Physiological Effects of Exposure to Arsenic, Mercury, Antimony and Selenium in the Aquatic Moss Fontinalis antipyretica Hedw.

Article *in* Water Air and Soil Pollution · August 2013

DOI: 10.1007/s11270-013-1659-1

CITATIONS
10

READS
215

4 authors, including:

carballeira A
University of Santiago de Compostela
230 PUBLICATIONS **6,041** CITATIONS

SEE PROFILE

Water Air Soil Pollut (2013) 224:1659
DOI 10.1007/s11270-013-1659-1

Physiological Effects of Exposure to Arsenic, Mercury, Antimony and Selenium in the Aquatic Moss *Fontinalis antipyretica* Hedw.

Santiago Díaz · Rubén Villares · María D. Vázquez · Alejo Carballeira

Received: 20 February 2013 / Accepted: 16 July 2013

Abstract Laboratory experiments were carried out to determine the effects of exposure to different concentrations of As, Hg, Sb and Se on photosynthetic and respiratory rates and on photosynthetic efficiency in the aquatic bryophyte *Fontinalis antipyretica* Hedw. Specimens of the moss, collected from a clean site, were incubated in solutions of As, Hg, Sb and Se (at concentrations ranging from 0.1 μg l^{-1} to 10,000 μg l^{-1}) for up to 22 days. The photosynthetic and respiratory rates were then determined by the light/dark bottle technique, and the photosynthetic efficiency was measured by the saturation pulse method. Although different responses were observed in relation to the concentration of the elements, clear responses in net photosynthesis and photosynthetic efficiency were generally only observed in the moss exposed to the highest concentrations of these elements in solution. Mercury was apparently the most toxic of the elements studied. Net photosynthesis and photosynthetic efficiency were also related to tissue concentrations of these elements in the moss. Despite the higher toxicity of Hg, this element can be accumulated at high concentrations in moss, probably at extracellular sites. For Sb, the same tissue concentration had very different physiological effects depending on the initial concentration to which the moss was exposed in solution. Temporal trends in chlorophyll fluorescence were more stable than trends in net photosynthesis. The respiratory rate was very variable and was not clearly related to the concentration of elements in solution or in moss tissues.

Keywords Photosynthesis · Respiration · Photosynthetic Efficiency · Bryophytes

S. Díaz · A. Carballeira
Ecología, Facultad de Biología, Universidad de Santiago de Compostela,
Santiago de Compostela, Spain

R. Villares (✉) · M. D. Vázquez
Ecología, Escuela Politécnica Superior, Universidad de Santiago de Compostela,
Lugo, Spain
e-mail: ruben.villares@usc.es

1 Introduction

In this study, we investigated the physiological effects of arsenic, mercury, antimony and selenium in the aquatic moss *Fontinalis antipyretica*. These elements, which are all toxic at relatively low concentrations, have different (if any) functions in living organisms. Selenium is a micronutrient in many taxonomic groups, although it is not essential for plants (Schrauzer 2004). Data obtained in different studies suggest that As is also an essential element in many living organisms, although this has not yet been corroborated (Stoeppler 2004). Antimony and Hg have no known function in living organisms (Drasch et al. 2004; Rish 2004). All of these elements occur naturally in fresh water, usually at low concentrations (<10 μg l^{-1} for As and Se, and only a few nanograms per litre for Sb and Hg) (Koch et al. 1999; Drasch et al. 2004; Rish 2004; Schrauzer 2004; Stoeppler 2004). However, they can be found at much

Published online: 31 July 2013

higher concentrations in certain areas as a result of natural or anthropogenic sources. For example, high concentrations of arsenic (10–5,000 μg l^{-1}) have been detected in hot spring waters (Kanamori and Sugawara 1965; Miyashita et al. 2009), and high concentrations of Sb (more than 150 μg l^{-1}) have been measured in rivers in some mining areas (Fu et al. 2010).

Aquatic bryophytes have been used for many years to biomonitor water pollution (e.g. Empain 1973; Wehr and Whitton 1983; Cenci 2000; Cesa et al. 2009). The moss *F. antipyretica* is one of the species most commonly used for biomonitoring as it is widely extended in the northern hemisphere, has been shown to accumulate different contaminants at high levels and alterations in its physiology are known to be good indicators of water quality (Vázquez et al. 1999; Cenci 2000; Fernández et al. 2006). Thus, the effects of variables such as organic pollution (Martínez-Abaigar et al. 1993; Vázquez et al. 2013), temperature (Carballeira et al. 1998), metals such as Cd and Cu (Sommer and Winkler 1982), wood ash solution (Aronsson and Ekelund 2006), ammonium (Vieira et al. 2009) and desiccation (Cruz de Carvalho et al. 2011) have been studied in relation to photosynthesis and/or respiration in *F. antipyretica*. Stress also affects the chlorophyll fluorescence yield in plants (Branquinho et al. 1997a, b; Lichtenthaler and Miehe 1997), although few studies have dealt with this parameter in the species under consideration (Rau et al. 2007).

In the present study, we aimed to determine how exposure of *F. antipyretica* to As, Hg, Sb and Se affected several physiological variables: net photosynthetic and dark respiration rates and photosynthetic efficiency. The results obtained will increase our knowledge of the physiological effects of these elements and the risks associated with their presence at different concentrations in aquatic systems. This will also enhance the usefulness of this moss with a view to extending its application to assessing the quality of fresh water.

2 Materials and Methods

2.1 Plant Material

Moss samples were collected from an unpolluted site in the upper reaches of the river Lérez (Galicia, NW Spain). There is no industrial activity in the upper river basin and the population density is low in this area. The initial concentrations of As, Hg, Sb and Se in these samples were 1,680, 0.978, 199 and 483 ng g^{-1}, respectively. These concentrations are similar to or lower than those reported for aquatic mosses from clean sites or concentrations cited as background levels (Roeck et al. 1995; Carter and Porter 1997; SEPA 2000; Nimis et al. 2002; Pekka et al. 2008; Culioli et al. 2009; Cesa et al. 2010; Gapeeva et al. 2010). Only plants submerged to a certain depth were collected to avoid those that might have suffered stress by being emerged for a prolonged period (Wehr and Whitton 1983). The samples were rinsed on site with river water and transported to the laboratory in a portable refrigerator (5±2 °C). In the laboratory, the plants were carefully washed with dechlorinated water to remove epiphytes and debris. All experiments were performed with 2-cm-long apices to minimize errors due to variations in the accumulation capacities of different plant parts (Wehr and Whitton 1983; Wells and Brown 1990).

2.2 Experimental Conditions

The moss samples were incubated in glass tanks with 5 l of each of the following solutions of As, Hg, Sb and Se: 0 (control), 0.1, 1, 10, 100, 1,000 and 10,000 μg l^{-1}, prepared from standard solutions of, respectively, As_2O_5, $Hg(NO_3)_2$, $SbCl_3$ and SeO_2 (Merck and Panreac). The different dilutions were prepared with tap water (the characteristics of which are shown in Table 1) filtered through activated carbon. During the incubations, the water was aerated continuously by means of an air pump. All experiments were carried out in a cool chamber at 16±0.1 °C, with a 12:12 h day/night cycle (light intensity 80 μmol photons m^{-2} s^{-1}). Aliquots of the moss samples were removed from the solutions after 1, 2, 4, 7, 11, 16 and 22 days, and the physiological variables and the element concentrations were determined as explained below. The aliquots used to measure the physiological variables were discarded and were not returned to the tanks, i.e. repeated measurements were not made on the same apices over time.

2.3 Determination of Elemental Concentrations

The element concentrations in mosses were determined from 300±5 mg apical tissue samples, which were digested with nitric acid at high temperature and high pressure, as previously described (López and Carballeira

Table 1 Characteristics of the water used in the experiments. Source: Town Council of Santiago de Compostela

Parameter	Unit	
pH		7.1
Conductivity	μS cm^{-1}	100
As	μg l^{-1}	<0.2
Hg	μg l^{-1}	<0.2
Sb	μg l^{-1}	<0.2
Se	μg l^{-1}	<0.2
Ca^{+2}	mg l^{-1}	2.3
Na^{+}	mg l^{-1}	14.0
Cl^{-}	mg l^{-1}	22.8
SO_4^{-2}	mg l^{-1}	3.5
NH_4^{+}	mg l^{-1}	<0.10
NO_2^{-}	mg l^{-1}	<0.05
NO_3^{-}	mg l^{-1}	2.8

1993; Wehr and Whitton 1983). The concentrations of As, Sb and Se were determined by atomic fluorescence spectroscopy (PSA Excalibur). The concentration of Hg was determined by atomic fluorescence spectroscopy (PSA Merlin Plus). For analytical control of the process, samples of certified reference material (European Community Reference Bureau No. 61: *Rhynchostegium riparioides*, an aquatic moss) were analysed at the same time as the other samples.

2.4 Determination of Physiological Variables

The net photosynthesis and dark respiration rates were determined by the light/dark bottle technique. Twenty apical segments of about 2 cm in length (approximately 0.05 g dw) were placed in each bottle (Karlsruhe, WTW KF-12, 0.31 l) and the bottles were incubated for 4 h at 16±0.1 °C and a light intensity of 80 μmol photons m^{-2} s^{-1}. The oxygen concentration was measured in each bottle before and after the assay with a shaking-head polarographic oximeter (Oxi96, WTW). At the end of the assay, the apical segments were dried at 105 °C to enable calculation of the dry weight.

Photosynthetic efficiency was evaluated by chlorophyll *a* fluorescence, a non-invasive technique. Chlorophyll fluorescence parameters were measured by the saturation pulse method (Schreiber et al. 1995) with a portable fluorometer (PAM-2000 photosynthesis yield analyser; Walz GmbH, Effeltrich, Germany). A pulse of saturating light (>5,000 μmol photons m^{-2} s^{-1}, 0.8 s pulse length, actinic white light) was applied through a fibre optic cable at an angle of 60° relative to the sample and at a distance of 12 mm from the moss surface. Prior to measurements, excess water was blotted from the moss and samples were dark-adapted for 20 min. This length of time is considered to be sufficient to allow all photosystem II (PSII) reaction centres to open (Fracheboud and Leipner 2003). Four replicate moss samples per treatment were then analysed to determine the maximum quantum yield of PSII as the ratio of $F_v/F_m=(F_m-F_0)/F_m$ (Bolhàr-Nordenkampf et al. 1989), where F_0 and F_m are, respectively, the minimal and maximal fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open (i.e. all primary acceptors oxidized). The F_v/F_m ratio indicates the efficiency by which the excitation energy is captured by open photosystem II reaction centres and represents the fraction of incident photon energy that is processed photochemically (Butler and Kitajima 1975; Krause and Weis 1991). This ratio, which is considered valuable in ecophysiology and stress physiology, decreases under stress conditions (Lichtenthaler and Miehé 1997; Csintalan et al. 1999). Unfortunately, the fluorometer broke down during the process of the study, thus preventing us from obtaining the data for Se. However, we think it is useful to present the results for the remaining elements.

2.5 Statistical Analysis

The relationships between the physiological variables and the independent variables *concentration in water* and *time* were modelled by multiple regression: linear and logarithmic fits were tested and the most significant fit was selected. If the fit for either of the independent variables was not significant, the variable was omitted from the analysis and a new fit was tested with the remaining independent variable by means of the curvilinear regression option of the IBM SPSS Statistics 20 software. The relationships between the physiological variables and the tissue concentrations were modelled in the same way.

3 Results

3.1 Effects on Net Photosynthetic Rate

The photosynthetic rates are expressed as percentages, calculated relative to the control for the same incubation

period. This procedure corrects for the "transplant effect," i.e. stress caused by relocation of specimens and by the laboratory conditions under which the experiments were performed. The response of net photosynthesis (NP) in relation to the concentration of the elements in solution varied depending on the element considered (Fig. 1). For Hg, this physiological parameter tended to decrease from low concentrations in solution. For As, the NP values were similar to the control values in the range 0.1 to 100 μg l^{-1} and were lower at higher concentrations of the element. For Se, there was also a clear decrease in the NP at the higher concentrations. Unusual behaviour was observed for Sb, with an increase in NP at concentrations of 100 and 1,000 μg l^{-1}, and a decrease at 10,000 μg l^{-1}. Although these data indicate a relationship between exposure concentration and photosynthetic rate, the effects were most evident at very high exposure concentrations (1,000–10,000 μg l^{-1}), which are not usually found in natural environments. However, for Hg, the relationship was manifested from lower concentrations.

The multiple regressions for NP in relation to the exposure concentration and time were established by taking into account all of the data for Hg because as already mentioned, it was the only element for which the NP values decreased continuously with increasing exposure concentration. For the other elements, which appeared to display a threshold response, only the final exposure concentrations were fitted. In all cases, time was not a significant variable (Table 2), and therefore, the fits were repeated by considering only the exposure concentration as an independent variable (Fig. 1). The most satisfactory fits were generally obtained with logarithmic equations, except for As, for which a inverse equation was most appropriate.

The median effective solution concentrations (i.e. the concentration of the element in solution that caused a 50 % reduction in net photosynthesis with respect to the control, $EC50_w$) were calculated from these regressions. The $EC50_w$ values followed the order Hg<As<Se≪Sb.

The relationship between NP and the tissue concentration of each element is shown in Fig. 2. From the

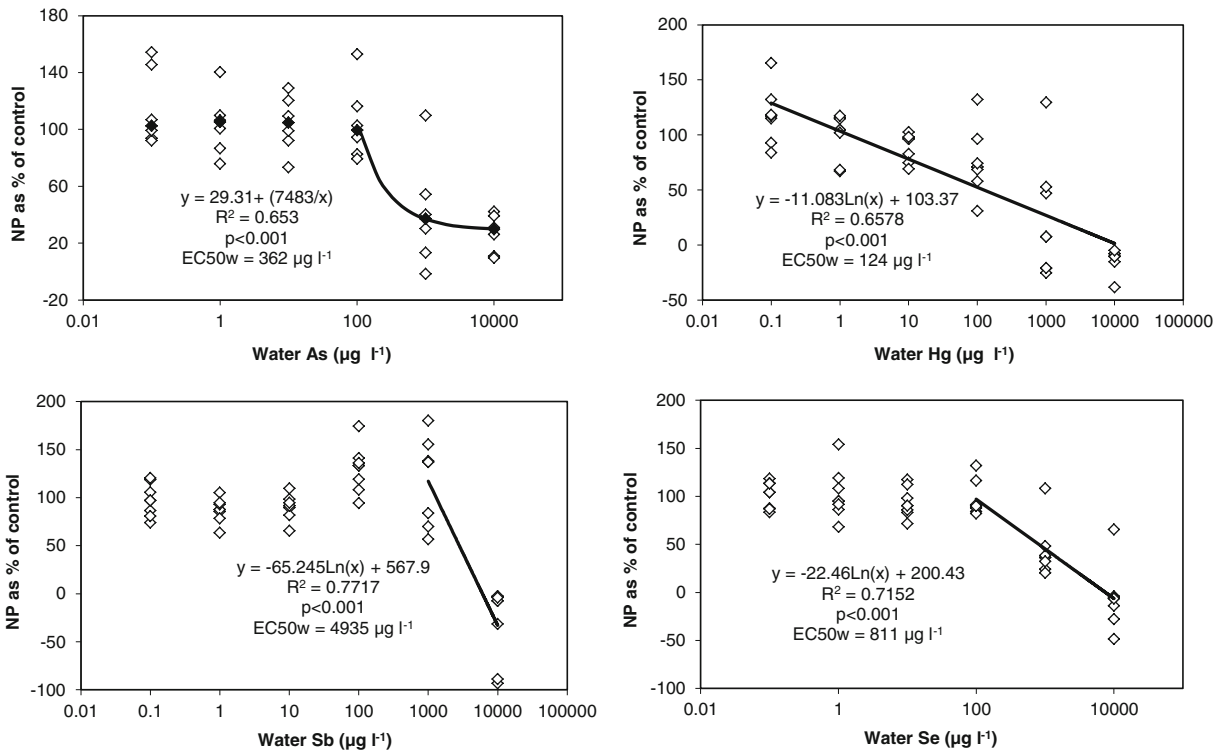

Fig. 1 Net photosynthetic rate (NP) of *F. antipyretica* previously exposed to different concentrations (0.1 to 10,000 μg l^{-1}) of As, Hg, Sb and Se in solution; the results are expressed as a percentage of the control value. *Black diamonds* represent the median values for each exposure concentration. The median effective solution concentrations for NP ($EC50_w$) calculated from regression equations are also shown

Table 2 Fits obtained by multiple regression for net photosynthesis and photosynthetic efficiency (F_v/F_m) in relation to the exposure concentrations and exposure times

		Coefficients	Sig.	Model R^2	Sig.
Net Photosynthesis					
As	Constant	161	<0.001	0.622	<0.001
	Ln X_1	−16.7	<0.001		
	X_2	1.23	0.156		
Hg	Constant	113	<0.001	0.669	<0.001
	Ln X_1	−11.1	<0.001		
	Ln X_2	−5.40	0.262		
Sb	Constant	139	<0.001	0.774	<0.001
	X_1	−0.017	<0.001		
	X_2	−0.614	0.725		
Se	Constant	220	<0.001	0.771	<0.001
	Ln X_1	−22.5	<0.001		
	Ln X_2	−11.3	0.051		
F_v/F_m					
As	Constant	121	<0.001	0.740	<0.001
	Ln X_1	−9.84	<0.001		
	Ln X_2	11.78	0.001		
Hg	Constant	108	<0.001	0.918	<0.001
	X_1	−0.007	<0.001		
	Ln X_2	−7.91	0.002		
Sb	Constant	100	<0.001	0.997	<0.001
	X_1	−0.010	<0.001		
	X_2	−0.024	0.762		

Fits are shown for exposure concentrations in water from 100 to 10,000 μg l^{-1}, except for net photosynthesis and Hg, for which fits were made with all data, and net photosynthesis and Sb, for which only data for exposure concentrations of 1,000 μg l^{-1} and 10,000 μg l^{-1} were used

X_1 exposure concentration, X_2 time

regression equation that best fitted the data, we calculated the median effective tissue concentrations ($EC50_t$), i.e. the concentrations of an element in the moss causing 50 % inhibition of NP with respect to the control. Best fits were achieved with logarithmic equations for As, Hg and Sb, and linear equation for Se. The fits for Hg and Se were better, and the coefficients of determination for As and Sb were quite low, although significant. The order of $EC50_t$ values was Se<As<<Hg<Sb.

3.2 Temporal Changes in the Net Photosynthetic Rate

In the moss samples exposed to As in solution, the NP decreased clearly over time only at the highest exposure concentration (Fig. 3). There was no evident trend, even at exposure to 1,000 μg l^{-1} of As, although the NP was almost always lower than in the control. For the other concentrations, the NP values were similar to the control values.

In the samples exposed to Hg, the NP values were similar to control values for exposure concentrations of up to 10 μg l^{-1}. In samples exposed to 100 μg l^{-1} of Hg, and particularly 1,000 μg l^{-1}, the NP decreased over time, and the values became negative for the longest exposure times. At the highest exposure concentration, and after an initial increase, the NP remained constant and negative. At this concentration, the moss appeared completely deteriorated from the first day of incubation, so that the value obtained may indicate a stressful situation with lethal effects. In fact, in laboratory studies, Samecka-Cymerman and Kempers (1995) calculated that the lethal concentration (24 h LC100) of Hg for the liverwort *Scapania undulata* was between 500 and 1,000 μg l^{-1}, i.e. within the same range of concentrations

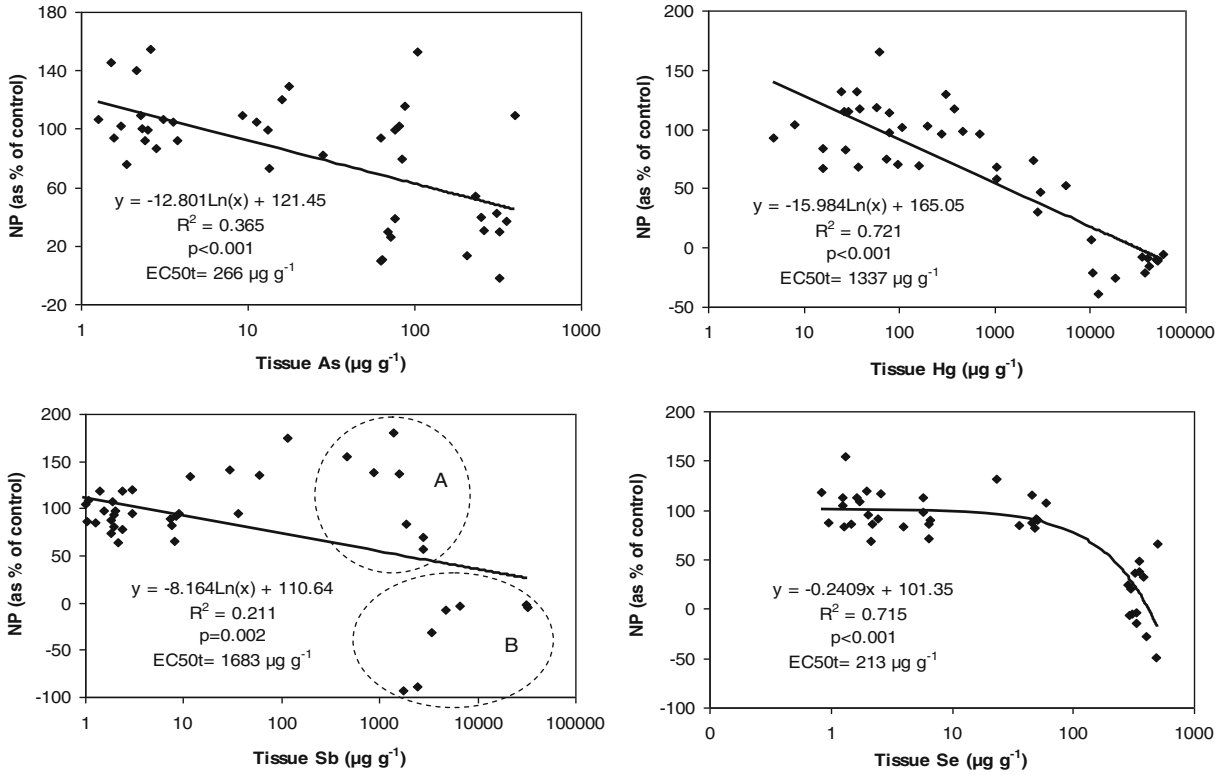

Fig. 2 Regressions between the net photosynthetic rate (*NP*), expressed as a percentage of the control value, and tissue concentrations of As, Hg, Sb and Se in the moss *F. antipyretica* after the exposure period. Median effective tissue concentrations for NP ($EC50_t$) estimated from regression equations are also shown. In the Sb graph, clusters *A* and *B* corresponds to samples exposed to 1,000 and 10,000 µg Sb l^{-1} respectively

at which there was a strong decrease in the NP in the present study.

For Sb, a decreasing trend in NP over time was only notable at 1,000 µg l^{-1}, whereas the effect of the maximum exposure concentration was similar to that observed for Hg. In the case of Se, there was no clear pattern, and the NP values were similar to controls at the first four concentrations. In the samples exposed to 1,000 µg l^{-1}, there was a sharp decrease in NP between first and second day of incubation, with constant values thereafter. At 10,000 µg l^{-1}, the response was similar, but with lower values of NP.

3.3 Effects on Dark Respiration Rate

Unlike net photosynthesis, the dark respiration (DR) rates were not clearly related to the exposure concentration. Although the median value tended to decrease, no clear trend was observed, as occurred in the case of the NP, and the values were very variable (data not shown). For As and Sb, the temporal changes in the DR for each exposure concentration were similar, with an initial increase in DR and a subsequent decrease. The initial increase, also observed in the NP, may be due to the stress suffered by the moss at the beginning of the exposure period. The moss then appeared to acclimatize to the new situation, which led to a delayed decrease in DR, and stabilization at values similar to control values for lower exposure concentrations. At the highest concentrations, the decreases were much more pronounced, probably because the physiology of the moss was seriously affected. For Hg, a clear trend was only observed at 10,000 µg l^{-1}, and there was a gradual decrease in respiratory rates, probably due to damage to the moss physiology and its subsequent death. The most clearly defined pattern of dark respiration rates was observed in moss samples exposed to Se, with values tending to increase with increasing time of exposure, which probably reflects the increased physiological stress over time. For the highest concentration, after a rapid initial increase, the values stabilized at levels well below the controls, probably indicating severe physiological

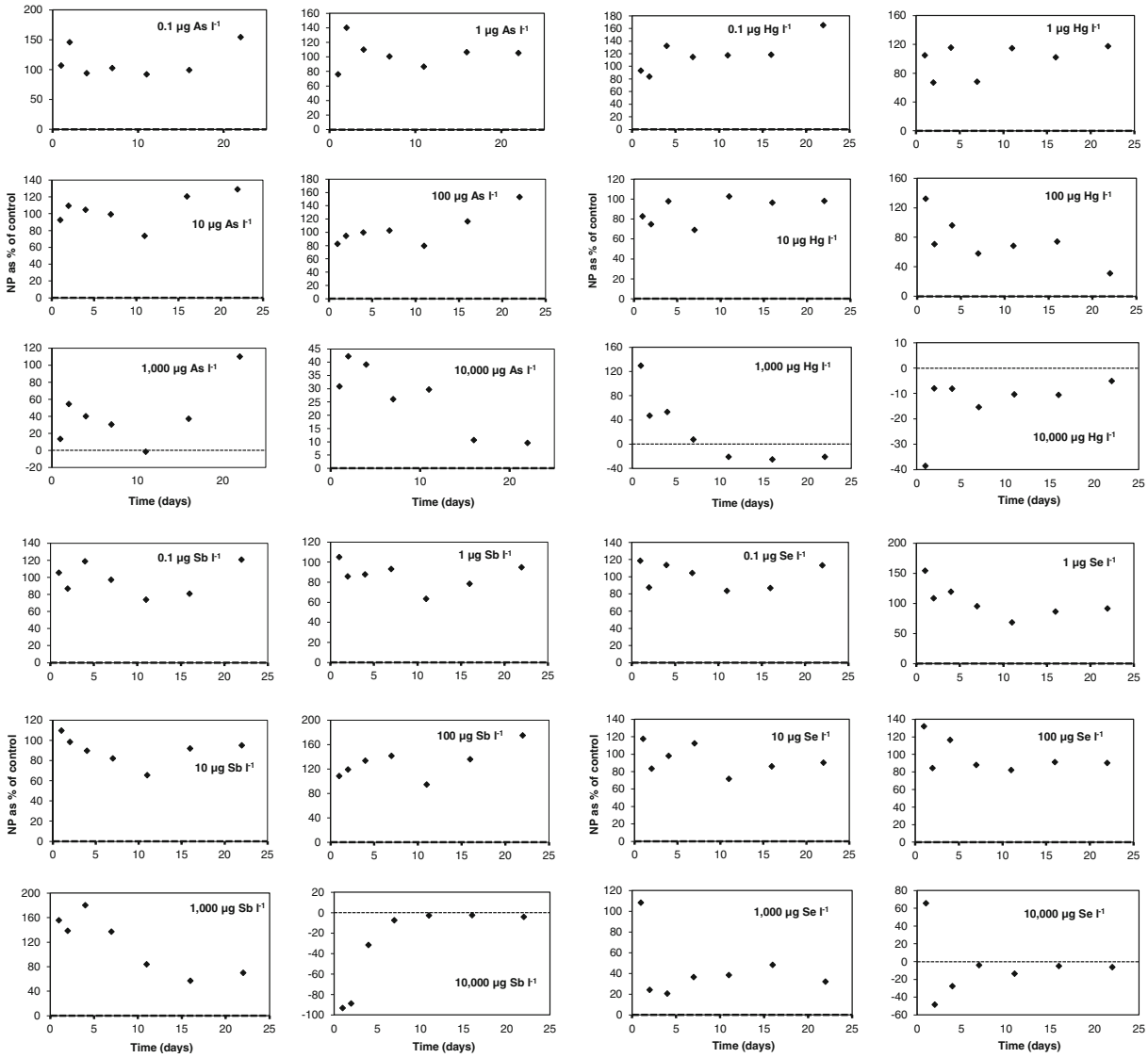

Fig. 3 Temporal changes in the net photosynthetic rate (*NP*), expressed as a percentage of the control value, in *F. antipyretica* exposed to different concentrations of As, Hg, Sb and Se in water. The *dotted line*, which represents NP=0, is shown for visual reference

damage. No clear trends in DR were observed in relation to the tissue concentrations of elements.

3.4 Effects on Photosynthetic Efficiency

The F_v/F_m ratio was only affected at the highest exposure concentrations (1,000 and 10,000 µg l^{-1}) of the elements (Fig. 4). The variable time had a significant effect for As and Hg (Table 2), so that the $EC50_w$ values for both elements depended on time. The $EC50_w$ values calculated for half of the exposure time (11 days) are shown in Fig. 4. In relation to tissue concentrations, the F_v/F_m ratio showed a clear pattern for Hg (Fig. 5), which began to decrease with tissue levels above approximately 1,000 µg g^{-1}. For As, the values began to decrease at tissue concentrations above approximately 60 µg g^{-1}, although the data are more widely scattered. The $EC50_t$ for Hg was much higher than for As, as found with $EC50_t$ calculated from net photosynthesis, although the differences were even greater. For Sb, the values for most of the samples (except for samples exposed to 10,000 µg l^{-1}) were similar to the control values.

The F_v/F_m ratio did not indicate any temporal trends for exposure up to 100 µg l^{-1} for any of the elements

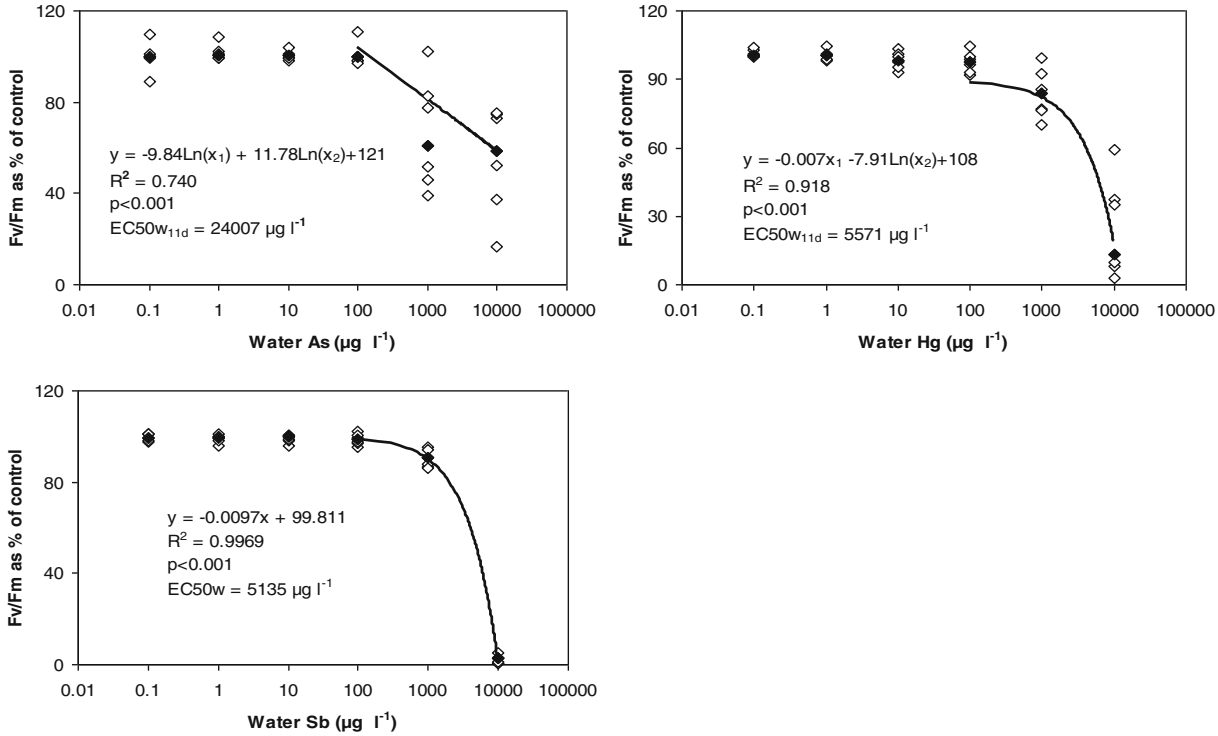

Fig. 4 Photosynthetic efficiency expressed as F_v/F_m of *F. antipyretica* previously exposed to different concentrations (0.1 to 10,000 µg l^{-1}) of As, Hg and Sb in solution; the results are expressed as a percentage of the control value. *Black diamonds* represent the median values for each exposure concentration. The median effective water concentrations for NP ($EC50_w$) calculated from regression equations are also shown, only the data for exposure concentrations between 100 and 10,000 µg l^{-1}were fitted. For As and Hg, the regression line shown is for time=11 days. X_1 exposure concentration. X_2 time

(Fig. 6), and values were very close to the control values. At the two highest exposure concentrations of As, the F_v/F_m ratio values tended to increase with the exposure time, although they were almost always lower than the control values. In contrast, at the two highest concentrations of Hg, the photosynthetic efficiency tended to decrease over time. The temporal trend in As and Hg concentrations was reflected in the multiple regressions shown in Fig. 4, in which as mentioned, the variable time was significant, with a positive slope for As and a negative slope for Hg.

4 Discussion

The net rates of photosynthesis and respiration in control mosses were within the ranges obtained in other studies of the same species (Aronsson and Ekelund 2006; Vázquez et al. 2013). A decreasing trend in net photosynthesis as the concentration of elements in the exposure solution increased was also observed with *F. antipyretica* exposed to Cd and Cu (Sommer and Winkler 1982) and with the terrestrial moss *Rhytidiadelphus squarrosus* exposed to Hg (Brown and Whitehead 1986).

The $EC50_w$ values based on the effect on net photosynthesis (Fig. 1) indicate that Hg was the most toxic of the elements under study (lower $EC50_w$), followed by As and Se. The least toxic was Sb, with a clearly higher $EC50_w$ value than the other elements. These results indicate that in the case of Hg, the effects on NP, although progressive, were evident from low concentrations. In the case of Sb, high concentrations in solution were required to produce a clear effect, and As and Se had intermediate effects. However, as this was a preliminary study of the toxicity of these elements, a wide range of exposure concentrations was used (logarithmic progression), and (except for Hg and NP) only two or three concentrations were used to calculate the $EC50_w$ values (for both NP and photosynthetic efficiency). Therefore, the $EC50_w$ values calculated in this study can only be used as guidelines. Future studies employing a wider range of concentrations in the area

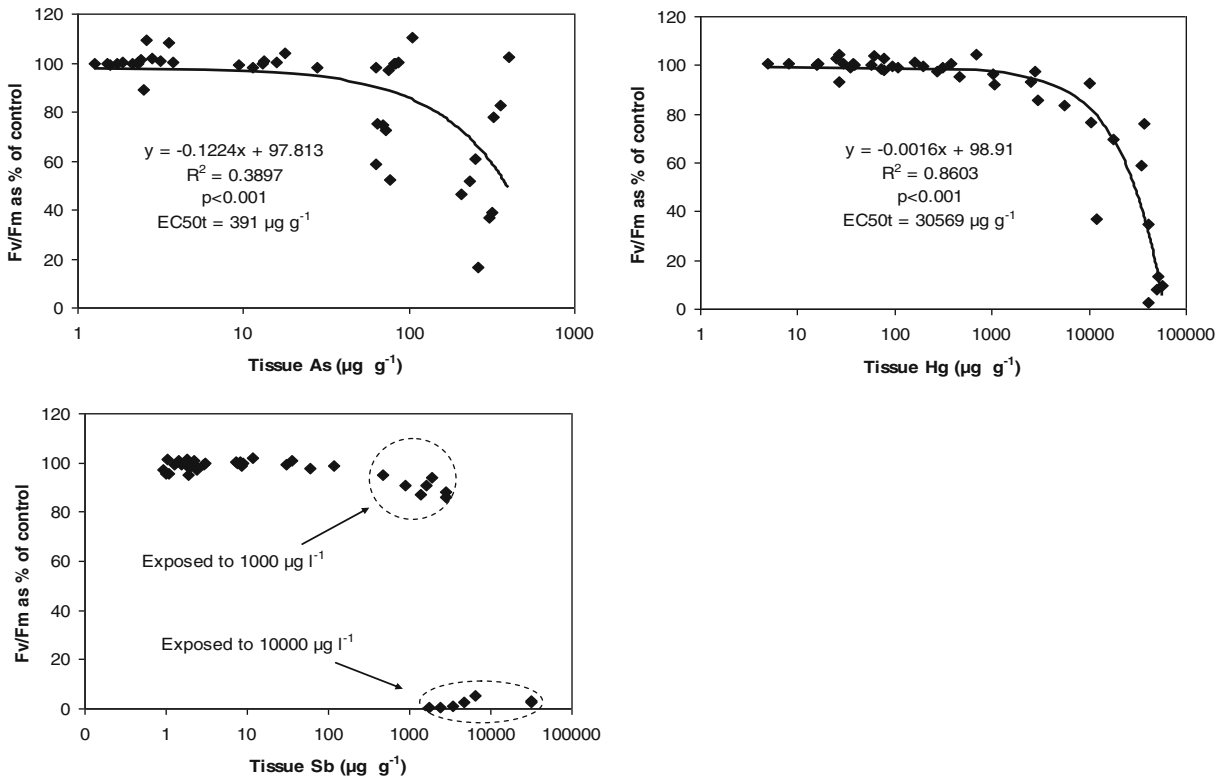

Fig. 5 Regressions between photosynthetic efficiency, expressed as F_v/F_m, and tissue concentrations of As, Hg and Sb in the moss *F. antipyretica* after the exposure period; the results are expressed as a percentage of the control value. Median effective tissue concentrations for NP ($EC50_t$) estimated from regression equations are also shown

where most variation in the results is expected, with the concentrations on a linear scale and use of replicates, would enable calculation of more accurate $EC50_w$ values.

The $EC50_t$ values (Fig. 2) show that low tissue concentrations of Se and As have the same effect on the NP in *F. antipyretica* as higher concentrations of Hg and Sb. Arsenic and Se tend to be bioconcentrated to a low degree but with important physiological effects, whereas Hg, which is easily bioconcentrated, is proportionally less toxic (a detailed description of the uptake kinetics found in the incubations has been published in Díaz et al. (2012)). These results clearly differ from those obtained for the concentrations in solution, which indicated (as explained above) that Hg was the most toxic element. The differences between both parameters ($EC50_w$ and $EC50_t$) may be related to the different proportions in which each element accumulates at the different cell sites. The cellular location of a toxic element is important in relation to the physiological effects, and elements accumulated in intracellular locations usually have the most negative effects (Brown and Wells 1990; Sidhu and Brown 1996; Branquinho et al. 1997a). Mercury is a type B element, with a high affinity for extracellular anionic sites in moss (Nieboer and Richardson 1980), so that it is easily accumulated at this site. The high $EC50_t$ values observed for Hg may therefore be attributable to its accumulation in the extracellular location, where the metal will have only a slight effect on the moss physiology, especially in the short term. This may explain why the effect of Hg was not as strong as might be expected in view of the tissue concentrations reached (Brown and Whitehead 1986). Unfortunately, no techniques had been developed to determine the cellular location of the studied elements in mosses when our study was carried out (see review by Pérez-Llamazares et al. 2011b), and a technique for extracting extracellular Hg from the terrestrial moss *Pseudoscleropodium purum* has only recently been developed (Pérez-Llamazares et al. 2009, 2011a). However, plants have mechanisms to combat intracellular metal toxicity: synthesis of antioxidants such as phenolic compounds, β-carotene and

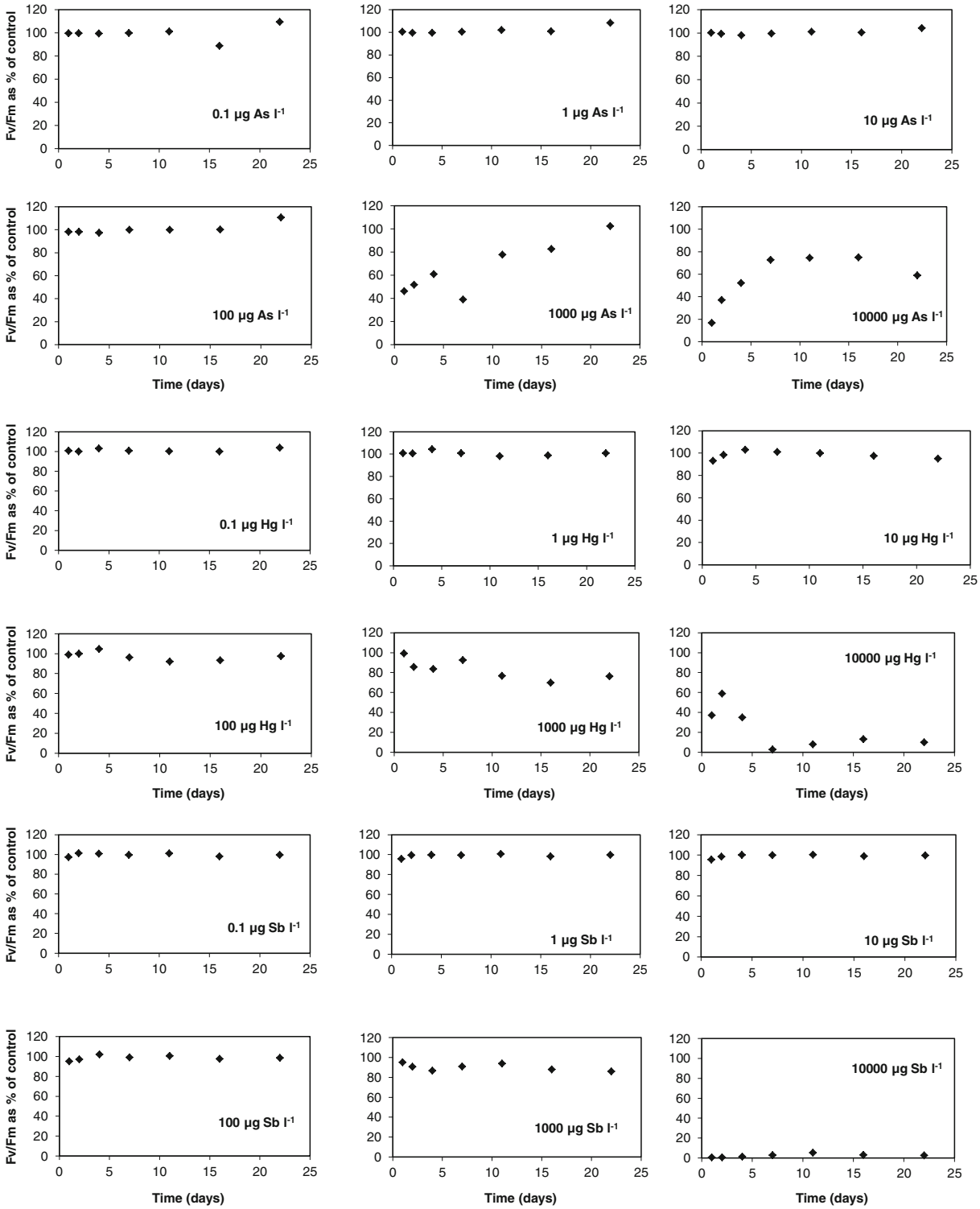

Fig. 6 Temporal changes in chlorophyll fluorescence (F_v/F_m), expressed as a percentage of the control value, in *F. antipyretica* exposed to different concentrations of As, Hg and Sb in water

glutathione; synthesis of phytochelatins that act as heavy-metal-binding peptides; or the accumulation of metals in vacuoles (Bruns et al. 1997; Carginale et al. 2004; Israr et al. 2006; Esteban et al. 2008). Moreover, the toxic Sb(III) form, which was the form used in the experiments, can be converted in plants to a less toxic Sb(V) form (Babula et al. 2008).

For Sb, at the highest concentrations in solution, the moss samples in which similar tissue concentrations were achieved showed different responses (Fig. 2). In those samples incubated in solutions containing 1,000 μg l^{-1} Sb, in which the tissue concentration was reached gradually, the NP was similar to that of the controls. However, in those samples exposed to 10,000 μg l^{-1} in solution, in which similar tissue concentrations were reached more quickly, the NP decreased greatly. Therefore, the moss appears to be able to adapt to low to moderate concentrations of this element in water.

The changes in NP over time again show the higher toxicity of Hg, as this is the only element that caused a gradual decrease over time in the NP at 100 μg l^{-1} in water (Fig. 3). For the other three elements, this concentration was not sufficient to affect the NP in 22 days of exposure. Antimony appeared to be the least toxic, as even at 1,000 μg l^{-1} the decrease in the NP was slow and gradual. Of the other elements, As appeared to have a more short-term effect, which was noticeable from the first day for concentrations of 1,000 and 10,000 μg l^{-1}, while on the first day, Se had no or only very slight effects, even at the two highest concentrations. Other authors have also found that the effects on the NP vary over time, for example, Brown and Wells (1990), who exposed the moss *R. squarrosus* to different concentrations of various metals, observed greater inhibition of photosynthesis in measurements made after 24 h than in measurements taken after 30 min.

The more variable response of DR than of NP is apparently usual and has been observed in other studies in which aquatic bryophytes have been exposed to different elements (Sommer and Winkler 1982; Brown and Wells 1990). The effect on DR varied widely, depending on the element considered and the concentration used. The observed increases in DR may be related to the energy costs associated with detoxification mechanisms (Connell et al. 1999). However, when the exposure continued (particularly at high concentrations), the DR decreased, possibly because of impairment of physiological and metabolic functions by the toxicant (Connell et al. 1999).

The F_v/F_m ratio varied between about 0.700 and 0.744 in the controls. A decrease to below 0.8 has been considered symptomatic of stress-dependent photoinhibition (Bjorkman and Demmig 1987; Maxwell and Johnson 2000), although the values obtained were similar to those reported by Rau et al. (2007) for control samples of *F. antipyretica* (0.72–0.75). The F_v/F_m data, especially for Hg and Sb, were less variable than the net photosynthesis data (Figs. 1 and 4). One possible explanation for this is that the fluorescence values are each means of four measures, as explained in the Section "Material and Methods." A great advantage of the fluorescence technique is that it is very easy to perform several measures on one sample, whereas to obtain several measures of photosynthesis by means of the light/dark bottle technique is more time consuming. The median effective water concentrations ($EC50_w$) at 11 days, calculated for F_v/F_m (Fig. 4), were much higher for As and Hg in comparison with net photosynthesis (Fig. 1); at 1 and 22 days, the $EC50_w$ values were also notably higher for F_v/F_m. However, for Sb, they were very similar for both physiological parameters. Antimony was identified as the most toxic element on the basis of photosynthetic efficiency (lower $EC50_w$) but it was the least toxic on the basis of NP (higher $EC50_w$). For both physiological parameters, Hg was more toxic than As. Moreno-Jiménez et al. (2009) also reported that Hg exerted more evident toxic responses than As in two species of shrubs growing in hydroponic culture. The increase in photosynthetic efficiency with increasing exposure time for the highest concentrations of As in water may be due to acclimatization of moss, and the value reached after 22 days of exposure to 1,000 μg l^{-1} was similar to that of the control.

For photosynthetic efficiency, the high value of the Hg $EC50_t$ signifies that *F. antipyretica* can bioconcentrate high levels of Hg before displaying physiological damage, which is a useful trait for a species used as a accumulative indicator. As already indicated for the effect on NP, this may be explained by the high proportion of this element that accumulates at the extracellular sites, where the effects on the cell physiology are limited. For Sb, two set of data can be distinguished; in the samples exposed to the highest concentration in water, the F_v/F_m values were almost 0 % of the controls, and the remaining data with values similar to the controls

(Fig. 5). For the samples in which tissue Sb reached 1,500–3,000 μg g^{-1}, the responses differed greatly depending on whether these concentrations were reached by exposure for several days to 1,000 μg Sb l^{-1} (with F_v/F_m values about 90 % of controls) or if the concentrations were reached more quickly after exposure to 10,000 μg Sb l^{-1} (with F_v/F_m values almost 0 % of controls). This is similar to the results for NP, although in this case, the difference was much more evident, which supports the previously expressed idea of acclimatization to this metalloid. In this case, the $EC50_t$ was not calculated, as fitting a regression line is meaningless for these data.

5 Conclusions

The physiological responses of mosses differ depending on the element, concentration, exposure time and physiological parameter considered. In general, the moss physiology only appears to be affected at the highest concentrations of the elements to which they were exposed. On the basis of the solution EC50 values for photosynthesis, Hg appears to be the most toxic element, followed by As. However, on the basis of the tissue EC50, Se had a larger effect in net photosynthesis, followed by As. These differences can be attributed to the different proportions in which each element is accumulated in the different cellular locations. Dark respiration does not appear to be a useful physiological response to stress because of a lack of any clear trend in relation to the exposure concentrations in solution or tissue concentrations. The results for the order of toxicity of the studied elements, evaluated from the chlorophyll fluorescence data, are consistent with those estimated from photosynthesis, except for Sb. Temporal trends in fluorescence were less variable than the trends in photosynthesis.

Accumulation of As and Se was low, although the small amounts accumulated had important physiological effects. Mercury was accumulated in much higher amounts, with clear effects on the moss physiology; however, despite the high concentrations reached in the tissues, the effects were relatively low, possibly because of the high affinity of Hg for extracellular binding sites.

High tissue concentrations of Sb had different effects when reached by exposure to a concentration of 1,000 μg l^{-1} in solution than when reached by exposure to 10,000 μg l^{-1}, and in the former case, the moss appear to acclimatize to this metalloid.

References

Aronsson, K. A., & Ekelund, N. G. A. (2006). Effects on growth, photosynthesis and pigments of the freshwater moss *Fontinalis antipyretica* Hedw. after exposure to wood ash solution. *Science of the Total Environment, 372*, 236–246.

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L., & Kizek, R. (2008). Uncommon heavy metals, metalloids and their plant toxicity: a review. *Environmental Chemical Letters, 6*, 189–213.

Bjorkman, O., & Demmig, B. (1987). Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta, 170*, 489–504.

Bolhàr-Nordenkampf, H. R., Long, S. P., Baker, N. R., Öquist, G., Schreiber, U., & Lechner, E. G. (1989). Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology, 3*, 497–514.

Branquinho, C., Brown, D. H., & Catarino, F. (1997a). The cellular location of Cu in lichens and its effects on membrane integrity and chlorophyll fluorescence. *Environmental and Experimental Botany, 38*, 165–179.

Branquinho, C., Brown, D. H., Máguas, C., & Catarino, F. (1997b). Lead (Pb) uptake and its effects on membrane integrity and chlorophyll fluorescence in different lichen species. *Environmental and Experimental Botany, 37*, 95–105.

Brown, D. H., & Wells, J. M. (1990). Physiological effects of heavy metals on the moss *Rhytidiadelphus squarrosus*. *Annals of Botany, 66*, 641–647.

Brown, D. H., & Whitehead, A. (1986). The effect of mercury on the physiology of *Rhytidiadelphus squarrosus* (Hedw.) Warnst. *Journal of Bryology, 14*, 367–374.

Bruns, I., Friese, K., Markert, B., & Krauss, G.-J. (1997). The use of *Fontinalis antipyretica* L. ex Hedw. as a bioindicator for heavy metals. 2. Heavy metal accumulation and physiological reaction of *Fontinalis antipyretica* L. ex Hedw. in active biomonitoring in the River Elbe. *Science of the Total Environment, 204*, 161–176.

Butler, W., & Kitajima, M. (1975). Fluorescence quenching in photosystem II of chloroplasts. *Biochimical & Biophysical Acta, 376*, 116–125.

Carballeira, A., Díaz, S., Vázquez, M. D., & López, J. (1998). Inertia and resilience in the responses of the aquatic bryophyte *Fontinalis antipyretica* Hedw. to thermal stress. *Archives of Environmental Contamination and Toxicology, 34*, 343–349.

Carginale, V., Sorbo, S., Capasso, C., Trinchella, F., Cafiero, G., & Basile, A. (2004). Accumulation, localisation, and toxic effects of cadmium in the liverwort *Lunularia cruciata*. *Protoplasma, 223*, 53–61.

Carter, L. F., & Porter, S. D. (1997). Trace-element accumulation by *Hygrohypnum ochraceum* in the upper Rio Grande

basin, Colorado and New Mexico, USA. *Environmental Toxicology and Chemistry, 16*(12), 2521–2528.

Cenci, R. M. (2000). The use of aquatic moss (*Fontinalis antipyretica*) as monitor of contamination in standing and running waters: limits and advantages. *Journal of Limnology, 60*(suppl. 1), 53–61.

Cesa, M., Bizzotto, A., Ferraro, C., Fumagalli, F., & Nimis, P. L. (2009). S.TR.E.A.M., system for trace element assessment with mosses. An equation to estimate mercury concentration in freshwaters. *Chemosphere, 75*, 858–865.

Cesa, M., Bizzoto, A., Ferraro, C., Fumagalli, F., & Nimis, P. L. (2010). Palladio, an index of trace element alteration for the River Bacchiglione based on *Rhynchostegium riparioides*. *Water, Air, and Soil Pollution, 208*, 59–77.

Connell, D. W., Lam, P., Richardson, B., & Wu, R. (1999). *Introduction to ecotoxicology*. Oxford: Blackwell Science.

Cruz de Carvalho, R., Branquinho, C., & Marques da Silva, J. (2011). Physiological consequences of desiccation in the aquatic bryophyte *Fontinalis antipyretica*. *Planta, 234*, 195–205.

Csintalan, Z., Proctor, M. C. F., & Tuba, Z. (1999). Chlorophyll fluorescence during drying and rehydration in the mosses *Rhytidiadelphus loreus* (Hedw.) Warnst., *Anomodon viticulosus* (Hedw.) Hook. & Tayl. and *Grimmia pulvinata* (Hedw.) Sm. *Annals of Botanny, 84*, 235–244.

Culioli, J.-L., Fouquoire, A., Calendini, S., Mori, C., & Orsini, A. (2009). Trophic transfer of arsenic and antimony in a freshwater ecosystem: a field study. *Aquatic Toxicolgy, 94*, 286–293.

Díaz, S., Villares, R., & Carballeira, A. (2012). Uptake Kinetics of As, Hg, Sb, and Se in the aquatic moss *Fontinalis antipyretica* Hedw. *Water, Air, and Soil Pollution, 223*, 3409–3423.

Drasch, G., Horvat, M., & Stoeppler, M. (2004). Mercury. In E. Merian, M. Anke, M. Ihnat, & M. Stoeppler (Eds.), *Elements and their compounds in the environment* (2nd ed., pp. 931–1005). Weinheim: Wiley-VCH.

Empain, A. (1973). Les bryophytes aquatiques utilisés comme traceurs de la contamination en métaux lourds des eaux douces. *Memoires de la Societe Royale Botanique de Belgique, 7*, 141–156.

Esteban, E., Moreno, E., Peñalosa, J., Cabrero, J., Millán, R., & Zornoza, P. (2008). Short and long-term uptake of Hg in white lupin plants: kinetics and stress indicators. *Environmental and Experimental Botany, 62*, 316–322.

Fernández, J. A., Vázquez, M. D., López, J., & Carballeira, A. (2006). Modelling the extra and intracellular uptake and discharge of heavy metals in *Fontinalis antipyretica* transplanted along a heavy metal and pH contamination gradient. *Environmental Pollution, 139*, 21–31.

Fracheboud, Y., & Leipner, J. (2003). The application of chlorophyll fluorescence to study light, temperature, and drought stress. In J. R. DeEll & P. M. A. Toivonen (Eds.), *Practical Applications of Chlorophyll Fluorescence in Plant Biology* (pp. 125–150). Dordrecht: Kluwer Academic Publishers.

Fu, Z., Wu, F., Amarasiriwardena, D., Mo, C., Liu, B., Zhu, J., et al. (2010). Antimony, arsenic and mercury in the aquatic environment and fish in a large antimony mining area in Hunan, China. *Science of the Total Environment, 408*, 3403–3410.

Gapeeva, M. V., Dolotov, A. V., & Chemeris, E. V. (2010). Prospects of using mosses (*Fontinalis antipyretica* Hedw. and *Pylaisia polyantha* (Hedw.) Bruch et al.) as indicators of environmental contamination with heavy metals. *Russian Journal of Ecology, 41*(1), 28–31.

Israr, M., Sahi, S., Datta, R., & Sarkar, D. (2006). Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. *Chemosphere, 65*, 591–598.

Kanamori, S., & Sugawara, K. (1965). Geochemical study of arsenic in natural waters. I. Arsenic in rain and snow. *Journal of Earth Science, Nagoya University, 13*, 23–35.

Koch, I., Feldmann, J., Wang, L., Andrewes, P., Reimer, K. J., & Cullen, W. R. (1999). Arsenic in the Meager Creek hot springs environment, British Columbia, Canada. *Science of the Total Environment, 236*, 101–117.

Krause, G. H., & Weis, E. (1991). Chlorophyll fluorescence and photosynthesis—the basics. *Annual Review of Plant Physiology and Plant Molecular Biology, 42*, 313–349.

Lichtenthaler, H. K., & Miehé, J. A. (1997). Fluorescence imaging as a diagnostic tool for plant stress. *Trends in Plant Science, 2*, 316–320.

López, J., & Carballeira, A. (1993). Interspecific differences in metal bioaccumulation and plant–water concentration ratios in five aquatic bryophytes. *Hydrobiologia, 263*, 95–107.

Martínez-Abaigar, J., Núñez-Olivera, E., & Sánchez-Díaz, M. (1993). Effects of organic pollution on transplanted aquatic bryophytes. *Journal of Bryology, 17*, 553–566.

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence: a practical guide. *Journal of Experimental Botany, 51*, 659–668.

Miyashita, S., Shimoya, M., Kamidate, Y., Kuroiwa, T., Shikino, O., Fujiwara, S., et al. (2009). Rapid determination of arsenic species in freshwater organisms from the arsenic-rich Hayakawa River in Japan using HPLC-ICP-MS. *Chemosphere, 75*, 1065–1073.

Moreno-Jiménez, E., Esteban, E., Carpena-Ruiz, R. O., & Peñalosa, J. M. (2009). Arsenic and mercury induced phytotoxicity in the Mediterranean shrubs *Pistacia lentiscus* and *Tamarix gallic*a grown in hydroponic culture. *Ecotoxicology and Environmental Safety, 72*, 1781–1789.

Nieboer, E., & Richardson, H. S. (1980). The replacement of the nondescript term "heavy metals" by a biologically and chemically significant classification of metal ions. *Environmental Pollution, 1*, 3–26.

Nimis, P. L., Fumagalli, F., Bizzotto, A., Codogno, M., & Skert, N. (2002). Bryophytes as indicators of trace metals pollution in the River Brenta (NE Italy). *Science of the Total Environment, 286*, 233–242.

Pekka, L., Halmeenpaa, H., Ecke, F., Vuori, K.-M., Mokrotovarova, O., Ohlander, B., et al. (2008). Assessing pollution in the Kola River, northwestern Russia, using metal concentrations in water and bryophytes. *Boreal Environmental Research, 13*, 15–30.

Pérez-Llamazares, A., Fernández, J. A., Aboal, J. R., & Carballeira, A. (2009). A search for an extracellular extractant of Hg for use in the sequential elution technique with *Pseudoscleropodium purum*. *Journal of Bryology, 31*, 23–29.

Pérez-Llamazares, A., Aboal, J. R., Fernández, J. A., & Carballeira, A. (2011a). Sequential elution technique in moss *Pseudoscleropodium purum*: comparison between the commonly used extracellular extractant $NiCl_2$ and other new extractants. *Water, Air, and Soil Pollution, 215*, 561–572.

Pérez-Llamazares, A., Fernández, J. A., Carballeira, A., & Aboal, J. R. (2011b). The sequential elution technique applied to cryptogams: a literature review. *Journal of Bryology, 33*, 267–278.

Rau, S., Miersch, J., Neumann, D., Weber, E., & Krauss, G.-J. (2007). Biochemical responses of the aquatic moss *Fontinalis antipyretica* to Cd, Cu Pb and Zn determined by chlorophyll fluorescence and protein levels. *Environmental and Experimental Botany, 59*, 299–306.

Rish, M. A. (2004). Antimony. In E. Merian, M. Anke, M. Ihnat, & M. Stoeppler (Eds.), *Elements and their compounds in the environment* (2nd ed., pp. 659–670). Weinheim: Wiley-VCH.

Roeck, U., Glasser, N., & Trémolières, M. (1995). Seasonal variations in mercury accumulation by the aquatic moss *Fontinalis antipyretica* Hedw. *Acta Botanica Gallica, 142*(6), 741–749.

Samecka-Cymerman, A., & Kempers, A. J. (1995). Preliminary investigations into the bioaccumulation of mercury by the liverwort *Scapania undulata* L. (Dum). *Ecotoxicology and Environmental Safety, 31*, 57–61.

Schrauzer, G. N. (2004). Selenium. In E. Merian, M. Anke, M. Ihnat, & M. Stoeppler (Eds.), *Elements and their compounds in the environment* (2nd ed., pp. 1365–1406). Weinheim: Wiley-VCH.

Schreiber, U., Bilger, W., & Neubauer, C. (1995). Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis. In E.-D. Schulze & M. M. Caldwell (Eds.), *Ecophysiology of Photosynthesis* (pp. 49–70). Berlin: Springer.

SEPA (Swedish Environmental Protection Agency). (2000). Environmental quality criteria. Lakes and watercourses. Report 5050. Swedish Environmental Protection Agency.

Sidhu, M., & Brown, D. H. (1996). A new laboratory technique for studying the effects of heavy metals on bryophyte growth. *Annals of Botany, 78*, 711–717.

Sommer, V. C., & Winkler, S. (1982). Reaktionen im gaswechsel von *Fontinalis antipyretica* Hedw. nach experimentellen belastungen mit schwermetallverbindungen. *Archiv für Hydrobiologie, 4*, 503–524.

Stoeppler, M. (2004). Arsenic. In E. Merian, M. Anke, M. Ihnat, & M. Stoeppler (Eds.), *Elements and their compounds in the environment* (2nd ed., pp. 1321–1364). Weinheim: Wiley-VCH.

Vázquez, M. D., López, J., & Carballeira, A. (1999). Uptake of heavy metals to the extracellular and intracellular compartments in three species of aquatic bryophyte. *Ecotoxicology and Environmental Safety, 44*, 12–24.

Vázquez, M. D., Villares, R., & Carballeira, A. (2013). Biomonitoring urban fluvial contamination on the basis of physiological stress induced in transplants of the aquatic moss *Fontinalis antipyretica* Hedw. *Hydrobiologia, 707*, 97–108.

Vieira, A. R., Gonzalez, C., Martins-Louçao, M. A., & Branquinho, C. (2009). Intracellular and extracellular ammonium (NH_4^+) uptake and its toxic effects on the aquatic biomonitor *Fontinalis antipyretica*. *Ecotoxicology, 18*, 1087–1094.

Wehr, J. D., & Whitton, B. A. (1983). Accumulation of heavy metals by aquatic mosses. 2. *Rhynchostegium riparioides*. *Hydrobiologia, 100*, 261–284.

Wells, J. M., & Brown, D. H. (1990). Ionic control of intracellular and extracellular Cd uptake by the moss *Rhytidiadelphus squarrosus* (Hedw.) Warnst. *New Phytologist, 116*, 541–553.