)
Pseudoscleropodium purum (B)	K, Hg	$Co_{(1)}$	L	Pérez-Llamazares *et al.* (2009)
		$Ni_{(1)}$ $Pb_{(2)}$ $Sr_{(1)}$ EDTA Dimercaprol Penicillamine	F	
Pseudoscleropodium purum (B)	Cu, K, Zn	$Ni_{(1)}$ EDTA	L	Fernández *et al.* (2010)
Pseudoscleropodium purum (B)	K, Mg, Zn	$Co_{(1)}$	L	Pérez-Llamazares *et al.* (2010)
		$Ni_{(1)}$ $Pb_{(2)}$ $Sr_{(1)}$ EDTA Dimercaprol Penicillamine		
Pseudoscleropodium purum (B)	Ca, K, Zn	$Ca_{(1)}$	L	Pérez-Llamazares *et al.* (2011a)
		$Hg_{(1)}$ $Au_{(1)}$ $Ni_{(1)}$	F	
Pseudoscleropodium purum (B)	Zn, K	$Ni_{(1)}$	L	Pérez-Llamazares *et al.* (2011b)
			F	

Note: The elements analysed and the extracellular extractants used in each case are indicated, as well as whether the studies were laboratory-based (L), field-based (F), or reviews (R). 1: metal chloride; 2: metal nitrate; *: extractant not specified. EDTA, ethylenediaminetetraacetic acid.

efficiency of the binding occurs in the following order: class A<borderline divalent<divalent class B, although the affinity of elements for cation exchange sites may vary depending on the species (Brown & Wells, 1990b).

Intracellular location

Intracellular elements may occur in three different forms within cells (Brown & Wells, 1990b): (1) as soluble elements in the cytoplasm; (2) bound to the inner face of the plasma membrane; and (3) dissolved within vacuoles or other organelles (e.g. chloroplasts or mitochondria). As it is impossible to distinguish between these three locations, all of the above types of elements are considered as intracellular.

Intracellular uptake requires that the elements pass through the hydrophobic plasma membrane. The process of intracellular uptake involves molecules that act as membrane transporters (carriers) and display different degrees of selectivity. The uptake is determined by the affinity of the elements for the transporters, the speed of transport of the carriers, the difference in the intracellular and extracellular charge, and the presence of other competing elements. The rate of uptake is much slower than the extracellular binding and may be controlled by inputs of energy, which are required for correct functioning of the transporter (Brown & Bates, 1990).

Control of uptake to the inside of the cell is influenced by different factors. When specific

transporters are present, one of the factors to take into account is the intracellular concentration existing at that moment. Thus, even when the concentration in the exterior is high, if the intracellular concentration is also high, uptake may not occur. However, for elements for which there are no specific transporters, such as Cd, and for which input is not controlled, the intracellular concentration may increase, independently of the previously existing intracellular concentration (Brown & Bates, 1990).

With regard to biological interpretation of the data, the elements present inside the cells are those that provide most information, as they: (1) have a potentially immediate effect on the metabolism of the organism, and are therefore more likely to cause toxic effects than the extracellular fractions; (2) provide a more reliable indication of the mean concentrations in the environment in which the organism grows, as the changes in the environmental chemistry may be reflected in the cell wall but not within the cell; and (3) reflect the nutritional demand of the cells.

Particulate fraction

The elements included in this fraction may occupy different positions: (1) bound to particles deposited on the surface of the organism; and (2) bound to particles included inside the cells.

With respect to the capacity for retention of particles, different studies (e.g. Richardson & Nieboer, 1980; Nieboer *et al.*, 1982; Giordano *et al.*, 2009; Pérez-Llamazares *et al.*, 2011b) provide a firm basis for the suggestion that lichens and bryophytes are effective at trapping mineral-rich particles of between 0.01 and 100 μm (Chamberlain & Little, 1981). The capacity to retain particles depends on the morphology of organisms, and varies depending on the species considered (Carballeira *et al.*, 2008), the level of hydration (Brown, 1984), and their possible handling during processing of the samples (Giordano *et al.*, 2009).

Description of the SET

Prior to the use of the SET, and to be able to affirm that the concentrations measured actually correspond to each of the above-described fractions, it must be ensured that the permeability of the plasma membrane is not altered. Otherwise, some of the elements included in the intracellular fraction may be quantified as extracellular. The integrity of the plasma membrane is ensured by knowing how some nutrients (i.e. K, Ca, and Mg) are distributed between the extra- and intracellular fractions when the membrane is in perfect condition. Displacement studies have shown that, in general, K is located at intracellular sites while Ca is mostly bound to the extracellular exchange sites (see e.g. Brown & Buck, 1979; Bates, 1982); Mg shows intermediate distribution patterns (Bates & Brown, 1974; Pérez-Llamazares *et al.*, 2010).

The distribution between the extra- and intracellular fractions is affected by species and by environmental conditions (Bates & Brown, 1974). Among the possible environmental conditions that may affect this distribution, the most important is perhaps the desiccation that some terrestrial organisms may suffer. In this case, unless severe damage occurs, the changes in permeability may be reversed, as the membranes may rapidly recover their optimal conditions. Brown & Buck (1979) demonstrated that storage of moss samples in conditions of 100% relative humidity enables the membranes to recover their normal permeability. Fernández *et al.* (2010) have shown that when moss samples were not maintained for a period in a moisture-saturated atmosphere, washing of the materials led to loss of K, as the membranes were altered after a period of drought in the field.

After demonstrating that the membrane permeability is not altered, the first step in the SET is to wash the material with deionized water (see e.g. Brown & Wells, 1988; Brown, 1995) or bidistilled water (see e.g. Vázquez *et al.*, 1999a; Fernández *et al.*, 2004) to obtain the soluble intercellular fraction. It is possible that this washing step eliminates some of the particles from the surface of the organism.

The second step consists of submerging the sample in a solution containing an agent capable of displacing the extracellularly bound elements. Selection of an appropriate agent is essential, as it must enable effective extraction of the elements in the fraction, without altering the membrane permeability. Few studies have been carried out to date with the aim of selecting appropriate extracellular extractants (Branquinho & Brown, 1994; Brown & Brūmelis, 1996; Branquinho *et al.*, 1997a; Vázquez *et al.*, 1999a; Pérez-Llamazares *et al.*, 2009; 2010; 2011a), and the following section of this review will be dedicated to these studies.

The third stage refers to quantification of the intracellular elements. For this, after the extracellular extraction, the cell membranes are ruptured, and the intracellular content is then extracted. For this purpose, a sequence of washing in boiling deionized water followed by total digestion was initially used (Brown & Buck, 1979), although in most later studies this was substituted by washing in dilute HNO_3. In exceptional cases, some authors have used ethylenediaminetetraacetic acid (EDTA) as an intracellular extractant (Branquinho *et al.*, 1999; Mikhailova & Sharunova, 2008).

The final step in the SET consists of obtaining the particulate material. In some studies, this fraction is determined together with the intracellular fraction (see e.g. Beckett & Brown, 1984a). When quantified separately, it is usually obtained by total acid

digestion (see e.g. Vázquez *et al.*, 1999b; Brūmelis *et al.*, 2004).

The first attempts to determine the cellular concentrations of different elements were carried out by Brown & Slingsby (1972) working with the lichen *Cladonia rangiformis*. The SET was later perfected and began to be used with other organisms. Different washing times and different extracellular and intracellular extractants were tested until the currently used technique was developed. The most commonly used protocol is that published by Brown & Wells in 1988. Slight modifications have been suggested, such as that proposed by Vázquez *et al.* (1999a), still in use today. It is recommended that the total content is determined in parallel, to be able to test whether the sum of the different fractions is equivalent to the total content, although this is not usually done. The extraction protocol consists of the following steps:

1. Intercellular extraction: the sample is washed (approximately 0.1 g dry weight) in 10 ml of bidistilled water for 30 s with shaking. The material is then removed and dried before subjecting it to the following stage of the SET and analytical determination of the extract.
2. Extracellular extraction: the sample is placed in 10 ml of the extraction medium, with shaking, in two successive steps (45+30 min); the extraction medium is renewed between the steps. The material is removed and dried before analytical determination of the extract.
3. Intracellular extraction: the sample is dried to constant weight (45°C) to rupture the cell membranes, and the dry weight is determined. The soluble intracellular metal is then dissolved by shaking for 30 min in 10 ml of 1 mol L^{-1} nitric acid. The material is removed before analytical determination of the extract.
4. Particulate fraction: the sample is dried to constant weight (at 45°C). The residual metal and insoluble particles are extracted or determined directly with the selected method (e.g. acid digestion). Analytical determination is then carried out.

Recently, Pérez-Llamazares *et al.* (2009) have proposed a new modification that consists of determining the contaminants directly in the sample and not in the extracts, thus preventing the analytical interference that may be produced between the elements and the extractants used (e.g. dimercaprol). After these modifications, the extraction protocol consists of the same stages, but always beginning by total extraction. The difference is that four times as much sample is used, and at each stage (total determination, 1–3), an aliquot corresponding to a quarter of the initial sample is reserved for posterior homogenization and analysis. The liquid extracts obtained can be discarded and the concentrations corresponding to each fraction of the different elements are calculated as follows:

1. particulate fraction: material determined after stage 3;
2. intracellular fraction: obtained by subtracting the sum of the concentrations of the intracellular and particulate fractions (material determined after stage 2), from the concentration of the particulate material (material determined after stage 3);
3. extracellular fraction: obtained by subtracting the sum of the concentrations of the extracellular, intracellular, and particulate fractions (material determined after stage 1) from the sum of the concentrations of the intracellular and particulate fractions (material determined after stage 2);
4. intercellular fraction: obtained by subtracting the total concentration from the sum of the concentrations of the extracellular, intracellular, and particulate fractions (material determined after stage 1).

In both protocols, it is recommended that at least three replicates are used for each extraction, in order to reduce the variability associated with the process.

Analytical determination

The concentration of elements can be determined by different analytical techniques. The most commonly used techniques are: flame atomic absorption spectrometry for major elements, and graphite furnace atomic absorption spectrometry for minor elements (e.g. Cd), with concentrations expressed in ppb.

Extractants Used

As already mentioned, in the SET, it is essential to use an extracellular extractant that effectively extracts the extracellularly bound elements but does not cause changes in the membrane permeability. One possible way of extracting the elements from the extracellular fraction would be to use cations that compete with the metals for the binding sites on the cell wall and external surface of the membrane. However, the use of a cation as an extractant would prevent the determination of that metal in the sample. Another option is to use chelating agents, which are compounds with a high affinity for metals.

The extracellular extracts used in studies that have been applied to the SET and are considered in the present review, are listed in Table 1. The number and proportion of studies in which these extractants have been used are shown in Figure 1, in which it can be seen that of the four most commonly used extractants, three are metallic cations: $NiCl_2$ (63% of studies), $SrCl_2$ (23%), and $Pb(NO_3)_2$ (8%). When the SET was first used, extracellular extraction was carried out with $SrCl_2$ (Bates & Brown, 1974), which is an effective displacing agent for class A cations (Bates, 1982, 1987). Later, when class B elements were studied, $NiCl_2$ was used as the extractant (Brown & Beckett, 1985; Wells & Brown, 1987). As Ni is a borderline element of low toxicity, it was chosen most frequently in recent studies to displace other cations (at a concentration of 20 mmol L^{-1}). One of the great advantages of $NiCl_2$ is that it can be used as a reference extractant for elements such as K and Zn, as it is known that Ni successfully displaces both elements when they are located extracellularly (Brown &

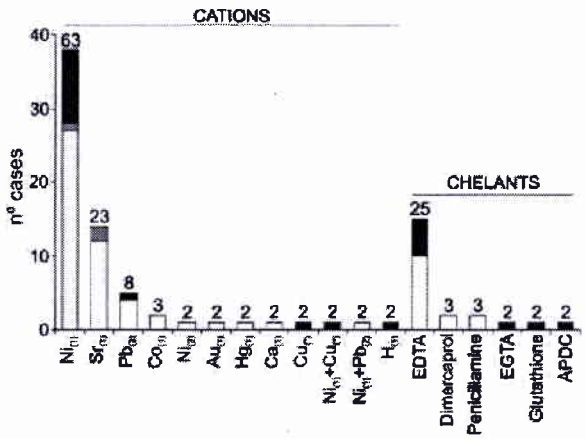

Figure 1 Number of cases in which each of the different extracellular extractants has been used in the sequential elution technique with bryophytes (white), lichens (black), or together (grey). The number above the bars indicates the percentage of studies in which each extractant has been used (note that the sum is >100% because several extractants are used simultaneously in different studies). 1: metal chloride; 2: metal nitrate; *: extractant not specified.

Brūmelis, 1996; Brūmelis *et al.*, 1999) and it would therefore serve to test the efficacy of other potential extractants (see e.g. Pérez-Llamazares *et al.*, 2010; Pérez-Llamazares, 2011a). Whereas $NiCl_2$ and $Pb(NO_3)_2$ have both been used in studies with bryophytes and with lichens, $SrCl_2$ has most commonly been used in studies with bryophytes (Figure 1).

The other cations have been used in only one study, with the exception of $CoCl_2$, which has been used in two studies. It has been shown that these cations are not sufficiently effective as extracellular extractants, even when used at high concentrations in the case of non-toxic elements (i.e. Ca), and their use is not recommended. Other cations have been rejected for use as extractants because of their high toxicities even at low concentrations, which produces alterations in membrane permeability (i.e. Au and Hg) (Pérez-Llamazares *et al.*, 2011a). Finally, combinations of Ni with other metal cations have sometimes been used for extracellular extraction (Figure 1; Table 1), although this does not always lead to an increase in the effectiveness of $NiCl_2$.

Chelating agents display a high affinity for cations bound to the cell wall and outer face of the plasma membrane and release them to the extraction medium. EDTA is the chelating agent most commonly used in the SET (in 25% of studies) and has been used with both bryophytes and lichens (Figure 1). Cations that are bound to exchange sites on the cell wall and plasma membrane may bind to OH functional groups of EDTA, thus releasing them to the extraction medium. The first study in which the efficacy of EDTA as an extracellular extractant was tested was carried out by Branquinho *et al.* (1997a) for the extraction of Cu, after which the compound became one of the most commonly used extracellular extractants. However, almost all authors recognize that EDTA causes alterations in the membrane, usually small breakages that do not greatly affect the final result of the study (Branquinho & Brown, 1994). The advantage of EDTA over the most commonly used extract (20 mmol L^{-1} $NiCl_2$) is that it enables the quantification of a larger number of elements, including Ni itself.

The other chelating agents have been used much less often than EDTA, only once or twice (Figure 1), in some cases because they do not produce better results, and in others because they have only recently been identified. The former include penicillamine, ethylene glycol tetraacetic acid, glutathione, and ammonium pyrrolidinedithiocarbamate, which are either not effective, are no better than EDTA (Branquinho & Brown, 1994) or cause problems such as analytical interference and the appearance of precipitates in the extracts, e.g. penicillamine (Pérez-Llamazares *et al.*, 2010). Among the chelating agents most recently used, dimercaprol has been found to be a good extractant for Pb and V, as well as Hg, and to date is the only extractant available for the latter element (Pérez-Llamazares *et al.*, 2009). The use of dimercaprol as a potential extraction agent for Hg arose because this compound is used to treat Hg poisoning in humans (Elberger & Brody, 1998). There may be other compounds with similar potential as extracting agents, although they have not yet tested in bryophytes or lichens, such as 2,3-dimercaptosuccinic acid, 2,3-dimercaptopropane-1-sulfonate, dimercaptopropanol-glycine, dimercaptosuccinic acid, and N-acetyl-DL-penicillamine.

All the extractants that have been used in the SET, as well as the elements that each extractant has successfully displaced, are shown in Table 2. The second column in the table includes previously untested compounds, used to extract different elements simply on the basis of their electrochemical characteristics (Brown & Wells, 1988). In these studies, it was deduced that the extractants being tested were capable of extracting certain elements, although no comparisons were made with other extractants and therefore the efficiency of the extraction could not be evaluated. As already mentioned, few studies have been carried out with the aim of discovering extracellular extractants suitable for extracting elements. Similar results were obtained with regard to the efficacy of the extractants for some elements but not for other elements, with the exception of K. This is because EDTA can produce alterations in the permeability of the plasma membrane, so that when it is used as an extractant, quantification of K may be erroneous, and therefore this is documented as an unsuccessful extraction in Table 2.

EDTA is presented as the most suitable extracellular extractant for most class A and borderline elements analysed to date (i.e. Al, Co, Cu, K, Mg, Pb, V, and Zn). However, Hg (a class B element) can only

be extracted with dimercaprol. The efficiency of extraction of extracellular Cd and Fe by EDTA has not been evaluated in any study, although the compound has been shown to be capable of extracting both elements in the studies in which it has been used (Mikhailova & Sharunova, 2008; Fernández *et al.*, 2004, respectively).

Some authors (Vázquez *et al.*, 1999a; Pérez-Llamazares *et al.*, 2010) have evaluated the efficiency of different concentrations of extracellular extractants. The concentration of $NiCl_2$ most commonly used for extracellular extraction is 20 mmol L^{-1}, and it has been found that this is an optimal concentration (Pérez-Llamazares *et al.*, 2010). With regard to EDTA, although the concentration most commonly used is 20 mmol L^{-1}, halving the concentration to 10 mmol L^{-1} does not reduce the effectiveness of extracellular extraction and has less effect on the membrane permeability (Vázquez *et al.*, 1999a; Pérez-Llamazares *et al.*, 2010), so that use of the lower concentration has been recommended in the later studies. With regard to $SrCl_2$, the concentration most commonly used has been 25 mmol L^{-1} (Farmer *et al.*, 1991; Bates, 1993), but the study of Pérez-Llamazares *et al.* (2010), in which different concentrations were evaluated, showed that the best concentration, on the basis of the results obtained with K, Mg, and Zn is 50 mmol L^{-1}. With regard to $Pb(NO_3)_2$, Vázquez *et al.* (1999a) chose a concentration of 50 mmol L^{-1}, whereas Pérez-Llamazares *et al.* (2010) recommended a concentration of 30 mmol L^{-1}. With regard to the chelating agents dimercaprol and penicillamine, use of concentrations of 30 and 50 mmol L^{-1}, respectively is recommended (Pérez-Llamazares *et al.*, 2010).

Those studies aimed at selecting suitable extractants have been carried out with different species (Table 1), which must be taken into account with regard to any species-related differences that may arise.

Studies Employing the SET

The number of studies in which the SET has been used to analyse different elements in bryophytes and lichens is shown in Figure 2. Among these, the nutrients Ca, K, and Mg have been the most commonly studied with the SET, and were the object of study during the earlier applications of the technique. In some 73% of the studies, in which the SET was used (Table 1), the concentration of K was determined because, as already mentioned, determination of the concentration of this element is useful to establish whether the membrane permeability is altered. The elements Ca and Mg were determined in approximately half of the studies (52 and 53%, respectively; Table 1). Although Ca has been analysed to observe its effect on the uptake of other elements, Mg has also been analysed (like K) to evaluate the membrane integrity.

After it was shown that the technique worked with nutrients, it began to be used with other metals of greater toxicological interest. Zinc is the metal most often determined in such studies (25%; Table 1, Figure 2), probably because of the high concentrations at which it occurs and the relative ease

Table 2 Efficacy of the extraction of different elements by different extracellular extractants used in the sequential elution technique

	Successful extraction		Extraction not successful
Extractant	Tested	Not tested	Tested
$Ni_{(1)}$	Cd^{k}, K^{k}, $Mg^{k,o}$, $Zn^{i,k}$	Cd^{e}, K^{o}, Mg^{c}, Sr^{c}, Zn^{f}	Al^{k}, Co^{k}, $Cu^{j,k}$, Hg^{n}, $Pb^{h,k}$
$Sr_{(1)}$	K^{o}, Mg^{o}	Al^{g}, Ca^{b}, Fe^{d}, K^{b}, Mg^{b}, Na^{b}	Hg^{n}
$Pb_{(2)}$	Al^{k}, $Cu^{j,o}$, K^{o}, Mg^{o}, Zn^{i}		Hg^{n}, Mg^{k}
$Co_{(1)}$	Mg^{o}		Hg^{n}
$Au_{(1)}$	...		Zn^{p}
$Hg_{(1)}$	...		Zn^{p}
$Cu_{(*)}$	...		Pb^{h}
$Ca_{(1)}$	...		Zn^{p}
$Cu_{(*)}+Ni_{(1)}$	...		Pb^{h}
$Ni_{(1)}+Pb_{(2)}$	Zn^{f}		...
$H_{(1)}$		Pb^{a}, K^{a}	
EDTA	Al^{k}, Co^{k}, $Cu^{j,k,o}$, K^{j}, Mg^{k}, $Pb^{h,k,o}$, V^{o}, $Zn^{i,o}$	Cd^{m}, Fe^{l}	Hg^{n}, $K^{k,o}$
Dimercaprol	Hg^{n}, K^{o}, Pb^{o}, V^{o}, Zn^{o}		Mg^{o}
Penicillamine	Cu^{o}, Hg^{n}, K^{o}		Mg^{o}
EGTA	...		Pb^{h}
Glutathione	...		Pb^{h}
APDC	...		Pb^{h}

Note: a. Brown & Slingsby, 1972; b. Bates & Brown, 1974; c. Brown & Buck, 1978a; d. Bates, 1982; e. Beckett & Brown, 1984a; f. Beckett & Brown, 1984b; g. Bates, 1993; h. Branquinho & Brown, 1994; i. Brown & Brümelis, 1996; j. Branquinho *et al.*, 1997a; k. Vázquez *et al.*, 1999a; l. Fernández *et al.*, 2004; m. Mikhailova & Sharunova, 2008; n. Pérez-Llamazares *et al.*, 2009; o. Pérez-Llamazares *et al.*, 2010; p. Pérez-Llamazares *et al.*, 2011a.
Tested: results obtained in studies in which the aim was to test the efficacy of extracellular extractants. **Not tested**: results obtained in studies in which different extractants were used without previously testing their efficacy for extractions, but which produced good results. 1: metal chloride; 2: metal nitrate; *: extractant not specified.

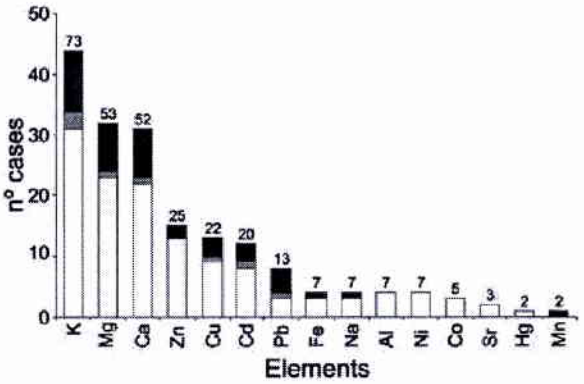

Figure 2 Number of cases in which the concentration of diverse elements in bryophytes (white), lichens (black), or in both (grey) has been determined by the use of the sequential elution technique. The number above the bars indicates the percentage of studies in which each element has been determined (note that the sum is >100% because in several studies various elements were determined simultaneously).

with which analytical determinations can be carried out, and because it is associated with many sources of metal emissions. The second most commonly analysed element is Cu (22%), followed by Cd (20%), which is also associated with processes of contamination and may also interfere in the uptake of other metals. Other heavy metals often analysed by SET are Pb and Fe (Figure 2).

With regard to the aims of the studies in which the SET has been used, a distinction may be made between those that focus on physiological aspects and those related to contamination and/or toxicity. The former include studies of the mobilization of elements inside bryophytes, mainly the moss *Hylocomium splendens* (Bates, 1987, 1992; Brūmelis & Brown, 1997; Brūmelis *et al.*, 2000, 2004), which confirm that transport occurs between the older regions and the apical zones. With regard to studies involving contaminating elements, these were initially carried out in the laboratory (Beckett & Brown, 1984b; Brown & Wells, 1990a). Most of these studies consist of exposing bryophytes or lichens to a solution of the element under study and determining, by use of the SET, the amount of the element that is incorporated into each of the cellular locations.

Once it was demonstrated that the SET could be used successfully in the determination of heavy metals in laboratory conditions, it has then been applied in field studies carried out in the surroundings of focal points of contamination (Brown & Brūmelis, 1996; Fernández *et al.*, 2004; Pérez-Llamazares *et al.*, 2009). Very few field studies or simultaneous laboratory and field studies have been carried out to date (18 and 12%, respectively; Table 1).

Use of the SET

Throughout its history, the SET has been applied in different ways, ranging from studies involving the effects that heavy metals may have on moss physiology (e.g. Brown & Whitehead, 1986; Brown & Wells, 1990a; Branquinho *et al.*, 1997b) to others concerning the tolerance of different mosses and/or lichens to desiccation (see e.g. Brown & Buck, 1979; Bates, 1997) or to the presence of heavy metals (e.g. Beckett & Brown, 1984b). In addition, various studies have investigated the uptake of different elements in mosses (see e.g. Bates, 1987; Wells & Brown, 1987; Bates, 1992; Vázquez *et al.*, 1999b; Fernández *et al.*, 2006) and lichens (e.g. Brown & Beckett, 1984), or have analysed the distribution of elements in the different cell locations, in laboratory studies (see e.g. Brown & Slingsby, 1972; Bates, 1976; Brown & Buck, 1978a; Branquinho & Brown, 1994) and, more recently, in studies with samples collected in the area of influence of different sources of contamination (e.g. Brūmelis *et al.*, 1999; Fernández *et al.*, 2004; Tretiach *et al.*, 2007). Finally, a small number of studies have focused on improving the use of the SET (Vázquez *et al.* 1999a; Pérez-Llamazares *et al.*, 2009; 2010; 2011a).

Problems Associated with the Technique

Several problems associated with the SET have arisen since the technique was first used, some of which have been resolved, e.g. the duration of intercellular washing should be <30 seconds so that there are no alterations in the equilibrium of extracellularly bound cations (Wells & Brown, 1990), and two consecutive washes are sufficient to obtain total content of the extracellular fraction (Vázquez *et al.*, 1999a). However, other problems remain to be resolved, including methodological-type problems, which are relatively easy to resolve, and problems inherent in the technique itself, which are more difficult to resolve.

Methodological-type problems are restricted to specific aspects related to use of the SET, which must be investigated in depth so that the technique can be standardized. These include the variability in the processes of extraction due to the heterogeneity of the sample and analytical errors, which will determine the number of analytical replicates required to obtain accurate results. To date, most authors have used three (see e.g. Vázquez *et al.* 1999a) or five replicates (see e.g. Branquinho *et al.*, 1997a, Pérez-Llamazares *et al.*, 2009), without any real justification for doing so. Another problem related to the methodology, restricted to terrestrial bryophytes, is the need to standardize the time that each species needs to be exposed to a moisture-saturated atmosphere to ensure that the permeability of the membrane is not altered. The duration of this period is very variable, between 24, 72, and 168 hours (Brūmelis & Brown, 1997; Pérez-Llamazares *et al.*, 2010; Pérez-Llamazares

et al., 2009; respectively). Moreover, as explained in the previous section, only a relatively small number of contaminants have been analysed by the SET to date, and therefore it remains to be demonstrated whether the extractants usually used are appropriate for other elements not yet analysed by the SET.

The problems inherent in the SET are all derived from the fact that the different cellular locations are defined by the analytical techniques used in their quantification. If there is no analytical technique that enables isolation of a certain cellular location, this will be quantified along with another, although from a biological point of view they may be different. In addition, it is not easy to test either the efficiency of the extractions or whether the element determined actually corresponds to the location under consideration. Very few studies have been carried out to test the efficacy of the extracellular extraction process (Table 2), and this may be the aspect that is currently in most need of detailed research. In general, after incubating the moss in a solution of metal for a short time (i.e. 1 hour), almost all of the metal is bioconcentrated in the extracellular fraction, so that the efficacy of the extracellular extractant is based on the fact that it extracts an extremely high proportion of the element (i.e. >99%). However, as nothing is known about the simple diffusion kinetics of the elements across the membrane, this is merely an assumption, as it has not been verified that transport to the interior of the cell does not occur, and therefore extraction of e.g. 98% of an element may not represent total extraction, as the remaining elements may be present in the intracellular compartment.

It is therefore necessary to use different techniques simultaneously to be able to determine whether the uptake of metal is extracellular or not. These additional techniques include histochemical techniques and electron microscopy with microanalysis. With regard to the former, the techniques developed for lichens, by Rinino *et al.* (2005), may also be suitable for bryophytes. Secondly, electron microscopic techniques may be an effective way of testing metal uptake and have been used to locate metals in different cellular compartments, in both lichens (Williamson *et al.*, 2004) and mosses (Bruns *et al.*, 2001; Basile *et al.*, 2001; Giordano *et al.*, 2005; Rau *et al.*, 2007). However, one disadvantage of this technique is that it is semiquantitative and therefore does not enable determination of the exact concentrations of the elements studied, so that its use is limited to being a complementary technique. The technique has been used recently to test the efficiency of the SET (Pérez-Llamazares *et al.*, 2011b), with surprising results. These authors have shown that extraction of extracellular Zn with $NiCl_2$, in samples of moss incubated in Zn, is efficient when the samples do not contain this metal in particulate form. However, when there is a lot of particulate material on the external surface of the moss, $NiCl_2$ extracts Zn equally from these particles, thus overestimating the extracellular fraction and therefore the bioconcentrated fraction, and underestimating the particulate fraction that constitutes a less immediate toxicological risk. With respect to the efficiency of extraction of the intracellular fraction, no tests have yet been carried out.

As already mentioned, the problem is that the different cellular locations are defined by the corresponding analytical techniques. As there is no technique that allows exclusive determination of the particulate material that is deposited on the moss surface, given that there is no efficient technique available for eliminating this material, it remains on the surface, thus interfering with subsequent extracellular and possibly intracellular extractions. This material is later quantified along with the interior particulate material, which is the bioconcentrated material and for which biological interpretation is quite different. Investigation is required to establish how a prior cleaning/washing step can be carried out to enable direct quantification or calculation of the difference in concentration of the external particulate material. Ducceschi *et al.* (1999) recommended cleaning samples with a nitrogen jet at 1 bar pressure during a suitable period of time to remove particulate matter present on the moss surface, without affecting the equilibrium between the extra- and intracellular concentrations in the moss, the efficacy of which was demonstrated by microscopy (SEM and ESEM). Adaptation of this treatment as a step in the SET requires further investigation.

The existence of all of these problems may be one reason why the SET is not routinely used, and why it has not been widely applied in field studies (Table 1), despite the advantages over conventional analysis of the total concentration in bryophytes or lichens. Another reason may be that the technique has not been developed for other types of contaminants, either inorganic anions (e.g. fluorines) or organic contaminants (e.g. PCDD/DFs, PAHs, PCBs, etc.). This restricts interpretation of the total concentration of elements as carried out with both mosses and lichens.

Although the extracellular fraction can provide information about potentially toxic elements that may pass to the interior of the cell (and may even be more harmful than those associated with intracellular particles), the greatest problem still existing may be the possible overestimation of the extracellular fraction when the sample contains particulate material on the external surface. In light of the disadvantages of the quantification of the extracellular fraction, quantification of the intracellular fraction appears to offer great advantages: (1) it may not be affected by particles (if

extraction of metal by the extracellular extractant is totally efficient); (2) it enables evaluation of environmental risks; (3) it provides a better representation of the average conditions of contamination to which the organism is subjected; and (4) it enables better evaluation of the phytotoxicity, as it has been related to photosynthesis and respiration in several studies (Brown & Sidhu, 1992; Branquinho *et al.*, 1997a).

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