



## Metal and proton adsorption capacities of natural and cloned *Sphagnum* mosses



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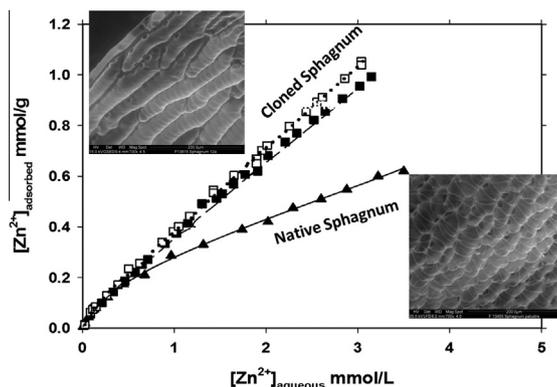
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### HIGHLIGHTS

- Natural and cloned mosses were compared for H and metal adsorption capacities.
- Metal adsorption capacity of mosses is related to DOC excreted by mosses.
- Cloned *S. palustre* has high specific surface area and metal adsorption capacities.
- *S. palustre* clones are optimal biosorbents for moss-bag technique.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Terrestrial mosses are commonly used as bioindicators of atmospheric pollution. However, there is a lack of standardization of the biomonitoring preparation technique and the efficiency of metal adsorption by various moss species is poorly known. This is especially true for *in vitro*-cultivated moss clones, which are promising candidates for a standardized moss-bag technique. We studied the adsorption of copper and zinc on naturally grown *Sphagnum* peat moss in comparison with *in vitro*-cultivated *Sphagnum palustre* samples in order to provide their physico-chemical characterization and to test the possibility of using cloned peat mosses as bioindicators within the protocol of moss-bag technique. We demonstrate that *in vitro*-grown clones of *S. palustre* exhibit acid–base properties similar to those of naturally grown *Sphagnum* samples, whereas the zinc adsorption capacity of the clones is approx. twice higher than that of the samples from the field. At the same time, the field samples adsorbed 30–50% higher amount of  $\text{Cu}^{2+}$  compared to that of the clones. This contrast may be related to fine differences in the bulk chemical composition, specific surface area, morphological features, type and abundance of binding sites at the cell surfaces and in the aqueous solution of natural and cloned *Sphagnum*. The clones exhibited much lower

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concentration of most metal pollutants in their tissues relative to the natural samples thus making the former better indicators of low metal loading. Overall, *in vitro*-produced clones of *S. palustre* can be considered as an adequate, environmentally benign substitution for protected natural *Sphagnum* sp. samples to be used in moss-bags for atmospheric monitoring.

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## 1. Introduction

The use of terrestrial mosses as bioindicators of metal pollutant deposition from the atmosphere has been known since late sixties, when mosses began to be used for monitoring air pollution in Europe [1]. At that time, the scientific community understood that atmospheric pollution constituted one of the greatest challenges for the health of future human generations and for the environment [2–4]. Terrestrial mosses are more frequently used than other sorbents due to their lower cost, widespread presence and high adsorption capacity of pollutants [5]. One of the most commonly used methods for atmospheric biomonitoring is the “moss-bag technique” [6]. Native *Sphagnum* mosses are the most frequently used in the moss-bag technique, followed by *Pseudoscleropodium purum* and *Hypnum cupressiforme* [7]. Despite the broad application of this technique over the past decades, there is still a lack of (i) standardization of sample preparation for the moss-bag technique and (ii) justification of the choice of one particular moss species under variable environmental conditions.

The FP7 European project “MossClone” was focused on the *in vitro*-production of a moss clone and on the standardization of its application in moss-bags for the biomonitoring of air pollution by metals and polyaromatic hydrocarbons (PAHs) (<http://www.mossclone.eu/>). Having standardized the biomaterial and the procedure of exposure, data collected in different locations worldwide could be directly compared to assess the degree of air pollution. For this, it is crucial to conduct a physico-chemical characterization of the species collected from nature in comparison with *in vitro*-cultivated samples. A recent comparative study of several mosses demonstrated that *Sphagnum* sp. exhibits higher stability and metal adsorption capacities than *P. purum*, *Hypnum* sp. and *Brachythecium rutabulum* [8]. In the present study, *Sphagnum denticulatum* from the field and clonal, *in vitro*-produced *Sphagnum palustre* samples [9] were compared in terms of their adsorption capacity with respect to two important metal pollutants, and at the same time, essential micronutrients,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . These metals are also highly contrasting in terms of their affinity to organic ligands, water residence time in the hydration sphere and thus the rates of interaction with organic surfaces. It is thus anticipated that these metals encompass a wide range of physico-chemical properties of other divalent metal cations. *Sphagnum* species are considered as most relevant for the moss-bag technique due to their morphology [10], specifically the high number of pores in the hyalocysts, which help to trap airborne particulates [11]. However, a clear advantage of using *in vitro*-produced *Sphagnum* biomass is due to the fact that peat mosses and peatlands are often protected, e.g. via the European Council Habitat Directive (92/43/EEC) or the Fauna–Flora–Habitat Directive (92/43/EWG).

In the paper at hand, we report on the results of a quantitative comparison between *S. denticulatum* from the field and two clones of *S. palustre* produced under laboratory conditions [9]. Specifically, we aimed at testing whether *S. palustre* clones can be used as surrogate of natural *Sphagnum* mosses in terms of (i) surface area and acid–base properties; (ii) elemental chemical composition of the biomass, and (iii) divalent metal adsorption capacity from aqueous solutions.

## 2. Materials and methods

### 2.1. Chemicals

Copper and zinc nitrate (Sigma–Aldrich) were used as sources of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . The adsorption experiments were carried out in 0.01 M  $\text{NaNO}_3$  and the solutions were prepared with Milli-Q water (18 M $\Omega$ ). Buffer solutions were used in order to keep a constant pH during the adsorption. The MES (Merck) at pH 5.5 and HEPES at pH 6.5 buffers (Sigma–Aldrich) were used for the  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  experiments, respectively.

### 2.2. Moss samples

The clones of *S. palustre* were produced at the University of Freiburg (Germany) from samples collected in the south of Sweden [9]. Two randomly selected clones, called clone 2a and clone 12a, originated from two different spores. The plants were grown in 5 L photobioreactors with liquid Knop medium [12] supplemented with micronutrients, sucrose and ammonium nitrate [9]. Because peat-forming natural *S. palustre* is protected in Europe (92/43/EEC, 92/43EWG), natural species, *S. denticulatum*, was collected in 2012 in NW Spain in a non-urban area. The samples, consisted of mostly apical, green parts, were rinsed 3 times in Milli-Q water and oven-dried at 120 °C.

### 2.3. Moss characterization

The elemental composition of peat mosses was measured after full acid digestion via microwave (MARS 5 system CEM) treatment in the A 10,000 Clean Laboratory Facilities of GET (CNRS-Toulouse). Full acid digestion was carried out on 100 mg of dried plant material with bidistilled  $\text{HNO}_3$  (9 mL), suprapure HF (0.2 mL; Merck) and suprapure  $\text{H}_2\text{O}_2$  (1 mL; Merck) in 20 mL Teflon containers. The digestion conditions were as follows: 1600 W for 8 min, keeping the temperature at 150 °C for 20 min. The standard used was BCR-482 lichen (Certified Reference Material, IRMM). The final solution (after digestion) was evaporated at 60 °C in a hot plate and re-diluted in 2%  $\text{HNO}_3$ . All major and trace elements except Si were measured by ICP-MS (Agilent 7500 ce series) with a detection limit of 0.001–0.0003  $\mu\text{g g}^{-1}$  and precision of  $\pm 5\%$ . Further details of moss analyses are given in Ref. [13].

The specific surface area (SSA) of peat moss samples was measured using the B.E.T.  $\text{N}_2$  multipoint adsorption technique using the Quantachrome Autosorb Automated Gas Sorption System with 10–14 adsorption points after 24 h of degassing at 120 °C. The typical uncertainty of these measurements was 10%.

The surface acid–base titration was conducted in 0.01 M  $\text{NaNO}_3$  at room temperature ( $20 \pm 1$  °C). Aqueous suspension of 1  $\text{g}_{\text{dw}} \text{L}^{-1}$  was conditioned for 1 h and pre-saturated with  $\text{N}_2$  at pH from 3 to 11. Acidic and basic titration was performed in two steps by adding aliquots of 0.1 M HCl and 0.1 M NaOH, respectively. All titration experiments were performed in triplicates. The reference solution was a biomass-free supernatant solution after 1 h conditioning under  $\text{N}_2$  bubbling. The pH was measured by a combined electrode (Mettler Toledo<sup>®</sup>) in a pH-meter ion analyzer (pHM250-Meterlab<sup>™</sup>) with an uncertainty of

$\pm 0.002$  units. The excess of charge was computed as the difference of the acid/base concentration in the suspension and in the reference solution according to common procedures of biomass titration [14,15]. Dissolved Organic Carbon (DOC) leached from the moss biomass during the experiments was monitored using a Carbon Total Analyzer (Shimadzu TOC-V<sub>CSN</sub>) with an uncertainty of 2% and a detection limit of  $0.1 \text{ mg L}^{-1}$ , as a function of pH, at experimental conditions similar to those of the titration and metal adsorption.

#### 2.4. Metal adsorption experiments

The peat mosses were rinsed 3 times with Milli-Q (18 M $\Omega$ ) and devitalized at 120 °C overnight [16]. Oven treatment allows devitalization of the biomass, thus eliminating the metabolic influence on the adsorption or uptake of metals, reducing the data variability [17–20]. Intact tissue was always used without grinding or disaggregation in order to meet the typical conditions of the moss-bag technique. The biomass was kept constant at  $1 \text{ g}_{\text{dry}} \text{ L}^{-1}$  for all the adsorption experiments.

The adsorption experiments were carried out following the protocol elaborated for field samples of mosses [8]. The adsorption of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  was investigated in 0.01 M  $\text{NaNO}_3$  as a function of pH and total metal concentration in solution. The aqueous solution was undersaturated with respect to the metal oxide or carbonate as verified by speciation calculations with the Visual Minteq computer code [21]. The experiments were conducted in polypropylene beakers at room temperature ( $20 \pm 1 \text{ }^\circ\text{C}$ ) and agitated with a suspended Teflon coated magnetic stirrer under continuous nitrogen bubbling.

The pH-edge adsorption experiments were performed at a constant initial concentration of  $\text{Cu}^{2+}$  ( $52 \text{ } \mu\text{mol L}^{-1}$ ) and  $\text{Zn}^{2+}$  ( $51 \text{ } \mu\text{mol L}^{-1}$ ). The pH ranged from 1.8 to 6.7 and from 1.8 to 9.9 for the  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  experiments, respectively. The pH was adjusted by adding aliquots of NaOH (0.1–0.01 M) or  $\text{HNO}_3$  (0.1–0.01 M). The adsorption of metals as a function of their concentration in solution was carried out in the range of  $5\text{--}3624 \text{ } \mu\text{mol L}^{-1}$  for  $\text{Cu}^{2+}$  and  $4\text{--}3500 \text{ } \mu\text{mol L}^{-1}$  for  $\text{Zn}^{2+}$ . The pH was kept constant at  $\sim 5.5$  and  $\sim 6.5$  for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , respectively. The elapsed time between each metal addition or the pH change was always 10 min, sufficient for establishing the adsorption equilibrium according to previous kinetic experiments [8]. The control solution for adsorption experiments was  $0.45 \text{ } \mu\text{m}$ -filtered moss leachate produced by interaction of the moss biomass with metal-free inert electrolyte during 1 h.

Sampled solution was filtered ( $0.45 \text{ } \mu\text{m}$ , acetate cellulose single used filter), acidified with bidistilled  $\text{HNO}_3$ , and analyzed for aqueous metal concentration using flame atomic adsorption spectroscopy (Perkin Elmer Analyst 400) with an uncertainty of  $\pm 2\%$  and a detection limit of  $0.05 \text{ mg L}^{-1}$ .

The results of the acid–base titration and metal adsorption experiments were treated with a Linear Programming Model (LPM) in order to compute the apparent equilibrium constants and the site densities for each individual experiment following the approaches elaborated for bacteria [14,15,22–25]. This model is highly appropriate for describing complex 3-D multi-layer systems with both organic components and rigid cell walls [23,26,27] and is capable of adequately describing proton and metal adsorption on natural mosses [8].

To account for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  complexation with organic ligands leached from the biomass, we used the NICA-Donnan model and Soil Humic Model (SHM) for natural organic complexation with metals (see Ref. [28] for application to aqueous solutions) implemented to vMinteq (version 3.1, Gustafsson [21]).

### 3. Results

#### 3.1. Specific surface area and elemental composition of the analyzed peat mosses

The clones exhibited a higher B.E.T. surface area (clone 2a =  $24 \pm 1 \text{ m}^2 \text{ g}_{\text{dry}}^{-1}$ ; clone 12a =  $28 \pm 1 \text{ m}^2 \text{ g}_{\text{dry}}^{-1}$ ) compared to natural samples ( $10.8 \pm 0.3 \text{ m}^2 \text{ g}_{\text{dry}}^{-1}$ ), as determined by 10–14 points adsorption isotherm, with a correlation coefficient that was always higher than 0.99. The differences between clones and natural samples were statistically significant ( $p < 0.05$ ). We found that the reliable SSA measurements require degassing at 120 °C overnight. Heating in vacuum at 80 °C did not yield sufficient degassing and either  $\text{N}_2$  adsorption isotherm could not be produced or significant ( $>20\%$ ) underestimation of  $\text{SSA}_{\text{B.E.T.}}$  occurred.

Elemental composition is one of the key factors controlling the adsorption capacity of different biosorbents, notably at low metal concentration in the environment. These results are listed in Table 1. For comparison, metal content of *S. denticulatum* from the field [8] and the average of 11 *Sphagnum* species from pristine western Siberian habitats [13] are also listed. The clones reflect the composition of the culture medium because the strains were not exposed to any atmospheric pollution. Among  $\sim 40$  major and trace elements, only nutrients P and Mo were slightly higher in the clones compared to the samples from nature. Another nutrient, K, exhibited a concentration in clones that was lower than that in field samples and Siberian *Sphagnum*. All other major and trace elements were significantly higher in natural *Sphagnum*, reflecting metal deposition from the atmosphere in their original location. In addition, the elemental composition of natural *Sphagnum* could be affected by soil particles and flowing surface waters as reflected in its elevated Al and Fe concentration (Table 1). Thus, *S. denticulatum* contained  $3.55 \text{ mg kg}^{-1}$  and  $36.0 \text{ mg kg}^{-1}$  of Cu and Zn, respectively, which is higher than both clones by a factor of 2–4. The other major and trace metals were lower by a factor of 2–10 in laboratory clones compared to field samples of *S. denticulatum*. The impoverishment of clones versus natural samples was especially remarkable for Al ( $\times 300$ ), V ( $\times 400$ ), Ga ( $\times 100$ ), As ( $\times 500$ ), Cd ( $>1000$ ), REEs ( $>1000$ ) and Pb ( $\times 400$ ).

#### 3.2. Acid–base titration

The results of the surface acid–base titration carried out at pH 3–10 in triplicates for 120 °C-devitalized mosses are depicted in Fig. 1. The pH value corresponding to zero net proton adsorption ( $\text{pH}_{\text{PZC}}$ ) was equal to  $4.5 \pm 0.1$  for the peat moss sample from the field, which was significantly lower than that of the clones (clone 2a:  $5.65 \pm 0.05$ , clone 12a:  $5.60 \pm 0.06$ ). The excess of charge was similar for both clones, and the magnitude of the surface charge of the clones was significantly higher than that of the field sample.

The experimental data of  $\text{H}^+/\text{OH}^-$  adsorption were fitted by LPM to calculate the acidity constants which could be linked to functional groups on the surface of the cells (Table 2). All analyzed peat mosses contained five possible functional groups: carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols with total binding sites of  $0.648 \text{ mmol g}_{\text{dry}}^{-1}$  (field sample),  $1.56 \text{ mmol g}_{\text{dry}}^{-1}$  (clone 2a) and  $1.31 \text{ mmol g}_{\text{dry}}^{-1}$  (clone 12a). The two clones had 1.04 and  $0.742 \text{ mmol g}_{\text{dry}}^{-1}$  of carboxyl/phosphodiester ( $\text{pK}_a = 3.3\text{--}5.8$ ) respectively, which was higher by a factor of 2–3 than that of *S. denticulatum*, having only  $0.355 \text{ mmol g}_{\text{dry}}^{-1}$ . In terms of the phosphoryl groups, clone 12a exhibited one order of magnitude higher value than that of clone 2a ( $0.817$  and  $0.079 \text{ mmol g}_{\text{dry}}^{-1}$ , respectively) and the field sample ( $0.043 \text{ mmol g}_{\text{dry}}^{-1}$ ). The clones also exhibited a 1.5 times higher concentration of surface amines ( $0.16\text{--}0.18 \text{ mmol g}_{\text{dry}}^{-1}$ ,

**Table 1**

Major and trace elemental composition of natural *Sphagnum denticulatum* (collected from the field as described in [8]), natural *Sphagnum* sp. collected in pristine regions of western Siberia across a 1800 km profile (average of 11 sites with 2 s.d., [13]) and laboratory clones *S. palustre* (2a and 12a). The detection limit is approx. 0.0003 mg kg<sup>-1</sup>. N.D. stands for non-determined.

Element	[Me] mg kg <sup>-1</sup>			
	Siberian <i>Sphagnum</i>	Field samples	Clone 2A	Clone 12A
Be	N.D.	0.23	<0.05	<0.05
B	7.0 ± 4.3	0.471	0.576	0.557
Na	337 ± 155	525	0.726	N.D.
Mg	572 ± 183	1630	378	387
Al	1336 ± 1143	1048	2.30	2.56
P	360 ± 133	390	502	545
K	1917 ± 1265	2595	1151	1567
Ca	1696 ± 749	3365	846	906
Ti	89 ± 72	16.5	0.611	0.744
V	2.4 ± 2.0	1.21	0.003	0.002
Cr	2.2 ± 1.4	0.460	0.282	0.107
Mn	82.7 ± 73	162	30.6	33.1
Fe	1224 ± 908	1146	150	141
Co	0.51 ± 0.32	0.238	0.034	0.034
Ni	1.77 ± 1.0	0.935	0.176	0.085
Cu	2.66 ± 1.4	3.55	0.803	0.605
Zn	27.9 ± 11.1	36.0	18.1	19.0
Ga	0.41 ± 0.3	0.232	0.002	0.003
Ge	N.D.	0.059	<0.01	<0.01
As	1.11 ± 0.4	0.524	0.001	<0.001
Rb	6.69 ± 4.25	10.22	0.047	0.063
Sr	11.2 ± 9.4	43.79	0.334	0.348
Zr	2.3 ± 1.8	0.287	0.039	0.039
Nb	N.D.	0.105	0.003	0.002
Mo	0.24 ± 0.2	0.204	1.567	1.853
Ag	N.D.	0.011	0.002	0.002
Cd	0.43 ± 0.2	0.203	<0.001	<0.001
Cs	0.23 ± 0.1	0.228	<0.002	<0.002
Ba	22.7 ± 9.4	44.7	0.047	0.048
La	0.56 ± 0.4	32.1	0.011	0.010
Ce	1.16 ± 0.93	6.83	0.013	0.011
Pr	0.13 ± 0.1	3.357	0.002	0.002
Nd	0.51 ± 0.4	12.6	0.010	0.008
Sm	0.10 ± 0.08	1.68	0.002	0.001
Eu	0.02 ± 0.02	0.168	<0.0003	<0.0003
Gd	0.09 ± 0.07	1.65	0.002	0.001
Dy	0.081	0.833	0.001	0.001
Ho	0.014	0.144	<0.0003	<0.0003
Er	0.043	0.371	0.001	0.001
Tm	0.007	0.039	<0.0003	<0.0003
Yb	0.04	0.245	<0.0003	<0.0003
Lu	0.007	0.026	<0.0003	<0.0003
Hf	0.16 ± 0.15	0.019	0.002	0.001
Ta	N.D.	0.019	0.001	≤d.l.
Pb	8.6 ± 4.7	42.9	0.129	0.057
Th	0.08 ± 0.06	0.033	<0.0003	<0.0003
U	0.07 ± 0.06	0.043	<0.0003	<0.0003

$pK_a = 8.05\text{--}9.35$ ) and polyphenols (0.29–0.24 mmol g<sub>dry</sub><sup>-1</sup>,  $pK_a \sim 10.30$ ) relative to natural sample (0.09 and 0.16 mmol g<sub>dry</sub><sup>-1</sup>, respectively). Consequently, the peat moss grown under natural conditions demonstrated a 1.5–2 times lower mass-normalized binding site density compared to the clones, and thus their metal adsorption capacities should also be lower than those of clones.

### 3.3. Adsorption of Cu<sup>2+</sup> and Zn<sup>2+</sup> as a function of pH

Results of adsorption of Cu<sup>2+</sup> and Zn<sup>2+</sup> onto peat mosses as a function of solution pH are plotted in Fig. 2 and the LPM adsorption parameters are listed in Table 3. The adsorption of Cu<sup>2+</sup> achieved the maximum at pH 6.2–6.7 and was 73–75% of the initial amount for the clones and 82% for the field samples. The 50% removal of initially added metal occurred at pH 4.4 and 4.0–4.1 for both field sample and *in vitro*-cultivated clones. In the case of Zn<sup>2+</sup>, the maximum adsorption was achieved at pH 7.3–7.6 and ranged from 72%

(field sample) to 82% (clone 12a). The 50% of initially loaded Zn<sup>2+</sup> was adsorbed at pH 5.2, 5.4 and 3.6 for the field sample, clone 2a and clone 12a respectively.

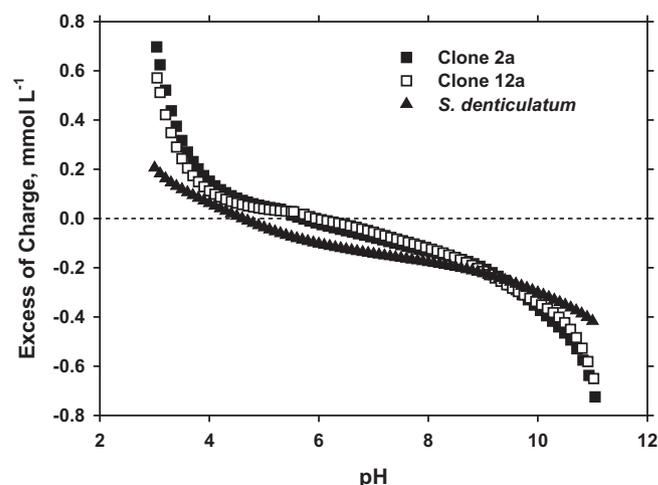
The analyses of obtained LPM parameters including the equilibrium constant ( $pK_s$ ) and available binding sites demonstrated that there are significant differences in Cu<sup>2+</sup> adsorption parameters between *Sphagnum* moss from nature and clones from *in vitro* culture: natural *Sphagnum* had two  $pK_s$  values (–2.20 and –0.15), while clone 2a exhibited three  $pK_s$  (–1.25, –0.15 and 2.60), and clone 12a had four  $pK_s$  (–1.45, –0.45, –0.20 and 3.0). Note that, within the LPM formalism, the lower the  $pK_s$  value, the stronger the interaction between the metal and the binding site. In this study, the lowest  $pK_s$ , at –2.20, was achieved for the field samples. The total concentration of Cu<sup>2+</sup>-binding sites was slightly higher in clones 2a and 12a (48 and 49  $\mu\text{mol g}_{\text{dry}}^{-1}$ , respectively) compared to natural *Sphagnum* (43  $\mu\text{mol g}_{\text{dry}}^{-1}$ ).

The equilibrium constants for the pH edge of Zn<sup>2+</sup> adsorption ranged from –1.55 to 1.65. Both *S. palustre* clones presented a slightly higher concentration of binding sites relative to the field sample: 32, 37 and 43  $\mu\text{mol g}_{\text{dry}}^{-1}$  for the field sample, clone 2a and clone 12a respectively. Clone 12a exhibited the lowest value of  $pK_s$  (–1.55) and the highest level of binding sites (42.5  $\mu\text{mol g}_{\text{dry}}^{-1}$ ).

The DOC concentration in pH-dependent adsorption experiments with these three studied peat moss samples is shown in Fig. 3. Natural *S. denticulatum* excreted a factor of 3–4 lower amount of DOC compared to the clones of *Sphagnum palustre*. Namely, peat moss from the field produced 14.5 mg L<sup>-1</sup> to 20.5 mg L<sup>-1</sup> of DOC from pH 1.7 to 10.9. For the same pH range (3.4–9.4), clone 2a excreted a slightly higher amount of DOC than clone 12a (41–49 mg L<sup>-1</sup>, and 28–37 mg L<sup>-1</sup>, respectively).

### 3.4. Adsorption of Cu<sup>2+</sup> and Zn<sup>2+</sup> as a function of metal concentration in solution

The results of Cu<sup>2+</sup> and Zn<sup>2+</sup> adsorption as a function of these metal concentrations in solution are shown in Fig. 4. The LPM fitting parameters are listed in Table 3. The “Langmuirian” adsorption isotherms were obtained at constant pH of ~5.5 and ~6.5 for Cu<sup>2+</sup> and Zn<sup>2+</sup>, respectively, and the metal concentration ranged from 4 to 3920  $\mu\text{mol L}^{-1}$ . The field sample demonstrated an approx. 30% higher adsorption capacity of Cu<sup>2+</sup> in comparison with *in vitro*-cultivated *S. palustre*. Although the total binding site concentration



**Fig. 1.** Surface acid–base titration of *S. denticulatum* and *S. palustre* clones in 0.01 M NaNO<sub>3</sub>. The conditioning time was equal to 1 h and the biomass was kept constant at 1 g<sub>dry</sub> L<sup>-1</sup>. All the measurements were triplicated.

**Table 2**

Surface acid–base titration and LPM model results for *S. denticulatum* and *S. palustre* clones produced in the laboratory reactors. The experiments were performed in 0.01 M NaNO<sub>3</sub> electrolyte solution with a constant biomass of 1 g<sub>dry</sub> L<sup>-1</sup> in triplicates.

Species	pK <sub>a</sub>	Binding sites (mmol g <sub>dry</sub> <sup>-1</sup> )	Functional groups
<i>Sphagnum denticulatum</i> (field sample)	3.50	0.205	Carboxyl-Phosphodiester
	4.85	0.108	Carboxyl
	5.85	0.042	Carboxyl
	6.55	0.020	Phosphoryl
	7.20	0.023	Phosphoryl
	8.05	0.028	Amine
	9.15	0.066	Amine
	10.30	0.156	Polyphenol
<i>Sphagnum palustre</i> (clone 2a)	3.30	0.920	Carboxyl-Phosphodiester
	5.80	0.120	Carboxyl
	7.35	0.079	Phosphoryl
	8.10	0.006	Amine
	9.35	0.154	Amine
	10.30	0.285	Polyphenol
<i>Sphagnum palustre</i> (clone 12a)	3.30	0.742	Carboxyl-Phosphodiester
	6.10	0.743	Phosphoryl
	7.25	0.074	Phosphoryl
	8.10	0.021	Amine
	9.05	0.161	Amine
	10.35	0.237	Polyphenol

was equal to 1.07 mmol g<sub>dry</sub><sup>-1</sup>, 24.0 mmol g<sub>dry</sub><sup>-1</sup> and 1.90 mmol g<sub>dry</sub><sup>-1</sup> for field samples, clone 2a and 12a respectively, the stabilities of these sites for the clones were lower, as reflected in their pK<sub>m</sub> values of Cu adsorption (Table 3). In the case of Zn<sup>2+</sup>, the clones demonstrated a factor of 2 higher adsorption capacities relative to the field samples. The total number of Zn-binding sites was equal to 94.1 mmol g<sub>dry</sub><sup>-1</sup>, 157 mmol g<sub>dry</sub><sup>-1</sup> and 750 mmol g<sub>dry</sub><sup>-1</sup> for the field sample, clone 2a and clone 12a respectively.

In addition to the LPM, experiments were fitted by a Langmuirian adsorption isotherm, often used for description of metal adsorption on biosorbents [29]. Here, it allowed rationalizing the adsorption data according to:

$$\frac{[Me^{2+}]_{aq}}{[Me^{2+}]_{ads}} = \frac{1}{K_L q_{max}} + \frac{[Me^{2+}]_{aq}}{q_{max}} \quad (1)$$

where K<sub>L</sub> is the Langmuir equilibrium (g<sub>dry</sub> mmol<sup>-1</sup>) constant and q<sub>max</sub> is the maximum adsorption capacity (mmol g<sub>dry</sub><sup>-1</sup>). All the experiments were adequately fitted by Eq. (1) with R<sup>2</sup> > 0.98; the results are listed in Table 4. The maximum adsorption capacity of copper was achieved for the field sample (1.25 mmol g<sub>dry</sub><sup>-1</sup>), and that of Zn<sup>2+</sup> was a factor of 4 higher for clones compared to the field sample.

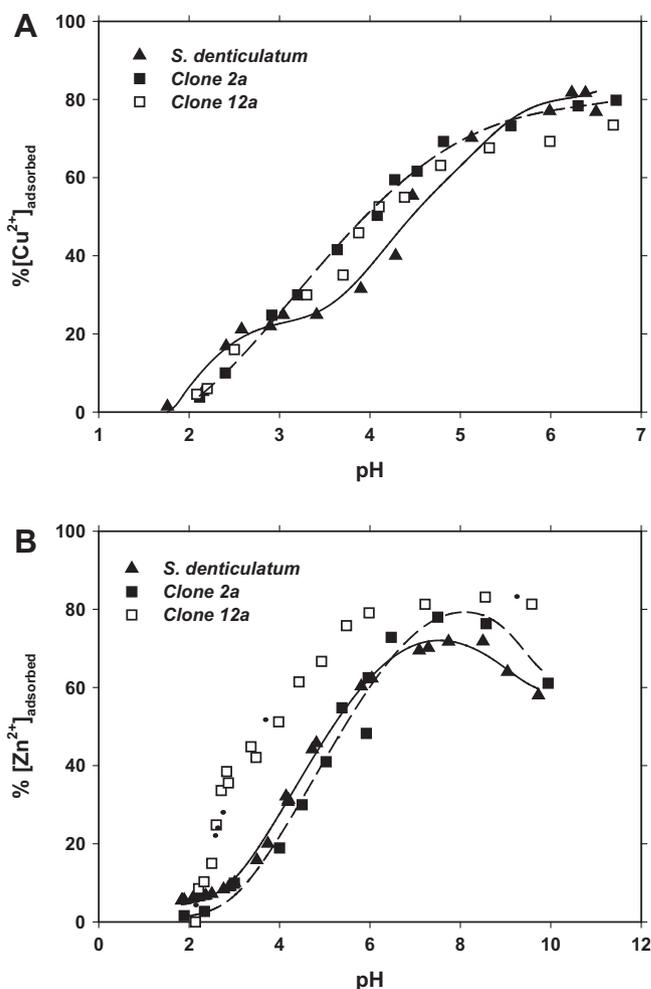
The results of thermodynamic modeling of Cu and Zn complexation with DOM leached from peat mosses at pH of 5.4 ± 0.1 and 6.5 ± 0.1 for Cu and Zn, respectively, are shown in Fig. 5. It can be seen that at typical metal concentration in solution (0.4 mmol L<sup>-1</sup>) the difference in Cu-free concentration between 15 and 45 ppm DOM solution is a factor of 1.7 while for Zn this factor is 1.4. At lower concentration of Cu, >99% of this metal may be complexed with aqueous organic ligands, whereas for Zn this amount never exceeds 84%. In other words, at otherwise similar aqueous metal concentration, the concentration of free Zn<sup>2+</sup> available for adsorption onto clones is more than an order of magnitude higher than that of free Cu<sup>2+</sup>. Note that the modeling of Cu and Zn complexation within SHM formalism yielded a very similar degree of complexation with DOM as a function of metal concentration. However, it is not ruled out that the organic complexes of metals

can also be adsorbed on the moss surfaces which make rigorous modeling of aqueous and surface complexation very difficult at the present time.

#### 4. Discussion

The B.E.T. measurements demonstrated that *in vitro*-grown clones had a factor of 2 higher specific surface areas than the field samples. According to morphological observations, shoots of *S. palustre* from the field are thicker and heavier than of those grown *in vitro* [9]. The average dry weight of field shoots (n = 10) was 14.76 ± 7.2 mg, while the *in vitro*-cultivated material had an average shoot (n = 13) weight of 2.72 ± 1.13 mg. Another rather variable morphological trait was the number of pores per hyalocyst: 3–16 pores for native moss and 2–3 pores for the clones of *S. palustre* [9]. However, in virtue of a high variability, the clones showed significantly lower and constant number of pores per hyalocyst to native ones. These results confirm that cloned species are less variable in surface morphology than natural samples. Therefore, cloned peat mosses are highly beneficial for the biomonitoring relative to the natural samples.

The SSA measured for *S. denticulatum* from the field (10.8 m<sup>2</sup> g<sub>dry</sub><sup>-1</sup>) is significantly higher than the geometric surface area of the

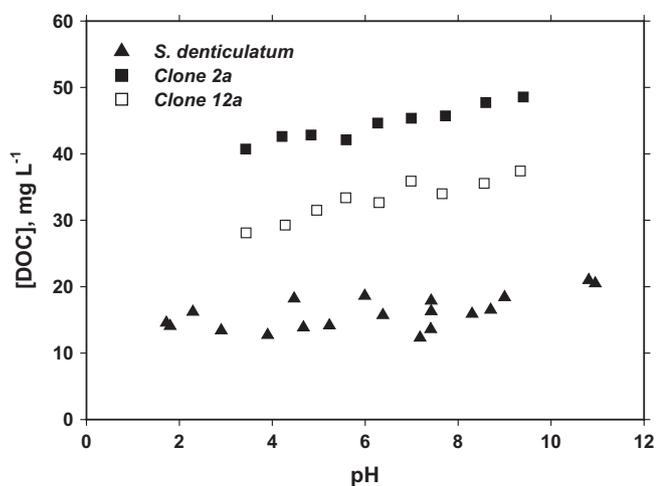


**Fig. 2.** Adsorption of Cu<sup>2+</sup> (A) and Zn<sup>2+</sup> (B) onto *S. denticulatum* and *S. palustre* clones as a function of pH, in 0.01 M NaNO<sub>3</sub> with constant biomass of 1 g<sub>dry</sub> L<sup>-1</sup>. The initial metal loading was 52 and 50.5 μmol L<sup>-1</sup> for Cu<sup>2+</sup> and Zn<sup>2+</sup>, respectively. The lines represent the LPM fit to the data.

**Table 3**

List of experiments performed in this study, experimental conditions and LPM parameters. All metal adsorption experiments were performed in 0.01 M NaNO<sub>3</sub> with a constant biomass 1 g<sub>dry</sub> L<sup>-1</sup>. K<sub>s</sub> and K<sub>m</sub> correspond with the equilibrium constant for the reaction between metal in solution and the available sites as a function of pH and metal aqueous concentration in solution, respectively.

Metal	Type of study	Species	pH-range	[Me <sup>2+</sup> ] <sub>0</sub> , μM	pK <sub>s</sub> /pK <sub>m</sub>	Binding sites (mol g <sub>dry</sub> <sup>-1</sup> )	
Cu	pH-edge	<i>Sphagnum denticulatum</i> (field sample)	1.8–6.5	51.94	-2.20	1.03 · 10 <sup>-5</sup>	
						-0.15	3.29 · 10 <sup>-5</sup>
						-1.25	2.80 · 10 <sup>-5</sup>
		<i>Sphagnum palustre</i> (Clone-2a)	2.1–6.7			-0.15	1.27 · 10 <sup>-5</sup>
						2.60	7.66 · 10 <sup>-6</sup>
						-1.45	2.07 · 10 <sup>-5</sup>
						-0.45	1.39 · 10 <sup>-5</sup>
						-0.20	2.60 · 10 <sup>-6</sup>
						3.0	1.18 · 10 <sup>-5</sup>
Zn	pH-edge	<i>Sphagnum denticulatum</i> (field sample)	1.8–9.7	50.48	-0.80	1.44 · 10 <sup>-5</sup>	
						0.55	1.79 · 10 <sup>-5</sup>
						-0.8	8.09 · 10 <sup>-6</sup>
		<i>Sphagnum palustre</i> (Clone-2a)	1.9–9.9			-0.35	8.98 · 10 <sup>-6</sup>
						0.95	2.10 · 10 <sup>-6</sup>
						-1.55	3.28 · 10 <sup>-5</sup>
						0.35	5.52 · 10 <sup>-6</sup>
						1.65	4.15 · 10 <sup>-6</sup>
Cu	Constant pH isotherm	<i>Sphagnum denticulatum</i> (field sample)	5.53 ± 0.03	5–3624	0.50	2.33 · 10 <sup>-5</sup>	
						2.45	1.05 · 10 <sup>-3</sup>
						0.6	4.39 · 10 <sup>-6</sup>
		<i>Sphagnum palustre</i> (Clone-2a)	5.4 ± 0.1	4–3920		2.05	4.57 · 10 <sup>-4</sup>
						5.30	2.35 · 10 <sup>-2</sup>
						1.60	2.03 · 10 <sup>-4</sup>
						3.25	1.67 · 10 <sup>-3</sup>
Zn	Constant pH isotherm	<i>Sphagnum denticulatum</i> (field sample)	6.7 ± 0.1	7–3500	2.75	2.16 · 10 <sup>-4</sup>	
						6.30	9.39 · 10 <sup>-2</sup>
						1.80	6.23 · 10 <sup>-5</sup>
		<i>Sphagnum palustre</i> (Clone-2a)	6.48–0.04	4–3145		3.60	1.33 · 10 <sup>-4</sup>
						6.10	0.157
						2.00	1.02 · 10 <sup>-4</sup>
						6.70	0.747



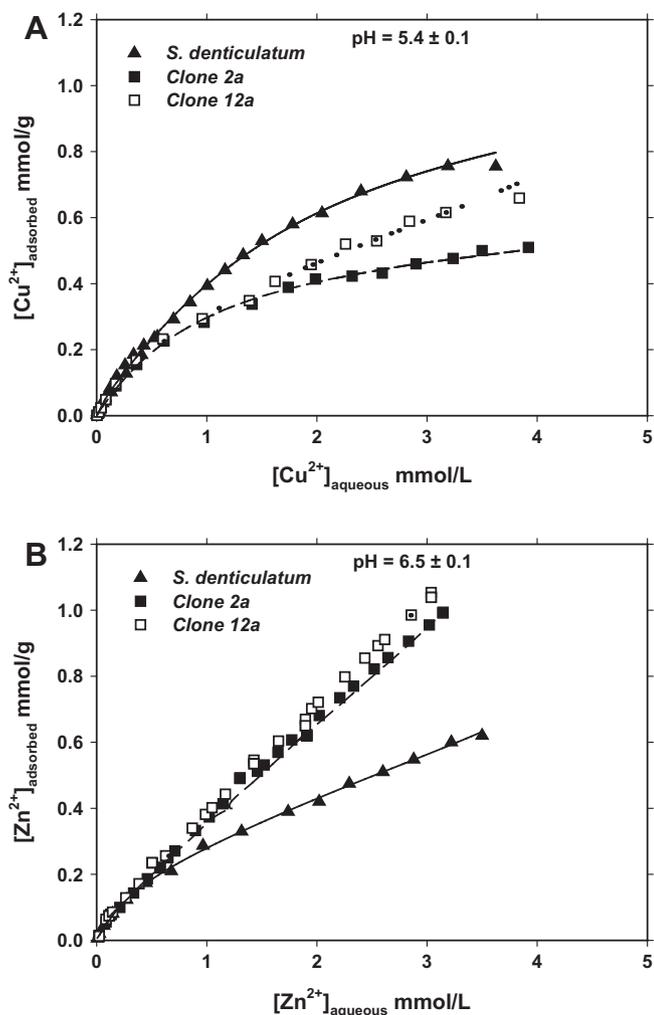
**Fig. 3.** Dissolved Organic Carbon (DOC) excreted by *S. denticulatum* from the field and *S. palustre* clones as a function of pH in 0.01 M NaNO<sub>3</sub> with constant biomass of 1 g<sub>dry</sub> L<sup>-1</sup>.

moss *H. cupressiforme* (0.136 m<sup>2</sup> g<sup>-1</sup>) and the lichen *Pseudevernia furfuracea* (0.027 m<sup>2</sup> g<sup>-1</sup>), Ref. [17], while it is comparable with SSA of Danish peat (13.3 m<sup>2</sup> g<sup>-1</sup>) and Heilongjiang peat (9.67 m<sup>2</sup> g<sup>-1</sup>), Ref. [30]. In terms of acidity constants, the functional groups were carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols, corresponding to pK<sub>a</sub> of ~3.30 to 3.50, 4.85–5.85, 6.10–7.35, 8.05–9.35 and 10.30–10.35, respectively. Note that the most relevant functional groups for metal binding on peat mosses are carboxylates and phenolates [31]. Natural *S. denticulatum* had ~55% less mass-normalized total binding sites compared to clones. This difference disappears when normalizing

the site number to the SSA<sub>B,E,T</sub>, which is a factor of 2 higher for clones compared to the field sample. As such, proton-binding properties of clone surfaces can be considered very similar to those of natural *Sphagnum*.

Langmuirian isotherms suggested that the field sample is able to adsorb 46–13% higher amounts of Cu<sup>2+</sup> ( $q_{\max} = 1.25 \text{ mmol g}_{\text{dry}}^{-1}$ ) compared to clones ( $q_{\max} = 0.67$  and  $1.09 \text{ mmol g}_{\text{dry}}^{-1}$  for clone-2a and 12a, respectively). However, the capacity of clones to adsorb Zn<sup>2+</sup> is 76–80% higher ( $q_{\max} = 3.87$  and  $4.60 \text{ mmol g}_{\text{dry}}^{-1}$  for clones 2a and 12a, respectively) than that of *S. denticulatum* ( $q_{\max} = 0.93 \text{ mmol g}_{\text{dry}}^{-1}$ ).

The Langmuirian equation, valid at specific and constant pH of aqueous solution, assumes that (1) adsorption is limited to the formation of a monolayer and (2) all surface sites have the same energy or equal affinity for metal [32,33]. The LPM can be considered as multi-langmuirian model with each site having its own adsorption energy and affinity [22]. In this regard, two approaches are not quantitatively comparable, and the sum of all binding sites in the LPM (Table 3, constant-pH isotherm) is always higher than the  $q_{\max}$  value of Eq. (1) (Table 4). In fact, the contribution of each site in the LPM may be pronounced at very narrow pH or [Me<sup>2+</sup>] concentration, since their complexation constants differ by 3–4 orders of magnitude. Therefore, the sum or the average of multiple LPM parameters cannot be compared with one single site number and stability constant of the langmuirian formalism. Note however, that the results of the present study are consistent with previous evaluations of langmuirian adsorption parameters of biological surfaces, as compiled in Refs. [34,35]. For example, the values of  $q_{\max}$  of this study are also comparable with those reported for *P. purum*, *Hypnum sp.* and *B. rutabulum* [8] and higher than that of aquatic plants [36–38], fungi [39,40], bacteria [41,42] and land plants [43].



**Fig. 4.** Adsorption of  $\text{Cu}^{2+}$  (A) and  $\text{Zn}^{2+}$  (B) onto *S. denticulatum* from the field and *S. palustre* clones as a function of metal concentration in 0.01 M  $\text{NaNO}_3$  with constant biomass ( $1 \text{ g}_{\text{dry}} \text{ L}^{-1}$ ). The pH was fixed at  $\sim 5.5$  and  $\sim 6.5$  for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , respectively. The aqueous metal concentration ranged from 4 to 3920 and from 4 to  $3500 \mu\text{mol L}^{-1}$  for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , respectively. The lines represent the LPM fit to the data.

The solution pH is one of the most important parameters affecting the biosorption of heavy metal ions [44,45]. Increasing the pH in solution leads to deprotonation of the available sites on peat moss surface layers, which thus increases the adsorption capacity with the pH rise. The adsorption of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  followed the “universal pH-edge adsorption pattern” already identified for mosses [8], cyanobacteria [25], phototrophic bacteria [15], heterotrophic bacteria [46–48] and diatoms [49]. The most significant

**Table 4**

Langmuir parameters computed from the experiments at different aqueous metal concentrations listed in Table 3 and plotted in Fig. 3 (Langmuirian isotherm).

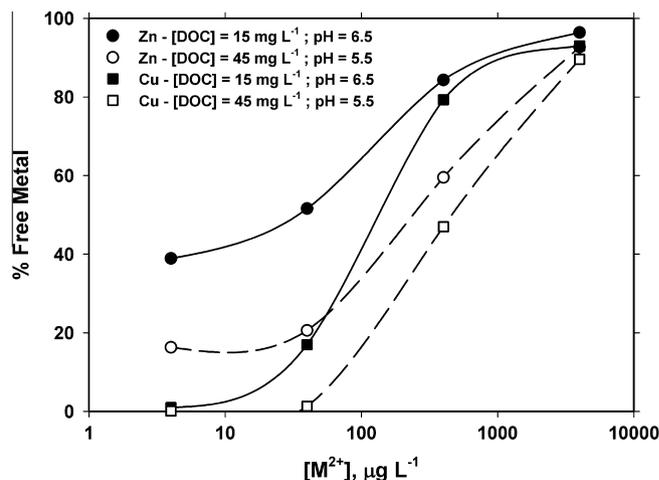
Species	$q_{\text{max}}$ ( $\text{mmol g}_{\text{dry}}^{-1}$ )	$K_L$ ( $\text{g}_{\text{dry}} \text{ mmol}^{-1}$ )
<b>Copper</b>		
<i>Sphagnum denticulatum</i> (field)	1.25	0.47
<i>Sphagnum palustre</i> (clone 2a)	0.67	0.78
<i>Sphagnum palustre</i> (clone 12a)	1.09	0.39
<b>Zinc</b>		
<i>Sphagnum denticulatum</i> (field)	0.93	0.44
<i>Sphagnum palustre</i> (clone 2a)	3.87	0.11
<i>Sphagnum palustre</i> (clone 12a)	4.60	0.09

$\text{Zn}^{2+}$  adsorption capacity was found for clone 12a, when 50% of  $\text{Zn}^{2+}$  was adsorbed at pH 3.6, which are 2 pH units lower than that for clone 2a and the samples from nature. The clone 12a also exhibited the lowest  $\text{pK}_s$  value ( $-1.55$ ) and the highest amount of total binding sites ( $49.0 \mu\text{mol g}_{\text{dry}}^{-1}$ ).

The production of DOM can affect the speciation of metals in solution and thus interfere in the adsorption process via decreasing the activity of free metal available for adsorption [50]. The nature of this DOM cannot be characterized unambiguously but presumably comprise cell exometabolites and nutrient organic compounds trapped in the surface pores. Given that the  $\text{SSA}_{\text{B.E.T.}}$  of clones is a factor of 2 higher than that of native *Sphagnum*, the retaining DOM capacity of clones is significantly higher. Copper is known to be strongly bound by organic ligands excreted by organisms [51]. These authors compiled the following order of the metal affinity complexation by natural organic ligands:  $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ , with the conditional stability constant of  $\text{Cu}^{2+}$  complexes being several orders of magnitude higher than that of  $\text{Zn}^{2+}$  ( $\log K$  of 13.5–16 and 7.8–9.5, respectively). Hirose [52] also reported a much stronger affinity of  $\text{Cu}^{2+}$  to organic ligands compared to that of  $\text{Zn}^{2+}$ . Moreover, Smith, Bell and Kramer [53] compiled the data from 13 carboxylic, 4 amine, 19 amino-carboxylic and 5 sulfidic-thiolate ligand compounds and demonstrated that the stability constant was significantly higher for  $\text{Cu}^{2+}$  than for  $\text{Zn}^{2+}$ .

The concentration of DOC excreted by the studied peat mosses (Fig. 3) thus may explain the difference in the adsorption capacity with respect to the two studied metals (Fig. 4A). The speciation calculation demonstrated that  $\text{Cu}^{2+}$  was significantly more bound to organic ligands produced by peat moss reaction with aqueous solution than  $\text{Zn}^{2+}$ . For this reason, only small fraction of total dissolved  $\text{Cu}^{2+}$  was present in free forms, available for adsorption (Fig. 5). This amount, however, was much higher for natural sample versus clone due to 2–3 times lower DOC concentration in the former (Fig. 3). As such, natural *Sphagnum* could adsorb more  $\text{Cu}^{2+}$  from solution than the clones. Based on the results of these calculations, and considering the divalent metal affinity to organic ligands [51], we expect that the majority of other metals (Co, Cd, Pb, alkaline-earths) will follow Zn rather than Cu and will be adsorbed more strongly by clones compared to *Sphagnum* collected from the field.

In addition to carboxylate groups, representing the major part of proton and metal binding sites, more specific and metal-selective moieties like phosphoryl, amine, and sulfhydryl also play



**Fig. 5.** Results of the thermodynamic modeling of  $\text{Cu}^{2+}$  (squares) and  $\text{Zn}^{2+}$  (circles) complexation with DOM leached from peat mosses (NICA-Donnan FA complexes in 0.01 M  $\text{NaNO}_3$ ), at pH 5.5 and 6.5 for Cu and Zn, respectively. Closed symbols were computed for  $[\text{DOM}] = 15 \text{ mg L}^{-1}$  and open symbols are for  $[\text{DOM}] = 45 \text{ mg L}^{-1}$ .

important roles in the adsorption of protons and metals within the 3-D porous layer of biological surfaces (e.g., Refs. [54,55]). However, these groups located both at the surface and within the porous surface layer could be screened (protected) by mineral coating formed during lifetime of natural samples. Therefore, an additional explanation for the lower adsorption capacity of naturally grown peat moss compared to clones is the significantly higher Fe and Al concentration in the field samples (Table 1). Fe and Al hydroxides and clay aluminosilicates, deposited from the atmosphere, are capable of blocking, as a protective layer, the active binding sites and decrease the concentration of specific organic moieties controlling metal complexation at the surface. In fact, thin hydroxide/clay coating, although being an efficient sorbent, may not allow protons and metals to penetrate within the moss surface layer, where they can interact with multiple surface sites, as it is known for other biological surfaces [49,54]. However, given the lack of direct evidence on hydroxide coating on mosses, this assumption remains to be verified by surface-sensitive techniques such as X-ray Electron Spectroscopy (e.g., Ref. [55]).

It follows that the use of cloned moss samples instead of natural species for passive biomonitoring and atmospheric pollution assessment has double benefit. First, clones exhibit a lower variability in both chemical and physical properties, allowing highly homogeneous stock of virtually identical biosorbents. Second, the concentrations of metal pollutants, metalloids and trace elements in cloned moss biomass are significantly lower than those in native samples, thus enabling efficient use of the clones during short periods of exposure to capture short-term pollution events.

## 5. Conclusions

This study provides physico-chemical background for unifying the method of atmospheric metal biomonitoring using the moss-bag technique. For this, we quantified the metal and proton adsorption of *in vitro*-cultivated *Sphagnum* moss as well as the species collected from the field, as a function of pH and  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  concentration. We demonstrated that *S. palustre* clones exhibit prominent physico-chemical characteristics to be used instead of environmentally-protected natural *Sphagnum* mosses. In particular, clones have a larger specific surface area and greater mass-normalized adsorption capacities compared to field samples, especially for  $\text{Zn}^{2+}$  and presumably for many other divalent metals. These differences in metal and proton adsorption between clones of *S. palustre* and *S. denticulatum* can be understood in terms of the composition of the cell walls, type and abundance of ligands in the cell walls, and the morphological features of field samples and *in vitro*-cloned samples. Laboratory clones contain a factor of 10–1000 lower concentrations of many atmospheric pollutants, compared to natural samples, and as such they can be used even for short period of biomonitoring in low-contaminated environments. Further work is necessary in order to reveal the mechanisms of surface and bulk enrichment in metals of natural mosses compared to cloned samples. Overall, the *in-vitro* production of *S. palustre* clones can provide an optimal biosorbent material for monitoring atmospheric metal deposition using the standardized moss-bag technique.

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