Chemosphere 236 (2019) 124375

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Biosurface properties and lead adsorption in a clone of *Sphagnum palustre* (Mosses): Towards a unified protocol of biomonitoring of airborne heavy metal pollution

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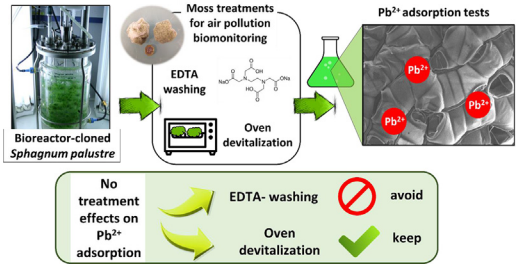
HIGHLIGHTS

- Surface properties and Pb^{2+} adsorption ability of a cloned moss were investigated.
- Clone and conspecific field-grown moss displayed comparable adsorption constants.
- EDTA-treated clone had the highest negative surface charge and the lowest pH_{PZC}.
- EDTA washing did not affect the ability of cloned moss to adsorb Pb^{2+}.
- Oven-devitalization is proposed as unique treatment for clone to use as biomonitor.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 May 2019
Received in revised form
5 July 2019
Accepted 14 July 2019
Available online 17 July 2019

Handling Editor: Lena Q. Ma

ABSTRACT

Although mosses are widely used for active biomonitoring of air pollution, a unified protocol for their treatment before exposure in bags is still lacking. Here we used field- and laboratory-grown *Sphagnum palustre* L. moss, respectively, treated by EDTA and devitalized by oven drying at 100 °C, to elaborate a consistent procedure of metal and proton adsorption on moss surfaces. Acid-base titrations and Pb^{2+} adsorption experiments at different pH values and Pb^{2+} concentrations in solution were performed with both field-collected and laboratory cloned mosses. Devitalization and EDTA treatments did not produce any measurable difference in terms of H^+ and Pb^{2+} adsorption capacities of moss surfaces. The stability constants for Pb^{2+} adsorption onto moss surfaces as a function of pH (pH-dependent adsorption edge)

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https://doi.org/10.1016/j.chemosphere.2019.124375

Keywords:
Biosorption
Peat moss
Langmuir isotherm
Moss-bags
Kinetics
Heavy metals

and at constant pH (5.5 and 6.5) as a function of Pb^{2+} concentration ("langmuirian" adsorption isotherm) were rather similar between different treatments. A Linear Program Modeling (LPM) of adsorption reactions revealed high similarity of adsorption constants regardless of treatments for both field-grown and cloned mosses. Therefore, in view of the use of *S. palustre* clone for biomonitoring lead in the environment, we recommend devitalization at 100 °C as unique treatment to perform with the aim to preserve the biomonitor before and after its exposure in bags.

1. Introduction

The atmospheric pollution and the improvement of air quality still represent huge and pressing issues worldwide. The presence of heavy metals in the atmosphere involves the whole environment and human life and it leads to a multitude of adverse consequences to humans, ecosystems and climate. Heavy metals like Cd, Hg and Pb, derived from both natural and anthropogenic sources, are persistent in the environment. Their occurrences as long-range transboundary air pollutants and wet and dry deposits of atmospheric emissions represent a serious risk factor for human health by inhalation or entrance and bioaccumulation in food chains, with a consequent increase of acute and chronic diseases (EEA, 2015). The toxicity effects by heavy metals for humans are often metal-specific and related to the exposure conditions. Particularly Pb exposures have developmental and neurobehavioral effects on fetuses and children, and increase the risk of high blood pressure and kidney damage in adults (WHO, 2007).

Anthropogenic emissions of Pb, mainly derived from fossil-fuel combustion, waste incineration and production of non-ferrous metals, iron, steel and cement (EEA, 2016), are regulated by several policy instruments on air pollution within the EU. Particularly with the 2008/50/EC air quality directive (EU Directive 2008/50/EC), the EU has set a limit value for annual Pb emission of 0.5 $\mu g\, m^{-3}$ and of 1.0 $\mu g\, m^{-3}$ near notified industrial sources.

Progress in reducing heavy metal pollution in most EEA-33 countries has been led by targeted international and EU legislations. In particular, the Clean Air Policy Package (CCEP-COM/2013/0918), adopted by the European Commission in late 2013, aims to improve Europe air quality by 2030. Coupled with regulatory methods for the control and the abatement of heavy metal emissions, the monitoring of the air quality using moss bags provides qualitative and quantitative data using economic, easy-to-manage and eco-friendly procedures (e.g. Aničić et al., 2009; Ares et al., 2010; Giordano et al., 2005). Due to their peculiar morphological and physiological features, mosses are able to adsorb a wide variety of airborne inorganic and organic pollutants (Bargagli, 1998; Concha-Graña et al., 2015; De Nicola et al., 2013; Figueira et al., 2002; Giordano et al., 2013), notably the particulate matter (PM), which represents the main transport form of heavy metals in the atmosphere (Di Palma et al., 2017; Spagnuolo et al., 2013; WHO, 2013).

Advances in the moss-bag biomonitoring standardization were recently achieved by Ares et al. (2014, 2012), addressing the key aspects of moss sampling, moss treatments, bags preparation and exposure. Moreover, in order to overcome the limitation of the use of naturally grown mosses as transplants, the use of a cloned moss was proposed within the framework of the FP7 EU MossClone project (www.mossclone.eu). Indeed, the use of mosses collected in nature implies environmental impact and is biased by an intrinsic variability in terms of elemental and chemical composition of the biomonitor, thus yielding a high degree of uncertainty in the interpretation of the results (e.g. Couto et al., 2004; Tretiach et al., 2011; Zechmeister et al., 2003). *Sphagnum palustre* L. axenically grown in photobioreactors was the moss species selected for cloning (Beike et al., 2015). It was studied for its metals and polystyrene nanoparticles adsorption properties (Capozzi et al., 2018; González et al., 2016a), molecular and elemental profile (Di Palma et al., 2016). It was also tested in the field after transplanting in bags (Capozzi et al., 2017a). However, the physico-chemical mechanisms underlying the uptake of airborne pollutants by the clone once exposed in bags and the effect of pre-exposure treatments on metal adsorption properties of the clone still requires investigation. Among moss pre-treatments, the oven-devitalization ensures a chemical homogeneity of the biomonitor by preventing its degradation during exposure and making the metal uptake occurring exclusively via passive ways (Adamo et al., 2007; Fernández et al., 2010; Giordano et al., 2009) independently of moss vitality. To enhance metal adsorption, biosorbents are usually pre-treated (Michalak et al., 2013). For example, Ferreira et al. (2009) used EDTA to remove metals from surface exchange sites of the aquatic moss *Fontinalis antipyretica* before performing Cu^{2+} *in vitro*-bioadsorption experiments. Towards the methodological optimization of the moss bag technique, the use of EDTA as chelating agent for mosses to expose in bags was recommended by Ares et al. (2012, 2014). The EDTA washing procedure for mosses before transplanting in bags was firstly applied by Lodenius and Tulisalo (1984), and more recently by Capozzi et al. (2017b, 2016a, 2016b), Di Palma et al. (2017) and Iodice et al. (2016) on native mosses and by Capozzi et al. (2017a) on a *S. palustre* clone. In particular, Di Palma et al. (2016) investigated the variation of the element content in *S. palustre* clone samples after EDTA and devitalization treatments. However, the impact of EDTA treatment on the ability of mosses to uptake heavy metals *in vitro* or in field tests was never investigated so far, neither alone or in combination with devitalization.

This work intends to contribute to the understanding of the metal adsorption properties of the *S. palustre* clone in order to optimize its employment as new biomonitor for the moss bag technique. To achieve this intent, the surface acid-base properties of the clone and its ability to adsorb Pb^{2+} ions were studied and compared to those displayed by the conspecific field-grown moss. Mosses were studied both before and after pre-treatment by EDTA washing and oven devitalization. Adsorption capacity was studied at different pH values and Pb^{2+} ions concentrations. The results should help to elaborate a unified protocol of moss biomonitoring heavy metals in the environment.

2. Materials and methods

2.1. Moss samples

The biomonitor investigated in this work was the *Sphagnum palustre* L. moss cloned in photobioreactors under controlled conditions, as described in Beike et al. (2015) and Reski et al. (2016). Briefly, the mosses were grown in 5 L photobioreactors with liquid

Knop medium (Reski and Abel, 1985) and by adding micronutrients, sucrose and ammonium nitrate (Beike et al., 2015).

The experiments and the analysis were conducted on untreated cloned *S. palustre* (C-U; oven dried at 40 °C for 8 h) and on cloned moss samples after the following treatments: (1) EDTA washing and oven drying at 40 °C for 8 h (C-EDTA); (2) devitalization in oven at 100 °C (C-100); (3) EDTA washing and oven devitalization (C-EDTA100). The devitalization was done by 3 consecutive 8 h-drying steps at 50, 80 and 100 °C. The EDTA washing was performed as follows: 1 wash for 20 min with EDTA (disodium salt di-hydrate, Panreac; 1 L 10 mM EDTA/12.5 g of moss) followed by 3 washes of 20 min each with distilled water (1 L distilled water/10 g of moss). In comparison with clone samples, untreated (FS-U) and oven-devitalized (FS-100) moss samples of the field-grown *S. palustre* collected in Posta Fibreno, central Italy (Terracciano et al., 2012) were also examined, avoiding the use of additional *S. palustre* samples for EDTA treatments, due to the scarce presence of the moss at the sampling point (Terracciano et al., 2012) and to European regulations for its protection (92/43/EEC Directive). More details on moss materials can be found in Di Palma et al. (2016), along with their molecular profile and elemental composition.

2.2. Reagents and solutions

The Pb^{2+} adsorption experiments were carried out in 0.01 M $NaNO_3$ electrolyte solution at room temperature (20 ± 1 °C) in polypropylene beakers, under a gentle stirring with Teflon coated magnets. The pH of the solutions was continuously measured by a combined electrode (Mettler Toledo^R) in a pH-meter ion analyzer (PHM250-Meterlab™) with an uncertainty of ±0.002 units. Filter-sterilized deionized water (18 MΩ, Millipore, MA, USA) was used to prepare all reagents and solutions. A test solution of lead (II) nitrate (Sigma-Aldrich) was used as source of Pb^{2+}. The adsorption of Pb^{2+} was carried out at pH values of 5.5 and 6.5, as they define the pH range in which the maximum adsorption of Pb^{2+} can be reached during the pH-edge experiments. The MES (2-(N-morpholino)ethanesulfonic acid; Merck) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma-Aldrich) buffer solutions were used in order to keep a constant pH of 5.5 and 6.5, respectively, during the adsorption experiments. The biomass was kept constant at 1 g L^{-1} in all experiments.

2.3. Moss surface characterization

The acid-base titration of *S. palustre* surfaces was carried out in 0.01 M $NaNO_3$ at room temperature (20 ± 1 °C). Solutions were conditioned for 30 min by pre-saturation with nitrogen. All the titrations were performed under a N_2 atmosphere and continuous stirring. The acid and basic titrations were conducted separately, by adding known amounts of HCl and NaOH titrant solutions (from 0.01 to 0.1 N), and in triplicate for each moss sample, in a whole range of pH between 3 and 10. The reference solution was the supernatant solution after the conditioning time and removing the moss biomass. The excess of charge was computed as the difference of the acid–base concentration in the suspension and in the reference solution according to usual procedures of biomass titration (González et al., 2016a, 2010; Pokrovsky et al., 2013).

The B.E.T. (Brunauer–Emmett–Teller) N_2 multipoint adsorption technique (Brunauer et al., 1938) was used to measure the specific surface area (SSA) of untreated (C-U) and EDTA washed/devitalized (C-EDTA100) clones by the Quantachrome Autosorb Automated Gas Sorption System. After a 24 h-degassing at 120 °C, between 10 and 14 adsorption points were taken in the B.E.T. domain, with an uncertainty of 10%.

Table 1
List of Pb^{2+} adsorption experiments performed in a 0.01 M $NaNO_3$ solution and with a constant biomass of 1 g L^{-1}, along with related experimental conditions and LPM parameters. K_s and K_m 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. $[Pb^{2+}]_0$ = initial concentration of Pb^{2+}; FS = field-grown moss; C = cloned moss; U = untreated moss; 100 = devitalized moss; EDTA = EDTA-treated moss.

Type of study	Species/treatment	pH-range	$[Pb^{2+}]_0$ μM	pK_s/pK_m	Binding sites mol g^{-1}
pH-edge	FS-U	2.40–7.61	14.47	−3.05	$1.12\cdot10^{-5}$
	FS-100	2.30–8.81	14.45	−2.90	$1.04\cdot10^{-5}$
	C-U	2.37–9.56	14.46	−2.15	$1.21\cdot10^{-5}$
	C-100	2.34–8.18	14.46	−2.15	$1.03\cdot10^{-5}$
	C-EDTA	2.40–8.37	14.45	−2.55	$9.59\cdot10^{-6}$
	C-EDTA-100	2.32–8.30	14.45	−2.30	$1.41\cdot10^{-5}$
Constant pH isotherm	FS-U	5.46 ± 0.03	11.1–1263.6	0.75	$3.84\cdot10^{-4}$
				5.00	$2.72\cdot10^{-2}$
	FS-100	5.46 ± 0.02	9.2–854.1	0.65	$2.63\cdot10^{-4}$
				4.80	$1.65\cdot10^{-2}$
	C-U	5.47 ± 0.03	8.12–1169.1	0.1	$1.73\cdot10^{-4}$
	C-100	5.47 ± 0.02	8.12–1169.1	–	–
	C-EDTA	5.45 ± 0.04	9.1–1233.2	0.15	$1.95\cdot10^{-4}$
				1.45	$4.42\cdot10^{-5}$
	C-EDTA-100	5.47 ± 0.03	9.2–1180.3	0.45	$1.72\cdot10^{-4}$
				2.25	$8.60\cdot10^{-5}$
	FS-U	6.53 ± 0.02	7.4–799.8	–	–
	FS-100	6.50 ± 0.01	7.4–799.8	−0.15	$5.29\cdot10^{-5}$
				1.50	$4.64\cdot10^{-4}$
				5.50	$1.36\cdot10^{-1}$
	C-U	6.48 ± 0.02	4.7–672.3	0.10	$1.19\cdot10^{-6}$
				0.65	$7.15\cdot10^{-5}$
				2.45	$4.02\cdot10^{-4}$
	C-100	6.53 ± 0.01	4.2–871.4	1.65	$1.52\cdot10^{-4}$
				5.70	$1.60\cdot10^{-1}$
	C-EDTA	6.57 ± 0.01	5.4–690.5	0.20	$2.65\cdot10^{-6}$
				1.25	$2.37\cdot10^{-4}$
				2.95	$5.53\cdot10^{-4}$
	C-EDTA-100	6.57 ± 0.01	6.4–972.8	1.55	$3.81\cdot10^{-4}$
				5.70	$2.32\cdot10^{-1}$

2.4. DOC analysis

Parallel titrations of field-grown and cloned mosses were carried out to determine the concentrations of Dissolved Organic Carbon (DOC) released in solution by mosses during titration experiments, as the dissolved organic matter can form complexes with metals and, thus, interfere with their adsorption by moss surfaces (González et al., 2016a). For this purpose, several aliquots of about 5 mL were withdrawn from moss solutions throughout the pH range used for titration, filtered at 0.45 μm and analyzed by a Total Carbon Analyzer (Shimadzu TOC-VCSN) with an uncertainty of 2% and a detection limit of 0.1 mg L^{-1}. The same procedure was applied to the untreated clone (C-U) and to the C-EDTA100 samples after 10 washings (10 min each) with filter-sterilized deionized water (1 g mos/1 L water). The clones yielded an amount of DOC twenty-fold higher than that released in solution by field-grown mosses (ANCOVA + Tukey test, $p < 0.05$; Figure ESM-1A). According to our results (Figure ESM-1B), the DOC amount in solution can be progressively reduced with washings for about 88% of the initial concentration already after the second washing. Therefore, additional moss washings (≥3) with deionized water before performing adsorption studies are recommended.

2.5. Adsorption experiments

The Pb^{2+} adsorption on moss samples as a function of contact time, pH and metal concentration was studied via batch experiments. The experimental conditions are detailed in Table 1. Adsorption kinetic experiments were conducted at constant pH values of 5.5 and 6.5 with an exposure time from 1 min until 8 h to ensure the reaching of equilibrium; the Pb^{2+} initial concentration was set at 15 μmol L^{-1} and all experimental aqueous solutions remained undersaturated with respect to lead hydroxide.

Adsorption of Pb^{2+} as function of pH was tested setting the initial metal concentration at 15 μmol L^{-1} and in several pH ranges between the pH values of 2 and 9, according to each type of moss sample (Table 1). The pH was regulated by addition of acid or basic solutions (NaOH or HNO_3; 0.1–0.01 M). The adsorption experiments on mosses were also studied as function of Pb^{2+} concentrations ranging from 9.6 to 2400 μmol L^{-1}, and from 9.6 to 1500 μmol L^{-1}, respectively, at pH of 5.5 and 6.5 kept constant by MES and HEPES buffers.

For each experiments, a 5 mL sample of supernatant solution was withdrawn after a reaction time of 10 min, enough to reach the adsorption equilibrium, as previously described for mosses by kinetic experiments (González and Pokrovsky, 2014). These aliquots were then filtered by sterile syringe filters (0.45 μm pore size) and acidified with bi-distilled HNO_3 (~15 N) for storage. Blanks were performed in the same reactors and at similar pH and Pb^{2+} concentration ranges without moss biomass. The same experimental protocols were applied, in parallel, for blank solutions, obtained by filtration of all the moss solutions after a gentle stirring for 1 h.

The determination of Pb^{2+} concentration in working solutions was carried out by atomic absorption spectroscopy using a flame atomic absorption spectrometer (PerkinElmer AAnalyst 400) with an uncertainty of ±2% and a detection limit of 0.05 mg L^{-1}. An external calibration was applied by measuring standards containing from 0.5 to 20 mg L^{-1} of Pb^{2+} in an acidified $NaNO_3$ solution. The results were evaluated considering RSD values < 2%, and Pb^{2+} concentrations <20 mgL^{-1} and > LQ (limit of quantification).

2.6. Data treatment

The data on Pb^{2+} concentrations of all moss samples were reported on a dry weight basis. Data analysis and statistical elaborations were performed by using Microsoft Excel-XLSTAT and Synergy KaleidaGraph v. 4.0 Softwares. The Shapiro-Wilk and Levene tests were carried out to check the assumptions of data normality and homogeneity of variance, respectively. In case of non-normal distribution, data were log-transformed before to use parametric tests. For the titration data sets and the Pb^{2+} adsorption experiments as function of pH and metal concentration in solution, the significance of the effects of treatments and moss sample was tested by using the Analysis of Covariance (ANCOVA) *plus* Tukey test as *post-hoc*. For the ANCOVA test, regression analysis and parallelism test were applied to test the significance of the influence of pH and metal concentrations in solution as covariates. For the Pb^{2+} adsorption experiments as function of time, the Mann-Whitney test was used to compare FS versus C samples, and the Friedman test was carried out to check effect of treatments for the clone samples. The non-parametric Wilcoxon matched pairs test was applied to compare two groups (i.e. for FS-U versus FS-100 in the kinetic experiments, and for C-U versus C-EDTA100 for the analysis of DOC concentrations after washings) or as *post-hoc* for Friedman test.

Estimation of the moss surface binding sites concentrations and of the apparent equilibrium constants related to acid–base surface titration and adsorption experiments were computed by the Linear Programming Model (LPM), following the same procedures as described for bacteria and mosses (González et al., 2016a and references therein). The details of the LPM model are presented in the ESM-2.

3. Results

3.1. Moss surface characterization

The results of the moss surface characterization obtained via acid-base titration at pH 2–10 in triplicates for treated and untreated field-grown (FS) and cloned (C) mosses are given in Fig. 1.

The pH values corresponding to zero net proton adsorption

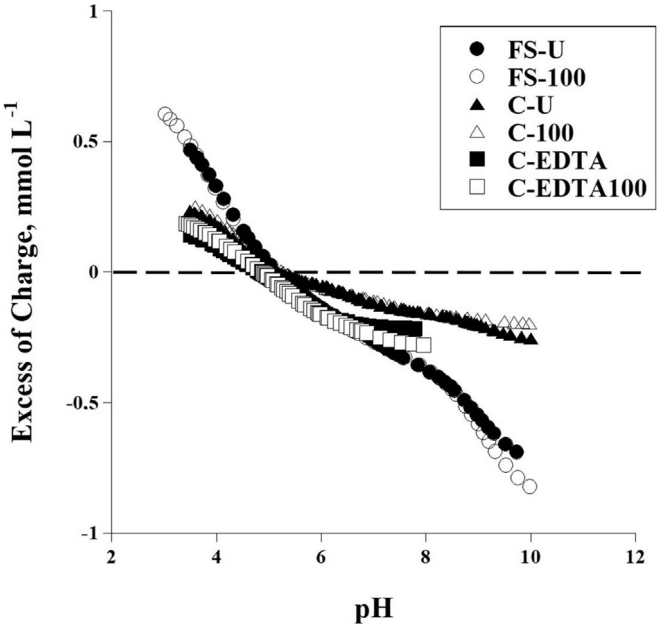

Fig. 1. Surface acid–base titration of field-grown (FS) and cloned (C) *S. palustre* moss samples in 0.01 M $NaNO_3$. The conditioning time was equal to 1 h and the biomass was kept constant at 1 g L^{-1}. All the measurements were triplicated. U = untreated samples; 100 = devitalized samples; EDTA = EDTA-treated moss.

(pH_{PZC}) were 5.14 and 5.09 for field-grown moss samples before (FS-U) and after devitalization (FS-100), respectively. Compared to the clones (ANCOVA + Tukey test, $p < 0.05$), field-grown mosses displayed the highest excess of adsorbed protons (on average, 0.536 mmol L^{-1}) and the highest negative surface charge. Comparing clones before and after treatments, significant variation (ANCOVA + Tukey test, $p < 0.05$) of pH_{PZC} and surface charges were produced only by EDTA washings. In fact, the pH_{PZC} of 5.23 for the untreated (C-U) clone and of 5.24 for the clone after devitalization (C-100) decreased by about one pH unit after EDTA treatment (i.e. pH_{PZC} values of 4.81 and 4.85 for C-EDTA and C-EDTA100, respectively). In the pH range 3.5–8.0, the highest excess of adsorbed protons (on average, 0.258 mmol L^{-1}) was exhibited by C-U and C-100, whereas EDTA-treated clones had the highest negative surface charge (on average, −0.274 mmol L^{-1}).

The experimental results were fitted by LPM model to calculate the acidity constants and potential functional groups on the moss cell surfaces (Table 2). Both untreated (FS-U) and devitalized field mosses (FS-100) were characterized by carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols as possible functional groups, with total binding sites of 1.333 mmol g^{-1} (FS-U) and of 1.580 mmol g^{-1} (FS-100). These values were higher 3 folds higher than those related to the cloned samples, whose tentative functional groups were mostly carboxyl and phosphoryl-like groups. Namely, the total number of binding sites computed for the untreated clone (C-U) was of 0.535 mmol g_{dry}^{-1}; after treatments, the total binding sites were 0.497, 0.395 and 0.513 mmol g^{-1}, respectively for C-100, C-EDTA and C-EDTA100. The FS-U and FS-100 moss samples had 0.754 and 0.901 mmol g^{-1} of carboxyl/phosphodiesters (pKa = 3.7–5.6) respectively, which was 2 folds higher than that of *S. palustre* clones, having on average 0.395 mmol g^{-1}. For field-grown samples, the amount of phosphoryl groups (pKa ~7.0) was on average 0.16 mmol g^{-1}, comparable with the amount of 0.14 mmol g^{-1} (pKa = 6.5–7.2) exhibited by the untreated clone, but higher by a factor of 1.7 than that related to C-100 and C-EDTA100 (0.097 and 0.094 mmol g^{-1}, respectively with pKa = 6.85–7.15). The surface amino groups (pKa = 9.0–9.2) had a total number of 0.40 and 0.54 mmol g^{-1} in field-grown mosses, 1.6 times higher than in devitalized clone C-100 (0.032 mmol g^{-1}).

The B.E.T. analysis of C-U and C-EDTA100 cloned mosses, showed an increase in moss $SSA_{B.E.T.}$ from 7.85 to 10.9 $m^2 g^{-1}$, as determined by the multipoint adsorption isotherm, with a correlation coefficient >0.99. Although this 39% increase is only slightly higher than the analytical uncertainty (±10%), it is possible that EDTA leads to some surface restructuring.

Table 2
Surface acid–base titration and LPM model results for field-grown (FS) and cloned (C) moss samples. The experiments were performed in 0.01 M $NaNO_3$ electrolyte solution with a constant biomass of 1 g L^{-1} in triplicates. K_a = proton dissociation constant. U = untreated moss; 100 = devitalized moss; EDTA = EDTA-treated moss.

Species/treatment	pK_a	Binding sites mmol g^{-1}	Tentative functional group
FS-U	4.10	0.557	Carboxyl/phosphodiester
	5.45	0.197	Carboxyl
	7.00	0.181	Phosphoryl
	9.00	0.398	Amine
FS-100	3.70	0.471	Carboxyl/phosphodiester
	4.40	0.216	Carboxyl
	5.55	0.214	Carboxyl
	7.05	0.139	Phosphoryl
	9.05	0.54	Amine
CU	4.45	0.337	Carboxyl
	5.50	0.0589	Carboxyl
	6.50	0.139	Phosphoryl
C-100	4.30	0.268	Carboxyl
	5.25	0.1	Carboxyl
	7.15	0.0971	Phosphoryl
	9.20	0.0319	Amine
C-EDTA	4.15	0.171	Carboxyl
	5.55	0.224	Carboxyl
C-EDTA100	4.00	0.182	Carboxyl/phosphodiester
	5.30	0.237	Carboxyl
	6.85	0.0942	Phosphoryl

3.2. Adsorption of Pb^{2+} over time

The Pb^{2+} adsorption on field-grown (FS) and cloned (C) mosses at pH 5.5 and 6.5, in a time range from 1 min to 8 h is shown in Fig. 2. The adsorption rate curves at pH 5.5 (Fig. 2A) had a sharp increase at the beginning of the reaction (around the first 10 min), in which field-grown and cloned mosses removed respectively about the 50 and 70% of the Pb^{2+} initially in solution (Mann-Whitney test, $p < 0.05$). In detail, in the first 5 min, the untreated cloned moss adsorbed a higher amount of Pb^{2+} than the untreated field-grown moss (75 versus 53% of the initial metal concentration). The EDTA treatment had no clear impact on the rate of adsorption, whereas the devitalization produced a significant effect (Friedman + Wilcoxon matched pairs tests, $p < 0.05$) by retarding the Pb^{2+} adsorption process on the surfaces of both field-grown and cloned mosses. Indeed, the devitalized samples reached the maximum absorption of Pb^{2+} only after 10–15 min and removed a lower percentage of initial Pb^{2+} compared to the corresponding untreated samples (i.e. 50% for FS-100 and 64% for C-100). The kinetic experiments conducted at pH 6.5 (Fig. 2B) revealed that for equal reaction time, cloned moss adsorbed 3 to 4 times higher Pb^{2+} than the FS mosses. Neither EDTA nor devitalization treatments of clones produced a significant (Friedman + Wilcoxon matched pairs tests, $p < 0.05$) effect on Pb^{2+} adsorption rates.

3.3. Adsorption of Pb^{2+} as a function of pH (pH-edge)

The effect of pH on adsorption of Pb^{2+} ions by field-grown and cloned *Sphagnum* mosses is shown in Fig. 3 and LPM parameters of Pb^{2+} adsorption as function of pH are listed in Table 1. As expected, the lead adsorption was pH dependent: at the lowest pH of around 2.3, mosses adsorbed very low amount of Pb^{2+} on their surfaces. With pH increase up to 4, lead adsorption steadily increased, reaching an adsorption plateau at pH around 5. Significant differences (ANCOVA + Tukey test, $p < 0.05$) were observed between field-grown and cloned mosses, with no effect induced by treatments. The Pb^{2+} adsorption yield of the cloned mosses increased from 15 to 80% with pH increase from 2 to 4, while the field-grown mosses yield 90% of adsorption in the same pH range. The maximal adsorption values (from 95 up to 99%) were reached at pH between 4.5 and 5.5 for field-grown mosses and between 5.5 and 6.5 for the clones. In detail, the field-grown mosses reached the 98% of Pb^{2+} adsorption at pH 5.0 while for all the cloned mosses this happened at pH 5.7, with the exception of the devitalized one (C-100), reaching only a 90% of Pb^{2+} adsorption at this pH value. The adsorption experiments, except the pH-edge, were carried out at pH values not higher than 5.5 and 6.5, to avoid Pb^{2+} hydrolytic reactions leading to the formation of lead precipitates.

Examining the LPM results (Table 1), field-grown (FS) and cloned (C) mosses displayed a comparable number of binding sites, ranging from $9.6 \cdot 10^{-6}$ mol g^{-1} for EDTA treated clone (C-EDTA) to $1.4 \cdot 10^{-5}$ mol g^{-1} for the EDTA/devitalized clone (C-EDTA100). The strongest interactions between surface binding sites and Pb^{2+} which correspond to the lowest p*Ks* values, were observed in both untreated (FS-U) and devitalized (FS-100) field mosses (p*Ks* = −3.05 and −2.90, respectively), followed by EDTA-treated clones (p*Ks* = −2.55 for C-EDTA and p*Ks* = −2.30 for C-EDTA100)

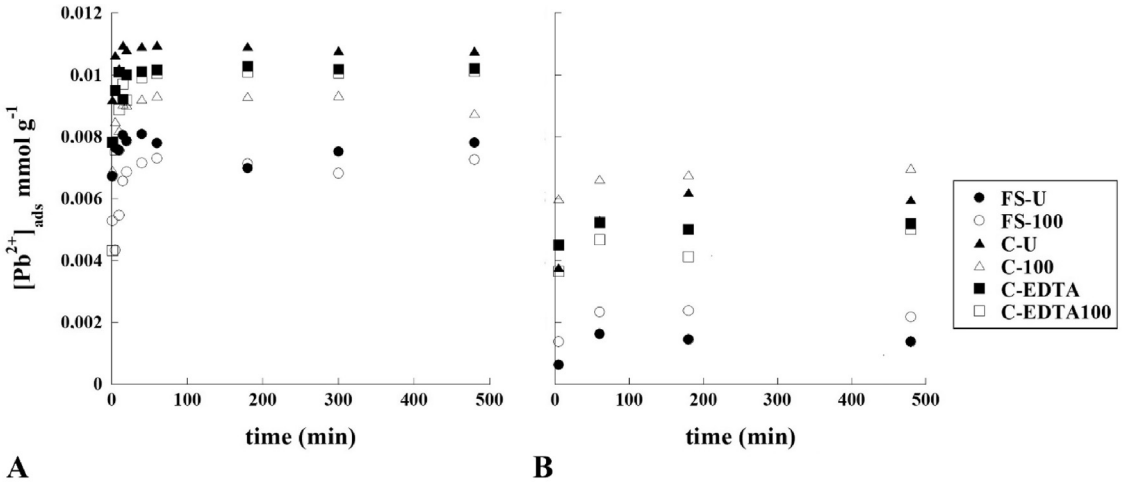

Fig. 2. Effect of time on Pb^{2+} adsorption onto field-grown (FS) and cloned (C) *S. palustre* surfaces in a 0.01 M $NaNO_3$ solution, at pH 5.5 (A) and pH 6.5 (B), at constant biomass of 1g L^{-1} and initial Pb^{2+} concentration of 15 μmol L^{-1}. U = untreated samples; 100 = devitalized samples; EDTA = EDTA-treated moss.

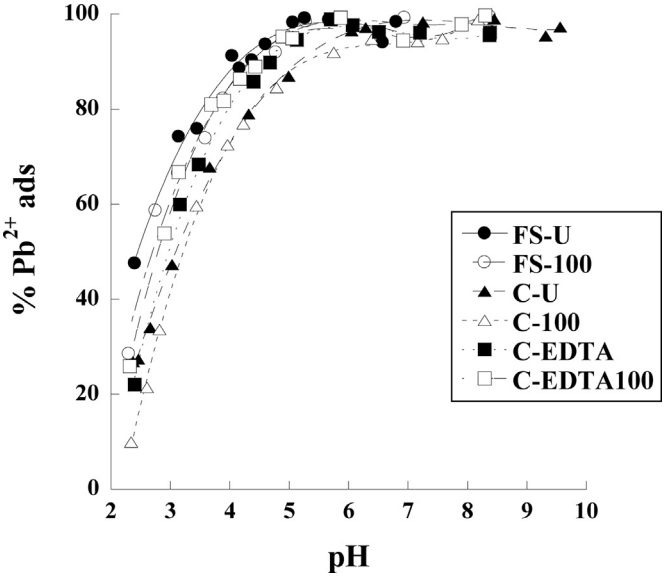

Fig. 3. Effect of pH on Pb^{2+} adsorption on field-grown (FS) and cloned (C) *S. palustre* surfaces in 0.01 M $NaNO_3$, at constant biomass of 1g L^{-1} and initial Pb^{2+} concentration of 15 μmol L^{-1}. U = untreated samples; 100 = devitalized samples; EDTA = EDTA-treated moss.

and by untreated and devitalized clones (p*Ks* = −2.15 for both C-U and C-100).

3.4. Adsorption of Pb^{2+} as a function of Pb^{2+} concentration in solution (langmuirian isotherm)

Fig. 4 shows the Pb^{2+} adsorption on moss surfaces as a function of lead concentration in solution at pH 5.5 and 6.5. The related LPM fitting parameters are reported in Table 1.

The Langmuir isotherm model was applied to describe the moss adsorption equilibrium, as described in González and Pokrovsky (2014) for mosses collected in nature, and by González et al. (2016a, 2016b) for field-grown and cloned mosses. Basically, the model assumes that the process of surface biosorption is limited to a sorbate monolayer coverage at sites, all possessing equal affinity for the sorbate. The linear form of the Langmuir model is given by Equation (1):

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

where Me^{2+} is a generic divalent cation, q_{max} is the maximum adsorption capacity (mmol g^{-1}), K_L the Langmuir equilibrium constant (g $mmol^{-1}$) related to the free energy of the biosorption process.

The Langmuir parameters computed for Pb^{2+}adsorption at pH 5.5 are reported in Table 3. The data of lead adsorption on field-grown and cloned mosses were adequately fitted by the Langmuir model, as indicated by the coefficient of determination $r^2 > 0.98$. Significant differences were observed between untreated field moss (FS-U) and all cloned (C) samples(ANCOVA + Tukey test, $p < 0.05$); the untreated field moss (FS-U) showed the highest maximum adsorption capacity (q_{max} value = 0.503 mmol g^{-1}), while the q_{max} values for all other mosses ranged between 0.205 mmol g^{-1} (FS-100) and 0.311 mmol g^{-1} (C-100). Neither the EDTA treatment nor the devitalization significantly affected the adsorption capacity of moss samples. Data from LPM (Table 1) computed for Langmuir Pb^{2+}adsorption at pH 5.5 showed that field mosses possessed the highest total binding site concentration, namely 27.58 and 16.76 mmol g^{-1} respectively for FS-U and FS-100, compared to the clones which displayed total amount of binding site equal to 0.223 mmol g^{-1} on average.

Fig. 4B and Table 2 report respectively the Langmurian isotherms and parameters for moss experiments conducted at pH 6.5 and in a Pb^{2+} concentration range of 9.6 μmol L^{-1} and 1.5 mmol L^{-1}. Also at pH 6.5, the untreated field-grown moss FS-U had the highest q_{max} value (2.797 mmol g^{-1}), followed by the devitalized field-grown moss FS-100; the lowest q_{max} value was attributed to the untreated clone (0.833 mmol g^{-1}). Significant differences (ANCOVA + Tukey test, $p < 0.05$) were observed when comparing FS with C mosses and, among clones, for the combined effects of EDTA and devitalization treatments.

The highest value of total binding site concentration computed by LPM for Langmurian isotherms at pH 6.5 (Table 1) was attributed to moss sample devitalized after EDTA treatment (i.e. 232 mmol g^{-1} for C-EDTA100), followed by C-100 (160 mmol g^{-1}) and FS-100 (137 mmol g^{-1}). Untreated (C-U) and EDTA washed (C-EDTA) clones had the lowest total number of binding sites, namely

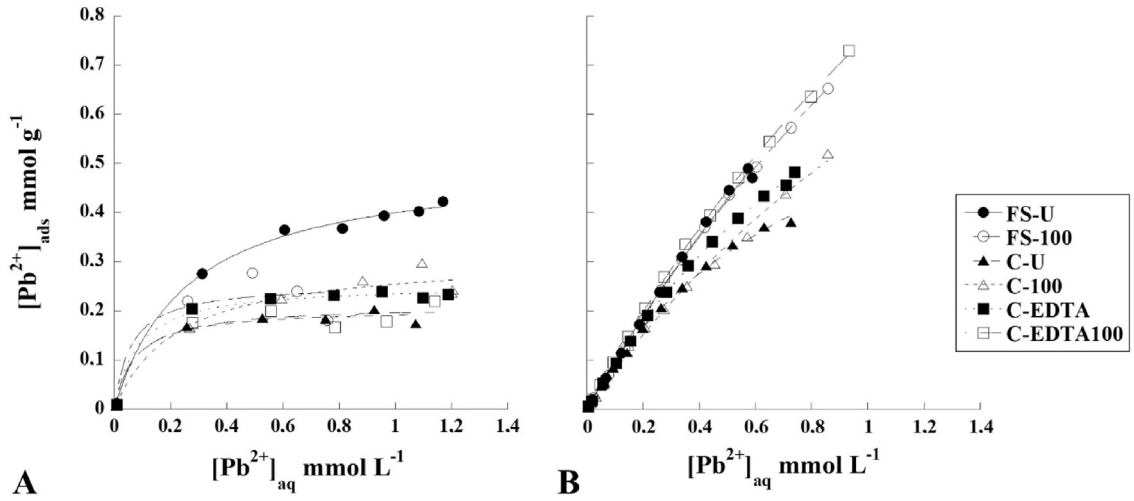

Fig. 4. Adsorption of Pb^{2+} on field-grown (FS) and cloned (C) *S. palustre* surfaces as a function of metal concentration in 0.01 M $NaNO_3$, at pH 5.5 (A) and 6.5 (B) and constant biomass of 1g L^{-1}. U = untreated samples; 100 = devitalized samples; EDTA = EDTA-treated moss.

Table 3
Langmuir parameters computed from the Pb^{2+} adsorption experiments at different aqueous metal concentrations and at pH 5.5 and 6.5, for field (FS) and cloned (C) moss samples. K_L = Langmuir equilibrium constant. q_{max} = maximum adsorption capacity. U = untreated moss; 100 = devitalized moss; EDTA = EDTA-treated moss.

pH	Species/treatment	q_{max} mmol g^{-1}	K_L g $mmol^{-1}$
pH 5.5	FS-U	0.503	3.643
	FS-100	0.256	5.405
	C-U	0.205	11.695
	C-100	0.311	6.110
	C-EDTA	0.254	11.128
	C-EDTA100	0.210	12.616
pH 6.5	FS-U	2.797	0.352
	FS-100	2.665	0.369
	C-U	0.833	1.192
	C-100	1.778	0.479
	C-EDTA	1.291	0.772
	C-EDTA100	2.618	0.402

0.634 mmol g^{-1}.

4. Discussion

Both field-grown and bioreactor-grown cloned *Sphagnum palustre* moss samples exhibited strong amphoteric properties, due to the acid-base dissociation of protonated organic moieties on their leaf and stem surfaces. The LPM model applied to the moss acid-base titration allowed to evaluate the acidity constants of potential functional groups (carboxyl/phosphodiester, carboxyl, phosphoryl, amine and polyphenols) occurring on the moss cell surfaces. *Sphagnum* mosses used in this study as biosorbents are well-known for the formation of peat, a complex mixture of organic compounds derived from incomplete degradation of plant materials and containing cellulose, lignin, humic and fulvic acids (Hodgkins et al., 2018; Klavins and Sire, 2010). Low molecular weight aliphatic compounds and simple phenolic molecules can be abundantly present in *Sphagnum* mosses concurring therefore to the retention of cations on moss surfaces through their functional groups. A significant role in the adsorption processes of lignin, a more complex phenolic molecule, may be tentatively taken into account, given that its presence in *Sphagnopsida* members is controversial (Klavina et al., 2012; Maksimova et al., 2013). However, the cuticle of the model moss *Physcomitrella patens* does not contain lignin but a phenol-enriched biopolymer that is regarded as evolutionary ancestor of lignin (Renault et al., 2017).

Typically, the moss species in the genus *Sphagnum* present dead and empty leaf cells (hyalocystes) communicating with the external environment through a variable number of pores for water and substances intake and storage. The fast adsorption of Pb^{2+}, almost completed within 10 min, suggests the absence of rate limitation by bulk and surface diffusion. Therefore, it is conceivable that both abaxial and adaxial leaf surfaces can be involved in metal adsorption processes. Although Beike et al. (2015) showed that field-grown and cloned *S. palustre* can display phenotypical differences, the sole observation of the morphological features can barely explain the differences between clones and field mosses in terms of metal adsorption ability.

Taking into account the pKa, the binding site concentrations and the possible functional groups computed from the application of the LPM model, there are no significant differences between the total concentration of binding sites for untreated and treated clones. The metal uptake rate is closely related to the efficiency of moss surface binding-sites to adsorb metals. The Pb^{2+} adsorption on field-grown and bioreactor-grown cloned moss surfaces as function of the time revealed a biphasic kinetic, with a fast uptake at the beginning of the process and a subsequent slower step. This behavior was already observed by Qin et al. (2006) and ascribed to the heterogeneity of the binding sites and to the porous nature of the peat moss cells, allowing a differentiation between an external and an internal diffusion of metal ions. In our study, no significant differences in terms of adsorption rate were observed between untreated and treated cloned mosses, consistently with the results of acid-base titrations.

The Pb^{2+} adsorption on moss surfaces was studied as function of pH because it is well known that the pH is the most important factor influencing the adsorption of heavy metals on all solid surfaces including plant biomass (Stumm, 1992), by affecting the degree of protonation of the functional groups occurring on the surfaces and, consequently, their availability to bind ions from solutions. Moreover, the adsorption of divalent heavy metal cations is mainly related to metal ion hydrolysis. Thus, the adsorption of heavy metals increases with increasing pH and with decreasing pK value of the metal ion hydrolysis reaction in the sequence Cd (10.1) < Ni (9.9) < Co (9.7) < Zn (9.0) ≪ Cu (7.7) < Pb (7.7) ≪ Hg (3.4) (Gryschko et al., 2005). Metal adsorption was studied from aqueous

solution assuming that either in water or in air environment it takes place between the aqueous metals and the moss surface. The adsorption of Pb^{2+} by moss surfaces strongly increased with pH in the pH range 2–9. The maximum adsorption of Pb^{2+} occurred at around pH 6.0 (namely, at pH 5.5 for field mosses and between 5.5 and 6.5 for cloned mosses). At these conditions, the DOC released in solution by mosses should not interfere with Pb^{2+} adsorption, as at pH 4.5 the DOM is highly protonated and not capable to form strong complexes with heavy metals (Kalbitz and Wennrich, 1998).

The maximum sorption capacities (q_{max} values) for Pb^{2+} of *S. palustre* mosses measured in the present study were not consistently different from those reported in the literature for other *Sphagnum* spp. (Table ESM-3). Exceptions (e.g. 1.109 mmol g^{-1} for *Sphagnum* spp.; González and Pokrovsky, 2014) might derive from differences in specie-specific characteristics, experimental conditions and sample preparations. Regardless the moss nature and status, a "universality" of adsorption parameters consisting in a similarity of the metal adsorption between different moss-biosorbents can be stated, as previously seen for other cations (González et al., 2016a, 2016b; González and Pokrovsky, 2014).

Moss treatment by devitalization has been proven to be a useful method both for field and cloned mosses to preserve their tissue integrity during exposure in bags, to avoid metabolism effects on pollutant uptake and to reduce the variability of results without significantly changing elemental composition of pre-exposed mosses (Adamo et al., 2007; Di Palma et al., 2016; Fernández et al., 2010; Giordano et al., 2009). This work shows that devitalization does not significantly enhance the Pb^{2+} adsorption capacity of mosses, even when it is followed by EDTA washings. This latter pre-treatment, although highly recommended for field-growing mosses to use for biomonitoring surveys (Ares et al., 2012), had no significant effect in increasing the ability of the cloned moss to *in-vitro* adsorb Pb^{2+} cations. This despite the slight increase of the $SSA_{B.E.T.}$ of cloned samples induced by EDTA washings combined with devitalization. EDTA pre-treatment seemed rather negatively affect the determination of cloned moss surface amphoteric properties and reduce the amount of Pb^{2+} available for adsorption on moss surfaces by forming complexes between Pb^{2+} and residues of EDTA released in solution. Moreover, it was already figured out that EDTA has no evident effect on the cloned moss elemental content, which is already very low compared to the mosses collected in nature, and that this pre-treatment can lead to Na content excess in clones, as well as to morphological damages to moss shoots and leaves (Di Palma et al., 2016). On the basis of our results, combined with the concept of the "universal metal adsorption edge" (i.e. adsorption of metals on mosses that follows an adsorption pattern comparable with other organic materials; González and Pokrovsky, 2014), it is conceivable that EDTA treatment used for moss clones can produce the same effect for other divalent metals.

5. Conclusions

The acid-base titration experiments and Pb^{2+} adsorption as a function of time, pH and Pb concentration in solution helped to characterize the surface chemical properties of the *S. palustre* clone developed in the Mossclone project. Moreover, this work provides new insights into the clone's ability to adsorb heavy metals (at least divalent cations as Pb^{2+}) once employed for the moss bag technique. Moss treatments with EDTA and devitalization by heating at 100 °C did not improve the ability of cloned moss to adsorb Pb^{2+}, and did not produce any substantial changes of their surface chemistry. However, we strongly recommend the use of devitalization as preserving method for moss clone during bag preparation and exposure. In contrast, washings with EDTA could be avoided for this new biomonitor, because it already has a very low metal concentration as a consequence of its *in vitro* cultivation in bioreactors. Overall, using oven-heating devitalization as sole treatment of cloned mosses appears to be the best strategy for biomonitoring applications, because it reduces the cost and time of moss bags preparation without negatively affecting the performance of the moss bags.

Acknowledgements

We thank Prof. Antonio Violante, University of Naples Federico II, for his helpful comments that greatly improved the manuscript. Part of this work was funded by FP7-ENV.2011.3.1.9-1, European Union (MOSSCLONE). Aridane G. González acknowledges a Post-doctoral grant of the Universidad de Las Palmas de Gran Canaria, Spain.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124375.

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