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ESTUDIO DE ALGUNOS ASPECTOS RELACIONADOS CON LA BRIO-MONITORIZACIÓN ACUÁTICA

Memoria presentada por
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para optar al Grado de Doctor en Biología

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HACEN CONSTAR

Que la presente memoria titulada *Estudio de algunos Aspectos Relacionados con la Brio-Monitorización Acuática* presentada por D. Santiago Díaz Barbeito para optar al Grado de Doctor en Biología, fue realizada en este departamento bajo su dirección.

Y considerando que representa trabajo de Tesis Doctoral, autorizan su presentación ante el tribunal correspondiente.

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Índice

Resumen

1. Introducción

La evaluación de la calidad de las aguas continentales en función de los clásicos análisis físico-químicos de agua presenta una serie de desventajas. Muchos contaminantes están presentes en concentraciones muy bajas (lo cual no implica que sean poco relevantes desde un punto de vista ecotoxicológico), esto entraña una serie de problemas analíticos, como son las mayores posibilidades de contaminar (positiva o negativamente) las muestras durante su recogida y posterior análisis o la necesidad de disponer de aparatos de análisis más costosos.

Los contaminantes pueden presentarse de distintas formas químicas, cada cual podrá tener su nivel de disponibilidad para los seres vivos. Es decir, un simple análisis de agua no nos va a reflejar la biodisponibilidad de los contaminantes detectados.

Los contaminantes suelen mostrar una alta variabilidad temporal en agua. Dependiendo de la época del año, la hora del día (debido a horarios de industrias, tareas domésticas), etc., las concentraciones de contaminantes en agua pueden ser muy diferentes. Además, vertidos esporádicos serán difíciles de detectar si no coinciden con el momento de recogida de las muestras. Por todo ello, para lograr tener una idea representativa de la situación real en función de una analítica de aguas es necesario realizar un esfuerzo de muestreo muy elevado.

Mediante el empleo de organismos indicadores se pueden solventar en teoría estos problemas, ya que éstos sólo van a incorporar contaminantes que por definición sean biodisponibles; su análisis corporal suministra una medida integrada representativa de las concentraciones fluctuantes en agua; y las concentraciones tisulares suelen ser mucho más altas (bioconcentración) que las del medio donde habitan. También su respuesta biológica será una medida integrada del efecto combinado de todos los compuestos a los que están expuestos simultáneamente bajo la acción de los factores ambientales.

Los briófitos acuáticos son organismos frecuentemente utilizados en estudios de biomonitorización fluvial debido a una serie de importantes características anatómicas y fisiológicas. Las hojas de muchos musgos así como las de las hepáticas foliosas presentan una única capa de células, esto hace que la superficie específica (superficie/masa o volumen) sea elevada, lo que facilita la bioconcentración de contaminantes. Dada la falta de raíces funcionales y sistema vascular (salvo algunas

excepciones), no obtienen nutrientes del sustrato, por lo que dependen única y exclusivamente del agua en la que viven sumergidos para su nutrición. Tienen un metabolismo activo durante todo el año, lo que es una ventaja frente a plantas superiores acuáticas que suelen permanecer en estado latente en invierno o algas que suelen tener ciclos de vida cortos. Existe un limitado número de especies en el hemisferio norte (lo que contrasta en ocasiones con su elevada biomasa) y suelen ser fáciles de identificar. Debido a la resistencia a ciertos contaminantes (las plantas vasculares suelen ser más sensibles a la contaminación) y a su enorme capacidad de concentración, ciertas especies de briófitos son ideales para su utilización como *indicadores acumuladores.* Sin embargo, debido a la gran sensibilidad que presentan ciertas especies a los tóxicos, también pueden ser utilizados como *indicadores sensitivos* (Zechmeister et al., 2003).

Esta Tesis se ha realizado dentro del Grupo de investigación en Ecotoxicología-USC y pretende dar respuesta a una serie de cuestiones pendientes dentro de la línea de investigación en brio-monitorización acuática, así como de abrir nuevas perspectivas. Estaba pendiente de abordar el estudio de los niveles de fondo, de las cinéticas y de las respuestas biológicas a Hg, As, Se y Sb; todos ellos considerados elementos ecotóxicos relevantes. El estudio del estrés térmico en briofitos es un aspecto totalmente novedoso en la literatura. De ahí surge el ambiguo título (*Estudio de algunos aspectos relacionados con la brio-monitorización acuática)* que se le ha dado a esta tesis, la cual está estructurada en 7 capítulos. De ellos, cinco capítulos corresponden a artículos publicados o pendientes de publicación en revistas pertenecientes al *Journal Citation Reports*. Otro capítulo ha sido presentado como comunicación a un congreso nacional y el restante es un trabajo en preparación.

En el primer capítulo se estudia, mediante experimentos de laboratorio, los efectos que tienen elevadas temperaturas sobre la fotosíntesis y composición de pigmentos fotosintéticos en el musgo *Fontinalis antipyretica*, uno de los briófitos más frecuentes en ríos gallegos y también una de las especies más utilizadas en bioindicación fluvial. Se ha demostrado que los briófitos acuáticos son muy sensibles a un incremento en la temperatura del agua. La actividad del enzima ribulosa bifosfato carboxilasa-oxigenasa es más reducida en plantas acuáticas que en terrestres, y dentro de las acuáticas, es más reducida en briófitos que en macrófitas vasculares (Proctor, 1981). Como resultado de esta baja actividad, la respiración rápidamente supera a la fotosíntesis al incrementarse la temperatura. Otras explicaciones de la sensibilidad de los briófitos acuáticos a altas temperaturas son las reducciones en actividades enzimáticas y el excesivo crecimiento

de bacterias y algas epífitas. Con este estudio se intentó evaluar la utilidad de *F. antipyretica* como indicador a largo plazo de contaminación térmica de baja magnitud, como frecuentemente sucede en ríos debido a vertidos de efluentes y descarga de aguas de refrigeración.

La acidificación es un problema frecuente en aguas continentales. Entre las causas principales están la deposición atmosférica, los lixiviados de escombreras de minas y los vertidos industriales. La toxicidad en los medios afectados por la acidificación está provocada por un lado por las elevadas concentraciones de protones, lo que origina el lixiviado de cationes de las células y cambios en el balance de iones, y por otro lado por el incremento de ciertos metales tóxicos como el aluminio. En el segundo capítulo se muestra un estudio en el que mediante experimentos de laboratorio se simularon reducciones de pH con diferentes concentraciones de aluminio. El objetivo fue estudiar las cinéticas de acumulación de aluminio en el musgo *Fontinalis antipyretica* incubado a diferentes concentraciones en agua y diferentes grados de acidez, así como observar el efecto del pH en las células del musgo en cuanto a la movilización de cationes esenciales (Ca, Mg y K).

Para una mejor interpretación de los estudios de biomonitorización se han desarrollado un buen número de trabajos para conocer la cinética de acumulación de numerosos metales en briófitos acuáticos. En el tercer capítulo se estudian las cinéticas de carga de un metal (mercurio) y tres metaloides (arsénico, selenio y antimonio) en el musgo acuático *Fontinalis antipyretica*. Estos elementos, exceptuando el antimonio, han recibido tradicionalmente gran atención debido a sus implicaciones ambientales. Aunque recientemente el antimonio se está mostrando como un elemento a tener en cuenta y el número de trabajos publicados sobre él han aumentado rápidamente (Filella et al., 2009). A pesar de la relevancia ambiental de estos elementos, si exceptuamos unas pocas excepciones con el mercurio, no existen estudios sobre las cinéticas de carga de ellos en briófitos acuáticos.

La selección de la especie/s a utilizar es un factor clave en cualquier estudio de biomonitorización. En caso de utilizar una única especie, con biomonitorización pasiva, el número de lugares de muestreo va a quedar restringido a aquellos donde dicha especie está presente. Este problema puede ser resuelto empleando más de una especie, aunque en ese caso se hace necesaria una intercalibración de las respuestas de las diferentes especies a los contaminantes a estudiar. Aunque los estudios de intercablibración son relativamente frecuentes en la biomonitorización de la

contaminación atmosférica con musgos terrestres (ej. Fernández et al., 2000; Carballeira et al., 2008), no sucede así con briófitos acuáticos. En el cuarto capítulo se investigan las diferentes capacidades de acumular arsénico y mercurio de cuatro especies de musgos y una hepática frecuentes en ríos gallegos creciendo en sus hábitats naturales. Para ello se trabajó con datos de campo de un muestreo extensivo, aprovechando el hecho de que en muchos puntos coexistía más de una especie.

Existen contaminantes, como pueden ser los metales, que están presentes de forma natural en el ambiente. En su estudio es interesante establecer su nivel corporal natural, conocido como nivel de fondo (*background*) y definido como la concentración natural de un elemento representativa de una zona de estudio, que a pesar de estar influenciada por la actividad humana, se mantiene bien conservada. En el quinto capítulo se presentan los niveles de fondo de mercurio y arsénico en diferentes especies de briófitos acuáticos que habitan en los ríos de Galicia estimados mediante diferentes aproximaciones estadísticas.

El sexto capítulo muestra los resultados de un estudio de *biomonitorización activa* de arsénico, mercurio y antimonio realizados con transplantes de *Fontinalis antipyretica* en el río Eume. La biomonitorización activa presenta ciertas ventajas frente al empleo de organismos nativos. Se pueden exponer organismos en lugares en los que no existen de forma natural. Se conoce el estado (concentración corporal o estado fisiológico) inicial de los organismos trasplantados y se puede controlar el tiempo de exposición. Dada la falta de adaptación a ambientes contaminados, los organismos trasplantados suelen presentar, en exposiciones cortas de duración, una mayor capacidad de bioacumulación de contaminantes y manifestar efectos fisiológicos negativos más intensos (Carballeira et al., 2003), ambos aspectos mejoran la sensibilidad de la biomonitorización.

En el último capítulo se estudian los efectos fisiológicos de arsénico, mercurio, antimonio y selenio sobre *Fontinalis antipyretica*. Con ese objetivo se realizan incubaciones en condiciones de laboratorio con el musgo a diferentes concentraciones de los elementos a estudiar y durante diferentes tiempos de exposición. Para cada tratamiento se evalúa posteriormente la fotosíntesis neta y la respiración mediante la técnica de la botella clara/oscura, también se mide la respuesta de la fluorescencia clorofílica, parámetro muy poco estudiado en *F. antipyretica*.

2. Métodos

Capítulo 1. Inercia y resilencia en las respuestas del briófito acuático *Fontinalis antipyretica* Hedw. a estrés térmico.

El material para los experimentos se obtuvo de tramos situados en tres ríos limpios, uno de ellos localizado aguas abajo de una fuente termal, con una temperatura ligeramente mayor a la normal en los ríos de la zona. Sin embargo, el musgo de este lugar no mostró respuestas fisiológicas significativamente diferentes, por lo que el material de los tres sitios fue tratado conjuntamente.

En el laboratorio, los ápices del musgo se incubaron con aireación para mantener el agua en condiciones de saturación de oxígeno, una irradiancia de 80 µmoles de fotones m^{-2} s^{-1}, suficiente para mantener una fotosíntesis próxima al máximo, y con un fotoperiodo 14:10 de luz:oscuridad. El musgo se incubó durante 25 días a varias temperaturas (de 16 a 34ºC). Cada 5 días se analizó el cociente de feofitinización, la fotosíntesis y la respiración.

Para evaluar la capacidad de rehabilitación (resiliencia) de *F. antipyretica* tras sufrir estrés térmico, primero se expusieron de 2 a 10 días las muestras de musgo a 30ºC y luego se transfirieron a cubetas con agua a 16ºC, donde estuvieron 40 días. Durante el período de rehabilitación a intervalos de tiempo regulares se determinaron las mismas variables fisiológicas que en el experimento anterior (conciente de feofitinización, fotosíntesis y respiración).

El cociente de feofitinización (D665/D665a) ha demostrado ser una medida fiable del estrés en briófitos acuáticos (López y Carballeira 1990; López et al. 1994). Para su cálculo se realiza una extracción de los pigmentos fotosintéticos de las muestras con acetona al 90%, siendo posteriormente determinada espectrofotométricamente su absorción a 665 nm antes y después de acidificar la muestra con 30 µl de HCl 1N por 3 ml de extracto. Viene a representar la relación entre clorofila y feofitina, y disminuye según aumenta el estrés en el musgo. Las tasas fotosintética y respiratoria se determinaron mediante el clásico método de la botella clara/oscura. Entre 0.7 y 0.9 g de peso fresco de musgo se colocaban en cada botella Karlsruhe de 0.31 l de capacidad. Las botellas se incubaron durante 4 horas a 16ºC. La concentración de oxígeno se midió antes y después de la incubación con un oxímetro polarográfico con agitador

incorporado. Después del ensayo, se calculaba el peso seco de las muestras para expresar los resultados por unidad de peso seco y tiempo.

Capítulo 2. Efecto del pH en las cinéticas de carga intra- y extracelular de Al en *Fontinalis antipyretica*. Cambios en los contenidos celulares de K, Mg y Ca.

El estudio se llevó a cabo en laboratorio con musgo procedente de un tramo de río limpio. De este musgo se separaron 500 ápices (2cm) que se pretrataron con $CdCl_2$ para evitar la interferencia de cationes previamente adheridos al briófito, con el objeto de estandarizar las condiciones iniciales en todas las muestras. Los ápices fueron incubados bajo concentraciones de 0, 0.1, 0.5, 1, 2 y 3 mg l^{-1} a pH de 3, 3.5, 4, 4.5, 5, 5.5, 5.8; durante 1, 6 o 24 horas. Las concentraciones de Al y los pH utilizados fueron similares a los encontrados en algunos ríos acidificados, debido fundamentalmente en Galicia a actividades mineras. Después del periodo de incubación, los metales bioconcentrados en cada compartimento celular fueron determinados mediante una técnica de elución secuencial. El metal *intercelular* fue eliminado inicialmente por lavado con agua destilada, de esta manera la primera fracción analizada fue la *extracelular* unida a los lugares de intercambio catiónico. Para ello se utilizó un catión con una mayor afinidad por los lugares de intercambio y/o que está a una mayor concentración que el que se pretende extraer; en este caso se utilizó $Pb(NO_3)_2$. El metal *intracelular* finalmente fue extraído secando el musgo a 60ºC e incubándolo posteriormente con HNO_3 1M.

Las cinéticas de carga de Al fueron parametrizadas mediante diferentes ecuaciones. La carga extracelular fue modelizada con una ecuación tipo Michaelis-Menten. Esta ecuación permitió el cálculo de la concentración máxima en el equilibrio y el tiempo requerido para alcanzarla. Para la cinética intracelular de Al se utilizaron ecuaciones lineales y logarítmicas, que dieron mejor resultado.

Capítulo 3. Cinéticas de carga de As, Hg, Sb y Se en el musgo acuático *Fontinalis antipyretica* Hedw.

Se realizaron experimentos en laboratorio con ápices de musgo colectado en un tramo de río limpio. Se incubaron en vasos de precipitados con aireación permanente y diferentes diluciones de los cuatro elementos estudiados: 0 (control), 0.1, 1, 10, 100, 1.000, 10.000 µg l^{-1}; cambiándose el agua cada 5 días. El musgo se mantuvo a 16ºC y con una irradiancia de 80 µmoles de fotones m^{-2} s^{-1} y un fotoperiodo de 12:12. Los tiempos de exposición fueron 1, 2, 4, 7, 11, 16 y 22 días. Al final de cada período de

exposición las muestras se atacaban con ácido nítrico en bombas de teflón en microondas. Las concentraciones finales alcanzadas fueron medidas mediante espectroscopía de fluorescencia atómica. Para facilitar las comparaciones entre los diferentes elementos y corregir la posible contaminación de los bajos niveles de estos elementos que podrían encontrarse en el agua de los experimentos, las bioconcentraciones fueron transformadas en Factores de Contaminación. Para ello se dividió la concentración tisular alcanzada de cada elemento en cada tratamiento por la concentración tisular alcanzada en el control para el mismo tiempo de incubación. Las cinéticas fueron ajustadas con diferentes ecuaciones, inicialmente debido a los resultados obtenidos en nuestro y en otros laboratorios, se intentó un ajuste a Michaelis-Menten. En los casos en los que los ajustes a este modelo no resultaron ser altamente significativos, se probaron ajustes a un modelo lineal y a diferentes modelos de regresión curvilínea. También se calculó el tiempo en alcanzar el equilibrio, el factor de contaminación en el equilibrio y la velocidad media de carga.

Capítulo 4. Arsénico y mercurio en briófitos acuáticos nativos: diferencias entre especies.

Se muestrearon 5 especies de briófitos acuáticos en ríos de toda Galicia: los musgos *Fontinalis antipyretica*, *Platyhypnidium riparioides*, *Brachythecium rivulare* y *Fissidens polyphyllus*, y la hepática *Scapania undulata*. En total se recolectaron 424 muestras de 218 estaciones de muestreo. Para homogeneizar las muestras se utilizaron únicamente los 2 cm apicales para evitar los posibles errores derivados de las diferentes capacidades de acumulación de las distintas partes de las plantas. Para su análisis los ápices se digirieron previamente con ácido nítrico en bombas de teflón en microondas para aumentar la agresividad del ataque. Los extractos fueron posteriormente analizados mediante espectroscopía de fluorescencia atómica. La calidad analítica fue evaluada mediante el análisis en paralelo del material de referencia certificado BCR-61 (*Platyhypnidium riparioides*).

Las diferencias en las capacidades de acumulación de las distintas especies que crecían en los mismos lugares fueron estudiadas mediante análisis de regresión. La hipótesis testada fue que diferentes pares de especies tenían la misma capacidad de bioconcentración, es decir, que el valor de la pendiente de la recta de regresión era 1. Las regresiones se calcularon únicamente cuando las especies coexistían en al menos 10

estaciones de muestreo. Dado que las regresiones así establecidas no tienen una variable independiente, los modelos de regresión fueron de tipo II, llevándose a cabo el ajuste mediante el método de regresión de eje mayor.

Capítulo 5. Niveles de fondo de mercurio y arsénico en briófitos de los ríos gallegos.

Las muestras de este estudio fueron las mismas que las empleadas en el capítulo 4. Es decir, se recogieron en ríos de toda Galicia un total de 424 muestras pertenecientes a 5 especies de briófitos acuáticos. Los análisis se realizaron mediante espectroscopía de fluorescencia atómica en extractos previamente obtenidos mediante digestión ácida en horno microondas.

Se determinaron los niveles de fondo mediante diferentes aproximaciones estadísticas. Un método fue utilizar el valor de la mediana más 2 desviaciones absolutas medianas (Reimann et al., 2005). Siendo la desviación absoluta mediana, la mediana de las desviaciones absolutas de los datos respecto a la mediana muestral. Otro método fue eliminar las concentraciones más elevadas hasta alcanzar un coeficiente de variación en el conjunto de muestras restantes próximo al 60% (Bonnefoy y Bourg, 1984). El nivel de fondo finalmente es estimado como el límite superior del intervalo de confianza al 95% de la media de la concentración del elemento en esas muestras. Otro sistema consistió en eliminar gradualmente las concentraciones más elevadas hasta que las muestras restantes alcancen la normalidad, correspondiendo el nivel de fondo a la mediana de los datos finales (Cesa et al., 2010). Reimann et al. (2005) proponen utilizar como nivel de fondo la valla interna superior de los diagramas de cajas en los que se representen los conjuntos de datos estudiados. Un método clásico en estudios geoquímicos es usar gráficas de frecuencias acumuladas (Sinclair, 1974), correspondiendo el primer tramo recto de estas representaciones a estaciones limpias. Mediante el último sistema utilizado se definió el nivel de fondo como la concentración correspondiente a la primera moda de los datos sometidos a un suavizado por núcleo (*kernel smoothing*).

Capítulo 6. Biomonitorización activa de arsénico, mercurio y antimonio en el curso bajo del río Eume mediante trasplantes del musgo acuático *Fontinalis antipyretica* Hedw.

Se trasplantaron en bolsas de malla muestras de *Fontinalis antipyretica* procedentes de un sitio limpio a 4 estaciones situadas en la cuenca del Eume, río abajo de los principales vertidos a este río, procedentes de la mina y escombrera de la central térmica instalada en As Pontes, así como de la propia población de esta localidad. Los tiempos de exposición fueron 4, 11, 19 y 28 días. El último día se recogió musgo autóctono de la misma especie a la utilizada en los trasplantes en 2 estaciones. Los análisis se realizaron mediante espectroscopía de fluorescencia atómica en extractos previamente obtenidos mediante digestión ácida en horno microondas.

Capítulo 7. Efectos fisiológicos de la exposición a arsénico, mercurio, antimonio y selenio en el musgo acuático *Fontinalis antipyretica* Hedw.

Las muestras de musgo procedían de un tramo de río limpio, del cual se utilizaron los 2 cm apicales. Se incubaron en vasos de precipitados con aireación permanente y diferentes diluciones de los cuatro elementos estudiados: 0 (control), 0.1, 1, 10, 100, 1.000, 10.000 μg l^{-1}; el agua se cambiaba cada 5 días. El musgo se mantuvo a 16ºC y con una irradiancia de 80 μmoles de fotones m^{-2} s^{-1} y un fotoperiodo de 12:12. Después de 1, 2, 4, 7, 11, 16 y 22 días se extraía una muestra para medir variables fisiológicas y concentraciones tisulares. Después de un ataque de las muestras con ácido nítrico a alta presión y temperatura, las concentraciones de As, Hg, Sb y Se se estimaron mediante fluorescencia atómica.

La fotosíntesis neta y respiración fueron determinadas por el método de la botella clara/oscura mediante incubación durante 4 horas a 16 ºC y 80 μmoles de fotones m^{-2} s^{-1} de irradiancia, midiendo la concentración de oxígeno antes y después del periodo de incubación.

La eficiencia fotosintética fue evaluada por fluorescencia de la clorofila *a* mediante un fluorómetro portátil. A las muestras, previamente adaptadas a la oscuridad para abrir todos los centros de reacción del fotosistema II, se les aplicaba un pulso de luz saturante. Se determinaban, para cada tratamiento, 4 réplicas de la relación $Fv/Fm = (Fm - F_0)/Fm$, siendo F_0 y Fm las producciones mínimas y máximas de fluorescencia respectivamente, de una muestra adaptada a oscuridad con todos los centros del

fotosistema II abiertos. La relación Fv/Fm representa la fracción de energía luminosa incidente que es procesada fotoquímicamente. Se considera un parámetro muy valioso para evaluar estrés en vegetales (Lichtenthaler and Miehé, 1997; Csintalan et al., 1999).

3. Objetivos y resultados principales

Capítulo 1. Inercia y resilencia en las respuestas del briófito acuático *Fontinalis antipyretica* Hedw. a estrés térmico.

El objetivo de este trabajo fue incrementar el conocimiento de la autoecología del musgo acuático *Fontinalis antipyretica*, y más específicamente, evaluar su utilidad como indicador de contaminación térmica de baja magnitud a largo plazo, fenómeno frecuente en ríos debido a actividades como el vertido de efluentes urbanos e industriales, o como aguas de refrigeración. Para ello se estudiaron en laboratorio las respuestas fisiológicas del musgo a altas temperaturas, desde 16ºC (control) hasta 34ºC. También se analizó su capacidad de recuperarse del estrés térmico.

La temperatura más alta empleada en los experimentos (33.5 ºC) tuvo un efecto claramente estresante, el cociente de feofitinización cayó drásticamente según aumentaba el tiempo de exposición. A 28.4 ºC se apreció una caída moderada de este índice, pero sólo a partir del día 15. A 24.9 ºC el efecto fue muy moderado, mientras que a 19.8ºC no hubo un efecto apreciable. A las dos temperaturas más moderadas, la fotosíntesis neta se incrementa inicialmente, para caer posteriormente, aunque hacia el final del estudio parecen aclimatarse a las nuevas temperaturas. Esta aclimatación probablemente se consigue mediante un cambio en el punto de compensación, ligado a una reducción de la tasa de respiración por la nueva temperatura. Aunque es necesario señalar que estos cambios observados en laboratorio pueden no tener lugar bajo las intensidades luminosas subóptimas que se suelen encontrar en el hábitat natural de estas plantas. También hay que tener en cuenta que los experimentos se realizaron con ápices, que tienen un metabolismo más activo que otras partes de la planta. Por tanto sería de esperar que la capacidad de *F. antipyretica* de adaptarse a altas temperaturas fuera menor en ambientes naturales que en condiciones ideales de laboratorio. Para cada temperatura y para cada variable fisiológica se calculó el tiempo subletal mediano

(ET50), es decir, el tiempo de exposición necesario para originar una caída del 50% en la variable. También se calculó para cada variable fisiológica la temperatura subletal mediana a 96 horas ($EC50_{96h}$). Este parámetro permite comparar los efectos de la temperatura; se observó que a pesar de las diferencias en los patrones de respuesta de las variables fisiológicas, los valores de $EC50_{96h}$ fueron en todos los casos muy parecidos (32-37ºC).

Los experimentos de recuperación mostraron que, atendiendo al cociente de feofitinización y a las tasas fotosintéticas y respiratorias, las plantas incubadas a 30ºC durante 10 días no se llegaron a rehabilitar. Las incubadas durante 4 y 2 días se recuperaban, en función del coeficiente de feofitinización, en unos 15 días después de ser transferidas a agua a 16ºC. En cambio atendiendo a las tasas fotosintéticas y respiratorias, no se recuperaron hasta el final del experimento (40 días). También se calculó la *amplitud* para cada variable fisiológica, definida como el máximo estrés (es decir, el mínimo valor D665/D665a) que permite una recuperación del 50% del valor normal de la variable en 10 días ($EC50_{10d}$). Los valores fueron muy parecidos para todas las variables fisiológicas (1.52-1.56).

En este estudio se aplicaron por primera vez técnicas ecotoxicológicas para el cálculo de diferentes parámetros (ET50, $EC50_{96h}$, $EC50_{10d}$) que permiten la caracterización simple y precisa de las respuestas de *F. antipyretica* a la temperatura en términos de resilencia (velocidad de recuperación y amplitud) e inercia. Consideramos que el empleo de estas técnicas son útiles para suministrar medidas normalizadas de las respuestas al estrés térmico, facilitando así las comparaciones entre poblaciones y entre especies, y entre respuestas fisiológicas.

Capítulo 2. Efecto del pH en las cinéticas de carga intra- y extracelular de Al en *Fontinalis antipyretica*. Cambios en los contenidos celulares de K, Mg y Ca.

En este estudio se analizaron las cinéticas y capacidades de acumulación de aluminio de *Fontinalis antipyretica* a diferentes niveles de acidez y concentraciones de este elemento. Además se investiga el comportamiento de tres cationes esenciales: Ca, Mg y K. Para todos los elementos estudiados se diferencian las concentraciones intra- y extracelulares.

El Al extracelular se acumuló principalmente durante la primera hora de incubación. Las mayores acumulaciones se produjeron a pH de 4.4, seguido de pH 4; tanto a pH más altos como más bajos la bioconcentración no fue tan importante. El pH resultó ser un factor más relevante en la acumulación extracelular que la concentración de Al en agua. En el modelado de las cinéticas, se encontraron buenos ajustes con la ecuación de Michaelis-Menten. El tiempo requerido para alcanzar el equilibrio también fue máximo entorno a pH de 4.4. Las tasas de carga en general fueron máximas también a este mismo pH de 4.4.

La concentración de Al intracelular inicial después de la preincubación con Cd fue aproximadamente el doble que la extracelular. Esto se debió al pretratamiento con Cd que desplazó ese metal extracelular, ya que normalmente los metales suelen estar más concentrados en el exterior. Durante las incubaciones a diferentes concentraciones de Al, la concentración de este metal dentro de las células aumentó, aunque en menor medida que en los lugares extracelulares. Al igual que sucedía con el Al extracelular, con el intracelular la máxima concentración se alcanzó también a pH de 4.4. Las cinéticas de carga siguieron modelos más variados que para las localizaciones extracelulares. La mayor parte de los ajustes se consiguieron con ecuaciones logarítmicas (con grandes acumulaciones durante la primera hora de incubación) o con ecuaciones lineales (con tasas de carga más constantes durante todo el periodo de incubación). La velocidad media de carga tendió a incrementarse con la concentración de Al en agua y con el incremento de pH (dentro del rango 3-4.4).

El Ca es un metal que se encuentra principalmente en las paredes celulares en una forma intercambiable, el K se encuentra casi por completo en lugares intracelulares y el Mg se encuentra en tanto en localizaciones intra- como extracelulares. Durante la preincubación, el Cd desplazó una gran cantidad del Ca y Mg de la pared celular. El Ca fue liberado de la pared celular en las condiciones más ácidas (pH 3.0-4.4) y fue acumulado a los pH más altos (pH 5.0-5.8). Probablemente exista competencia entre Ca^{+2}, H^{+} y Al^{+3} por los lugares de intercambio de la pared. A pH menores de 5, las altas concentraciones de protones y de Al en el agua, junto con su alta afinidad por los lugares de intercambio, provocó la liberación de Ca. A pH de 5 o mayor, el descenso en la concentración de H^{+}, junto con el descenso en la solubilidad de Al y un cambio en su especiación, deben ser suficientes para que el Ca presente en el agua compita con éxito por los lugares de intercambio. En condiciones más ácidas, mayores concentraciones de Al en agua provocan mayores lavados de Ca, aunque la liberación de Ca extracelular a

causa del Al es mucho menor que la debida al intercambio con H^+. En cuanto al Ca intracelular, al igual que sucedía con el extracelular, las incubaciones de pH más cercanos a la neutralidad provocaron una carga de este metal, mientras que a pH más bajos hubo un lixiviado progresivo de Ca intracelular. La concentración de Al en el agua no pareció interferir con la asimilación de Ca en el interior de la célula.

El K extracelular sufrió unas variaciones de poca importancia y no significativas durante todo el estudio. El K intracelular sufrió una pérdida del musgo en todas las incubaciones, lo que indicó un cambio en la permeabilidad de la membrana. Las pérdidas más grandes se produjeron en las soluciones más ácidas. El efecto del pH y la concentración de Al en el medio en la acumulación de Mg extracelular fue casi idéntico al encontrado para el Ca. El Mg intracelular, como el K, sufrió pérdidas, que fueron menos pronunciadas según aumentaba el pH, el Al tuvo poca influencia.

Capítulo 3. Cinéticas de carga de As, Hg, Sb y Se en el musgo acuático *Fontinalis antipyretica* Hedw.

El principal objetivo de este estudio fue conocer y modelizar las cinéticas de bioconcentración de un metal (mercurio) y tres metaloides (arsénico, antimonio y selenio), en una de las especies de musgo acuático más utilizados en estudios de monitorización fluvial. De estos cuatro elementos, y sobre todo de los metaloides, a pesar de su importancia ambiental como demuestran recientes estudios, existe una carencia importante de datos sobre su acumulación en briófitos acuáticos.

El modelo que mejor se ajustó a las cinéticas de carga fue el de Michaelis-Menten, seguido del modelo lineal y modelo logarítmico. En 6 casos ninguno de los modelos resultó adecuado debido a una caída importante en los factores de contaminación después de los primeros días de incubación. En estos casos sólo se ajustaron los primeros tiempos, en los que resultaron apropiados modelos lineales e inversos. La bioconcentración resultó especialmente rápida en los primeros 2 a 4 días. Arsénico y Se siguieron más de cerca una cinética tipo Michaelis-Menten. Para Hg este modelo únicamente se ajustó bien para la concentración de exposición más baja, encontrándose buenos ajustes con ecuaciones lineales y logarítmicas para otras concentraciones. Aunque los patrones de carga fueron bastante variables dependiendo de la concentración de exposición a este elemento, son de destacar los elevados Factores de

Contaminación (FC) alcanzados para todas las concentraciones de exposición de Hg desde el primer tiempo, siendo los más altos de los cuatro elementos estudiados. El Sb presentó un comportamiento muy irregular, lo que sugiere que *F. antipyretica* puede no ser un buen biomonitor de este elemento, sobre todo cuando sus concentraciones en agua son bajas.

El orden de afinidad de *F. antipyretica* por los elementos estudiados fue Hg>Sb>As≈Se. En cuanto al tiempo de equilibrio, se encontraron grandes diferencias dependiendo del elemento y de la concentración, una consecuencia aplicada de este hecho es que el tiempo de exposición recomendado en un experimento de biomonitorización activa (con trasplantes) podría ser muy variable: de unos pocos días hasta un mes. Los FCs máximos más bajos se encontraron para el As, que fue el elemento que mostró una caída más importante de los FCs (después de los primeros días) para la concentración de exposición más alta. Los siguientes FCs máximos más bajos se encontraron para el Se, para el cual también hubo una caída, aunque no tan acusada para la concentración de exposición mayor. Los FCs máximos más elevados se encontraron para Hg, para este metal la caída en los FCs a altas concentraciones de exposición fue bastante moderada.

Los Factores de Bioconcentración (FBCs), definidos como el cociente entre la concentración tisular de un elemento en una muestra (a la que previamente se le ha sustraído la concentración tisular de ese elemento en un control) y la concentración de ese elemento en agua, tendieron a disminuir según se incrementaba la concentración de exposición. Esta tendencia fue más clara para As, Hg y Se. Este comportamiento implica una menor sensibilidad de *F. antipyretica* de detectar concentraciones más altas en agua, aunque es necesario señalar que las concentraciones de exposición más elevadas en este estudio difícilmente se alcanzarían en el campo, salvo en situaciones de contaminación extrema. Son de destacar los elevados FBCs encontrados para el Hg, lo que pone de manifiesto la capacidad de *F. antipyretica* de magnificar las concentraciones de este metal en agua, característica que es de vital importancia en un buen biomonitor. Por ejemplo, después de un solo día de incubación a 0.1µg l^{-1} de Hg, las concentraciones tisulares se multiplicaron casi por 5.000.

Capítulo 4. Arsénico y mercurio en briófitos acuáticos nativos: diferencias entre especies.

El intercalibrado entre especies es un tema importante en estudios de biomonitorización, puesto que nos permite utilizar distintas especies conociendo de antemano sus diferentes capacidades de acumulación. En este trabajo se realiza un intercalibrado entre 5 especies de briófitos acuáticos en ríos gallegos respecto a su capacidad de acumular arsénico y mercurio.

Las concentraciones de As en los briófitos fueron unos 2 órdenes de magnitud mayores que las de Hg, lo cual puede parecer normal dada las mayores concentraciones que se suelen encontrar de forma natural de este metaloide, a pesar de la mayor capacidad de bioconcentrar Hg que tienen los musgos acuáticos (Díaz et al., 2012). Las concentraciones de As también fueron las que resultaron más variables. Al contrario que en otros estudios (ej. Samecka-Cymerman et al. 2007; Vuori and Helisten 2010), no se encontraron diferencias en las concentraciones de los briófitos en función de la litología de la cuenca en la que se desarrollaban. Las distribuciones kernel mostraron para todas las especies y ambos elementos un sesgo a la derecha, más acusado para As, también la relación entre la concentración máxima y el valor modal resultó mayor para As en todas las especies, lo cual podría indicar mayores problemas de contaminación por este metaloide. Los valores modales observados en estas distribuciones fueron similares a las concentraciones de estos elementos en briófitos acuáticos de lugares limpios, estas modas fueron empleadas como niveles de fondo, a partir de los cuales calcular Factores de Contaminación (concentración de un elemento en una muestra dividido por su nivel de fondo). Para As sólo algunas muestras se encuadraron dentro de las dos categorías de Factores de Contaminación más altos ("contaminación seria" y "contaminación extrema") establecidas por Mouvet (1986), por ninguna de Hg. Para ambos elementos la mayor parte de las muestras entrarían dentro de las dos categorías de contaminación más baja.

Los análisis de regresión realizados entre los diferentes pares de especies mostraron que en ningún caso se encontraron diferencias significativas respecto a una recta de pendiente 1, lo cual indica que no existen diferencias en la bioconcentración de As y Hg en las especies estudiadas. A pesar de los resultados de otros estudios que sí encuentran para otros elementos diferencias interespecíficas, el empleo conjunto de las especies consideradas parece válido para los elementos estudiados y para la zona de trabajo. Es

necesario considerar que en ríos con características diferentes a los de la zona de estudio, los resultados podrían no ser extrapolables.

Capítulo 5. Niveles de fondo de mercurio y arsénico en briófitos de los ríos gallegos.

El principal objetivo presentado en este capítulo fue el cálculo de los niveles de fondo en briófitos acuáticos de dos importantes contaminantes para los cuales todavía no existían datos en Galicia. Se discuten diferentes métodos estadísticos para el cálculo de dichos niveles. Finalmente se proponen como niveles de fondo de mercurio y arsénico las concentraciones correspondientes a la primera moda de la distribuciones de datos suavizadas por núcleo. Este método obtiene unos niveles razonables y no muestra problemas de subjetividad o de arbitrariedad que presentan otros métodos.

Capítulo 6. Biomonitorización activa de arsénico, mercurio y antimonio en el curso bajo del río Eume mediante trasplantes del musgo acuático *Fontinalis antipyretica* Hedw.

En este trabajo se estudia la idoneidad de *F. antipyretica* para ser utilizado en estudios de biomonitorización activa de arsénico, mercurio y antimonio. El musgo acumuló claramente As y Hg respecto a la concentración inicial, siendo la acumulación más rápida en el caso del Hg. Las concentraciones más elevadas para ambos elementos se encuentran en el punto que a priori debería estar más contaminado. Es posible que una parte importante del Hg acumulado por el musgo procediera de deposición atmosférica y no llegara por tanto en su totalidad de los focos de contaminación transportado por el agua. La acumulación de As y Hg fue mayor en el musgo trasplantado que en el autóctono, lo que corrobora la utilidad de la técnica. Para antimonio se encuentran unos resultados irregulares y a veces difíciles de explicar, por lo que no se recomienda el uso de *F. antipyretica* para su biomonitorización.

Capítulo 7. Efectos fisiológicos de la exposición a arsénico, mercurio, antimonio y selenio en el musgo acuático *Fontinalis antipyretica* Hedw.

En este trabajo se estudia la respuesta fisiológica (fotosíntesis neta, respiración y fluorescencia clorofílica) de *F. antipyretica* -una de las especies de musgo acuático más frecuentes en los ríos gallegos- cuando se expone a la contaminación por As, Hg, Sb y Se.

Para la fotosíntesis neta sólo con Hg se apreció una caída constante según se aumentaba la exposición desde bajas concentraciones. Para el resto de los elementos, sólo se encontraron respuestas claras frente a las concentraciones en agua más elevadas: ≥1000 μg l^{-1} para As y Se, y ≥10000 μg l^{-1} para Sb. Por ello las concentraciones en agua medianas efectivas ($EC50_w$), es decir, las concentraciones que provocan una reducción en la fotosíntesis neta del 50% respecto a los controles, calculadas por regresión, resultó menor en Hg y máxima para Sb, indicando por tanto una mayor toxicidad para Hg. También se estudió la relación entre fotosíntesis neta y la concentración tisular de los elementos estudiados, calculándose en este caso las concentraciones tisulares medianas efectivas ($EC50_t$). Se encuentra una gran diferencia con el Hg, que en función de su concentración tisular parece ser uno de los menos tóxicos. Con relación a la respiración, no se encontró una tendencia clara de este parámetro fisiológico en función de la concentración de exposición en agua, ni en función de las concentraciones tisulares.

La fluorescencia clorofílica estimada en función de la relación Fv/Fm sólo fue afectada por las concentraciones de exposición más elevadas. Si se compara con la fotosíntesis neta, muestra una menor variabilidad en los datos, probablemente debido a que éstos son el promedio de varias réplicas, mientras que para la fotosíntesis no se pudo realizar nada más que una medida por tratamiento. Para As y Hg las $EC50_w$ estimadas para la ratio Fv/Fm fueron mucho mayores que las estimadas para la fotosíntesis neta. Con relación a las concentraciones tisulares, destacar el elevado $EC50_t$ calculado para Hg, lo que nos indica una elevada capacidad de bioconcentración de este metal por *F. antipyretica* antes de que empiece a mostrar daños fisiológicos, lo que es una interesante característica en un buen bioindicador acumulador. Para Sb se pueden distinguir dos series de datos dentro del rango de concentraciones tisulares 1500 – 3000 μg g^{-1}: una que procede de musgo expuesto a 1000 μg Sb l^{-1}, con unos valores cercanos a los controles, y otra que procede de musgo expuesto a 10,000 μg Sb l^{-1} y con valores cercanos al 0% respecto a los controles. Una respuesta similar es observada para

fotosíntesis neta, aunque no de una forma tan clara como la observada para fluorescencia. Por tanto parece que el musgo es capaz de aclimatarse a concentraciones relativamente altas de Sb en agua, alcanzando altas concentraciones tisulares con pequeños efectos fisiológico. Pero no es capaz de aclimatarse a la concentración más alta testada, y la misma concentración tisular puede tener efectos prácticamente letales.

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Capítulo 1

Inercia y resiliencia en las respuestas del briófito acuático Fontinalis antipyretica *Hedw. a estrés térmico*

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Inertia and Resilience in the Responses of the Aquatic Bryophyte *Fontinalis antipyretica* Hedw. to Thermal Stress

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Abstract. The physiological responses of the aquatic bryophyte *Fontinalis antipyretica* Hedw. to high temperatures, ranging from 16°C (control) to 34°C, were investigated experimentally. Plant samples were maintained at the temperature under study for up to 25 days with regular determination of physiological variables (pigment ratio and photosynthetic and respiratory rates). Physiological responses to temperature did not differ significantly between mosses collected from a normal river site and from a river site with abnormally high temperature due to input from a hot spring. Simple curve-fitting procedures and summary statistics analogous to those used in toxicological research were employed to compare responses as revealed by the different physiological variables. In a second series of experiments, the capacity of *F. antipyretica* to recover from high-temperature stress was investigated by maintaining samples at 30°C for 2, 4, or 10 days, then transferring the samples to normal conditions (16°C) for 40 days. Physiological variables were again monitored at regular intervals throughout both phases of the experiment. In general, good recovery was observed even after exposure to high temperatures for 10 days. The results of these assays allow quantification of the relationship between a pigment ratio and net photosynthesis rate.

In rivers with rocky substrates in the Atlantic region of Northwest Spain, the moss *Fontinalis antipyretica* Hedw. is one of the principal components of the vegetation. This and other bryophyte species provide important microhabitats for aquatic invertebrates, which in turn are major determinants of a river's carrying capacity for fish (Gerson 1982; Slack and Glime 1985). *F. antipyretica* often shows luxuriant growth in nutrient-rich waters and may sometimes completely cover the river bottom (Say and Whitton 1983). However, relatively little is known about its importance as a primary producer. Similarly, and though some information is available on ecological requirements (largely inferred on the basis of geographical distribution; see Glime 1987), there have been few attempts to characterize its ecological profiles with precision (though see López *et al.* 1997).

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Most aquatic mosses are more tolerant of low than high temperatures. Within the genus *Fontinalis*, a number of species are highly resistant to both low temperatures and desiccation. Many species are capable of net positive growth at temperatures as low as 1°C (Priddle 1980a, 1980b; Maberly 1985a, 1985b), allowing them to maintain vegetative growth almost throughout the entire year. Some species occur at high altitudes (Geissler 1982; Peñuelas 1987), and even in polar regions (Longton 1982).

Aquatic bryophytes have been reported to be capable of surviving temperatures of up to 100°C for short periods, though in general they do not tolerate prolonged exposure to temperatures in excess of 20°C (Glime and Carr 1974; Glime and Acton 1979; Glime 1980; Glime and Raeymaekers 1987). Even short periods at temperatures in excess of 20°C may reduce growth rates (Glime 1992), which would explain the virtual absence of aquatic bryophytes from the tropics (Glime and Vitt 1984).

Species like *F. antipyretica*, which develop entirely below the water surface are generally subject to very minor temperature fluctuations because the water acts as a buffer. In accordance with this, the temperature range tolerated by aquatic mosses is much narrower than that of terrestrial mosses (Glime and Vitt 1984), and optimum growth typically occurs within the range 5–15°C (Dilks and Proctor 1975; Furness and Grime 1982).

Aquatic mosses have rather low rates of photosynthesis, probably because of the low ribulose biphosphate carboxylase-oxygenase (rubisco) activity (Proctor 1981). In general, the activity of this enzyme is lower in aquatic plants than in terrestrial plants (Bowes 1985), and lower in aquatic bryophytes than in vascular macrophytes (Farmer *et al.* 1986). As a result of this low rubisco activity, respiration rapidly exceeds photosynthesis with increasing temperature (Glime and Vitt 1984; Mouvet 1986). In *F. antipyretica*, for example, net photosynthesis is maximal between 10 and 15°C, while respiratory rate increases up to 20°C (Sommer and Winkler 1982). There are, however, other possible explanations for the observed sensitivity of aquatic bryophytes to high temperatures, such as reductions in enzyme activities (Fornwall and Glime 1982) or excessive growth of bacteria and epiphytic algae (Glime and Acton 1979; Glime and Vitt 1984).

Both the rate of photosynthesis and the photosynthetic pigment composition of aquatic mosses are to a great extent controlled by water temperature (Martínez and Núñez 1991). In

the present study, we performed laboratory experiments to quantify the effects of high water temperatures on these two parameters in *F. antipyretica*, with the aim of extending knowledge of the autecology of this species. More specifically, we aimed to evaluate the usefulness of *F. antipyretica* as an indicator of long-term, low-magnitude thermal contamination, as frequently occurs in rivers (whether in isolation or in combination with other forms of contamination) as a result of activities such as effluent disposal and cooling water discharge.

Materials and Methods

Plant Material

Fontinalis antipyretica was collected from three clean sites, each on the upper reaches of a small river (3–5 m width): the Brandelos (540 m above sea level), the Tinto (290 m above sea level), and the Caldo (670 m above sea level). The site on the Caldo was just downstream of the input from a hot spring that maintains summer water temperature at 21–23°C, slightly higher than normal summer river temperatures in this region. The moss was transferred in plastic bags to the laboratory in portable refrigerators at 5 ± 2°C. In the laboratory, the plants were carefully washed with dechlorinated water to remove epiphytes and debris (because such material may interfere with determination of photosynthesis and respiration). Before experiments, all plants were acclimatized to laboratory conditions, by maintenance for 24 h at 16°C in well-oxygenated water under a 14:10 h light:dark cycle. All experiments were performed with 2-cm-long apices to maximize uniformity of plant material.

Experimental Conditions

Both experiments (A and B) were carried out in glass tanks with recirculating purified water (bubbled with air to maintain 100% O_2 saturation; total water volume per tank 15 L, replacement rate 11 L/h), in a cold chamber with a 14:10 h light:dark cycle and ambient temperature of 16°C (though water temperatures were varied, see below). Light was supplied with warm white fluorescent tubes that supplied a photon flux of about 80 µmol/m²/s, which is sufficient to maintain near-maximal photosynthesis (Martínez and Núñez 1991). Water level was maintained constant (10 cm depth) to ensure permanent immersion of the moss samples.

The required water temperatures were maintained with the aid of a net mixer with inputs of water at 16°C and 50°C; water for control tanks (16°C) did not pass through the mixer.

Experiment A: Inertia

To investigate the effects of high temperature on physiology, moss apices were maintained for various periods (5–25 days) at various temperatures (16–33.5°C) prior to determination of pigment ratio, gross photosynthesis rate, respiration rate, and net photosynthesis rate. Batches of 50 apices were placed in 7 × 7 × 10 cm boxes (open-topped to ensure good illumination) made of rigid plastic mesh (pore size 2 mm). The moss batches were then placed in tanks with mean water temperatures (°C; n = 25 days of experiment) of 16 ± 1.0 (SD) (control), 19.8 ± 0.7, 24.9 ± 0.8, 28.4 ± 0.4, or 33.5 ± 0.5 (two tanks per temperature, 10 moss batches per tank). At 5, 10, 15, 20, and 25 days after the start of the experiment, one batch was removed from each tank for determination of physiological variables.

Annual mean river water temperatures in the study region range from 7.7 to 21.3°C and the mean is 15.4 ± 3.1°C (López and Carballeira 1993).

Experiment B: Resilience

To investigate the capacity of *F. antipyretica* to recover from periods of high-temperature stress, moss batches were maintained at 30°C for various periods (2–10 days), then returned to water at 16°C for up to 40 days. Samples were taken for determination of physiological variables at regular intervals throughout both phases of the experiment. The length of the period at 30°C was determined on the basis of three predefined cut-off levels: specifically, mosses were transferred to cold water when the mean D665/D665a ratio (see below) dropped below 1.60 (first group), 1.57 (second group), and 1.46 (third group).

Determination of Physiological Variables

All determinations were performed on samples of two moss batches (50 apices per batch). Chlorophyll phaeophytinization quotient (D665/D665a) has previously been shown to be a reliable measure of stress in aquatic bryophytes (Peñuelas 1984; López and Carballeira 1990; López *et al.* 1994); note that decreasing values indicate increasing stress. The ratio was determined as per López and Carballeira (1990); briefly, samples were extracted with 90% acetone and purified by centrifugation; D665/D665a ratio was then determined spectrophotometrically (Milton Roy Spectronic 3000 Array) as the ratio of absorbance at 665 nm to absorbance at 665 nm after acidification with 30 µl of 1 M HCl per 3 ml of the pigment extract.

Photosynthesis and respiration rates were determined by the light/dark bottle technique (four light bottles and two dark bottles per treatment). Between 0.7 and 0.9 g wet weight of moss (12–15 apices) was added to each bottle (Karlsruhe KF-12, 0.31 l). After temperature stabilization, bottles were incubated for 4 h at 16°C. Oxygen concentration was measured before and after the assay with a polarographic oxymeter with shaking head (Oxi96, WTW). Preliminary experiments showed that these assay conditions ensured sufficiently large changes in oxygen concentration (± 20%) for accurate determination of photosynthesis and respiration. After assay, the dry weights of the samples were determined to allow calculation of rates of oxygen exchange per unit dry weight per unit time.

Results

Preliminary experiments (results not shown) indicated that physiological responses to high temperature did not differ significantly between moss samples from the River Brandelos (normal temperature) and from the River Caldo (abnormally high temperatures) (analyses of variance; $p > 0.05$ in all cases). This suggests that *F. antipyretica* from the Caldo does not show any type of acquired tolerance as a result of persistent exposure to relatively high temperatures. In view of these results, material from the three sites was in subsequent experiments treated as identical and assigned randomly to the different treatments, thus increasing sample sizes and improving precision and reliability.

Inertia

The time-courses of D665/D665a ratio, gross photosynthesis, respiration, and net photosynthesis at the various water tempera-

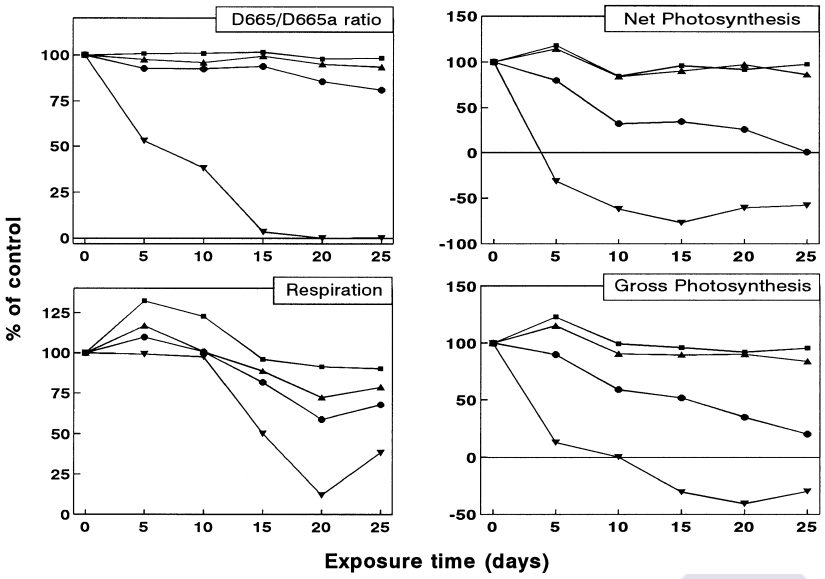

Fig. 1. Time-courses of physiological variables expressed as percentages of the corresponding control values obtained at 16°C at each of the temperatures studied in the inertia experiment A. Vertical axis: percentage of control value. Horizontal axis: exposure time in days. Temperature: 19.8°C squares, 24.9°C up-pointing triangles, 28.4°C circles, 33.5°C down-pointing triangles

Table 1. Time-courses of D665/D665a ratio (PQ), net photosynthesis (NP), respiration (R), and gross photosynthesis (GP) in plants maintained in the laboratory at 16°C as the control for the inertia experiment A

Time (Days)	PQ	NP	R	GP
PRE	1.66 ± 0.01	270.7 ± 39.5	145.1 ± 39.5	415.8 ± 44.8
0	1.65 ± 0.01	365.7 ± 57.3	169.5 ± 46.7	535.2 ± 62.9
5	1.63 ± 0.01	246.3 ± 43.9	126.3 ± 33.9	372.7 ± 44.5
10	1.64 ± 0.01	222.5 ± 56.6	142.8 ± 46.2	365.3 ± 58.9
15	1.63 ± 0.01	233.6 ± 58.4	135.9 ± 40.6	369.5 ± 65.8
20	1.64 ± 0.02	293.1 ± 48.9	111.9 ± 51.3	405.0 ± 41.9
25	1.65 ± 0.02	289.7 ± 36.8	120.1 ± 27.0	409.8 ± 32.3

PRE = values obtained immediately after transfer to the laboratory
Units for GP, R, and NP are µmol O_2/g dw/h
Values shown are means ± SD

tures are shown in Figure 1. In all cases, values shown are percentages of the corresponding control value (*i.e.* that obtained at 16°C; see Table 1); this procedure corrects for the ''transplant effect,'' *i.e.* stress due simply to relocation, which was particularly apparent at the beginning of the experiment.

Maintenance at 33.5°C had clearly stressing effects, as revealed by the steady decline in D665/D665a ratio throughout the experiment. At 28.4°C, a moderate drop in D665/D665a ratio was apparent from day 15 onward. Maintenance at 24.9°C appears to have had a slight effect on D665/D665a ratio; note that small but persistent effects on the pigment ratio may be associated with marked effects on photosynthesis and longer-term survival (see below). Maintenance at 19.8°C had no appreciable effect on D665/D665a ratio over the experimental period (25 days).

The mildest treatments (19.8 and 24.9°C) provoked an initial increase in net photosynthesis, followed by a clear reduction. By the end of the experimental period, however, the plants appeared to have become acclimatized to the new temperatures, because net photosynthesis was approximately normal.

For each temperature and each physiological variable, exposure time required to cause a 50% drop in that variable (mean sublethal time = ET50) was estimated by extrapolation from the data shown in Figure 1 (linear or curvilinear regression, whichever model gave the best fit; results not shown; in all cases the model selected gave $r^2 > 0.7$, $p < 0.05$). For each physiological variable, temperature required to cause a 50% drop in that variable within 4 days (96-h mean sublethal time = $EC50_{96h}$) was then estimated from curves fitted to the extrapolated data points (again by linear or curvilinear regression, whichever model gave the best fit; Figure 2). The $EC50_{96h}$ values obtained (Table 2) provide a means of comparing the effects of temperature: despite the different patterns of response of the different physiological variables, $EC50_{96h}$ values were in all cases similar (32–37°C).

Resilence

The results of this experiment, designed to investigate the capacity of *F. antipyretica* to recover from high-temperature stress, are shown in Figure 3. Again, values shown are percentages of the corresponding control value (*i.e.* that obtained at 16°C) (Table 3). Plants maintained at 30°C until the D665/D665a ratio dropped below 1.47 (*i.e.* 10 days) did not recover normal ratios within the period of the experiment. Plants maintained at 30°C until the D665/D665a ratio dropped below 1.57 (4 days) or 1.60 (2 days) recovered near-normal ratios within about 15 days of transfer to water at 16°C. In these less severely stressed plants, gross photosynthesis, respiration, and net photosynthesis all recovered more slowly than D665/D665a ratio, and did not return to normal until the end of the experimental period. In the severely stressed plants (10 days at 30°C), normal rates of photosynthesis and respiration were not recovered within the experimental period.

These data allow evaluation of the elasticity of the response of *F. antipyretica* to high-temperature stress, *i.e.* its rate of recovery. As can be seen from Figure 4, the percentage recovery observed 10 days after release from stress depends on the severity of the initial stress. As a summary statistic, we calculated the amplitude for each physiological variable, where amplitude is defined as the maximum stress (*i.e.* minimum

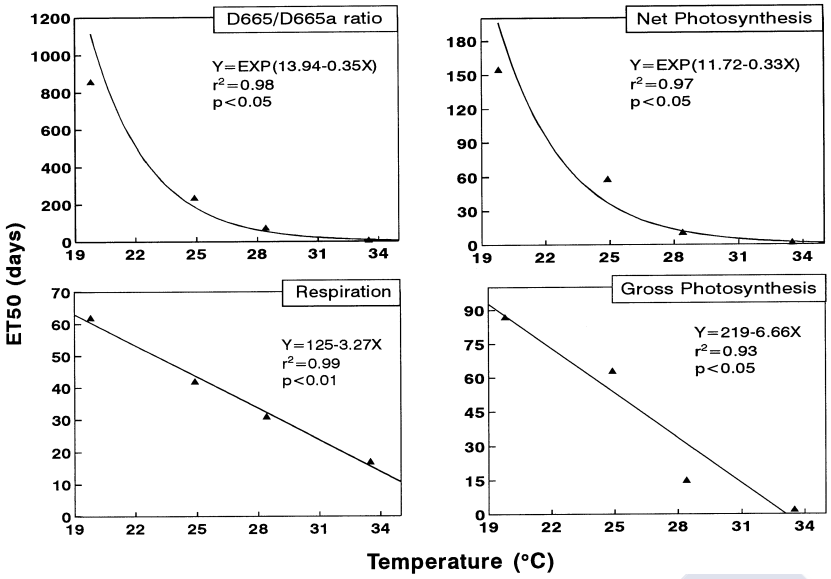

Fig. 2. Relationships between water temperature °C, horizontal axis and mean sublethal time ET50 days, vertical axis in *Fontinalis antipyretica*

Table 2. 96-hour mean sublethal temperature $EC50_{96h}$ for *Fontinalis antipyretica*

$EC50_{96h}$	PQ	NP	R	GP
Temperature °C	36.4	32.0	37.0	32.3

PQ = D665/D665a ratio
NP = net photosynthesis
R = respiration
GP = gross photosynthesis

D665/D665a ratio value) which permits recovery to 50% of the normal value for that variable within 10 days ($EC50_{10d}$). These values (Table 4) are again very similar (1.52–1.56) for all four physiological variables.

Discussion

Photosynthesis in aquatic bryophytes is largely controlled by three factors: temperature, light intensity, and carbon source availability (Maberly 1985a, 1985b; Dilks and Proctor 1975). As a consequence, the present results regarding the effects of temperature on photosynthesis in *F. antipyretica* are specific for the conditions under which our experiments were carried out (*i.e.* nonlimiting light and CO_2 availability); in all cases, rate of photosynthesis was higher after a few hours in the tanks than immediately after collection (Tables 1 and 3).

The net photosynthesis rates obtained in the present study are similar to those reported previously for aquatic bryophytes by Allen and Spence (1981), Peñuelas (1987), and Martínez *et al.* (1993), though slightly higher than those reported by Miyazaki and Satake (1985). Our results indicate that the net photosynthesis rate of *F. antipyretica* is higher than that of terrestrial bryophytes and submersed cormophytes, and similar to that of shade-adapted phanerogamic herbs (Larcher 1977). *Fontinalis antipyretica* can thus be considered to be a shade-adapted species with relatively low compensation and saturation points (Bowes 1985).

In general, aquatic bryophytes show a limited range of tolerance as regards mean water temperature; in most species, the range of tolerance below optimum temperature is broader than that above optimum temperature. Our results (Figures 1 and 3) confirm the sensitivity of *F. antipyretica* to increases in water temperature. Both long-term exposure to moderately high temperatures (*e.g.* 19.8 or 24.9°C for 25 days) and short-term exposure to higher temperatures (30°C for 2 days) had marked effects on physiology. Net photosynthesis was initially stimulated by high temperatures; however, this effect was rapidly counteracted by the increase in respiration so that the compensation point was typically crossed within a few days. Despite this, *F. antipyretica* is capable of acclimatization to moderately high temperatures (19.8 or 24.9°C) within 3 weeks. This compensatory alarm response is in accordance with Selye's general adaptation syndrome hypothesis (Gray 1989). No such acclimatization occurred at 28.4 or 33.5°C. Acclimatization is probably achieved by a shift in the compensation point (*i.e.* by a reduction in respiration rate at the new temperature, apparent in our results from about day 10 onward). Shifts in compensation point with respect to temperature appear to be common in aquatic bryophytes (Empain 1977), and are much more frequent in aquatic than semi-aquatic species (Mouvet 1986).

Saturation light intensity is in general reached much more rapidly by aquatic than by terrestrial mosses (Kallio and Kärenlampi 1975), probably because of the higher chlorophyll content of the former (López and Carballeira 1990). It should be stressed that the apparent shift in compensation point with respect to temperature observed in the laboratory experiments reported here may not occur under the suboptimal light intensities prevailing in the plant's natural habitat. It should also be kept in mind that this study was carried out with apical material, which has a more active metabolism than other parts of the plant (Miyazaki and Satake 1985). The capacity of *F. antipyretica* to adapt to higher temperatures is probably much weaker in the natural environment than under idealized laboratory conditions.

In general, the respiration rates obtained in the present study are within the ranges previously reported for aquatic bryophytes and some vascular aquatic macrophytes (Maberly 1985a,

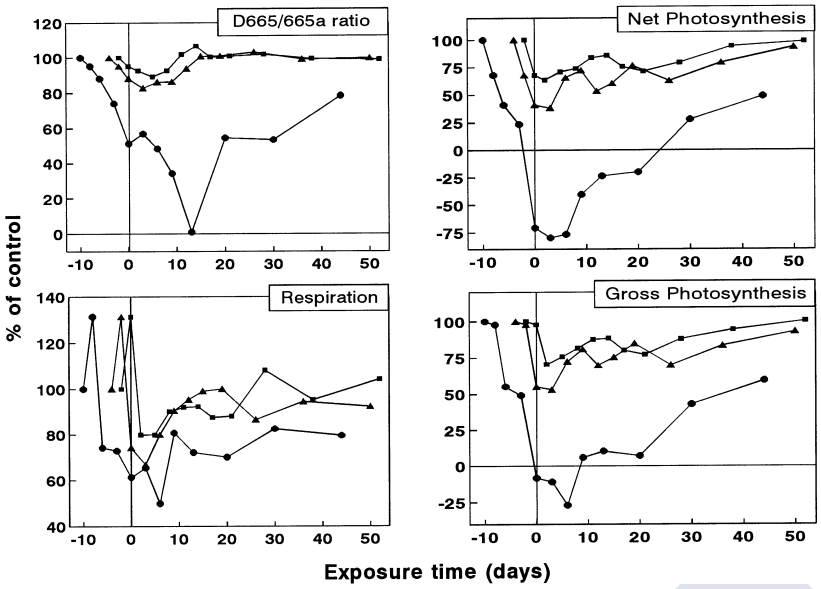

Fig. 3. Time-courses of physiological variables expressed as percentages of the corresponding control values obtained at 16°C in each of the treatments studied in the resilience experiment B. Vertical axis: percentage of control value. Horizontal axis: Number of days at 30°C before transfer to 16°C. Negative values: degradation or number of days after transfer to 16°C. Positive values: recovery. Plants were maintained at 30°C until D665/D665a ratio dropped below 1.60, *i.e.* 2 days: squares, 1.57, *i.e.* 4 days: triangles or 1.46, *i.e.* 10 days: circles

Table 3. Time-courses of D665/D665a ratio (PQ), net photosynthesis (NP), respiration (R), and gross photosynthesis (GP) in *Fontinalis antipyretica* maintained in the laboratory at 16°C as the control for the Resilience experiment B

Time (days)	PQ	NP	R	GP
PRE	1.65 ± 0.01	296.5 ± 42.6	150.1 ± 27.6	446.5 ± 51.2
0	1.63 ± 0.01	347.1 ± 47.7	166.0 ± 33.1	513.0 ± 41.4
2	1.62 ± 0.01	214.9 ± 34.3	190.9 ± 53.1	405.9 ± 30.6
4	1.61 ± 0.01	227.1 ± 35.4	170.3 ± 31.7	397.4 ± 41.3
6	1.61 ± 0.01	174.2 ± 55.0	188.8 ± 26.9	363.0 ± 47.6
9	1.62 ± 0.01	190.3 ± 49.3	171.5 ± 8.0	361.8 ± 51.2
12	1.61 ± 0.02	163.4 ± 32.5	147.3 ± 33.4	310.8 ± 39.2
16	1.63 ± 0.00	194.9 ± 31.7	126.5 ± 27.3	321.4 ± 48.1
19	1.61 ± 0.01	195.2 ± 37.1	121.4 ± 22.9	316.6 ± 36.1
23	1.63 ± 0.00	224.4 ± 41.2	122.8 ± 29.5	347.2 ± 42.8
30	1.62 ± 0.01	305.1 ± 29.5	132.6 ± 10.0	437.8 ± 29.1
40	1.63 ± 0.01	318.8 ± 37.8	125.5 ± 37.5	444.3 ± 40.2
54	1.64 ± 0.01	309.6 ± 38.0	162.6 ± 21.3	472.2 ± 42.6

PRE = values obtained immediately after transfer to the laboratory
Units for GP, R and NP are µmol O_2/g dw/h
Values shown are means ± SD

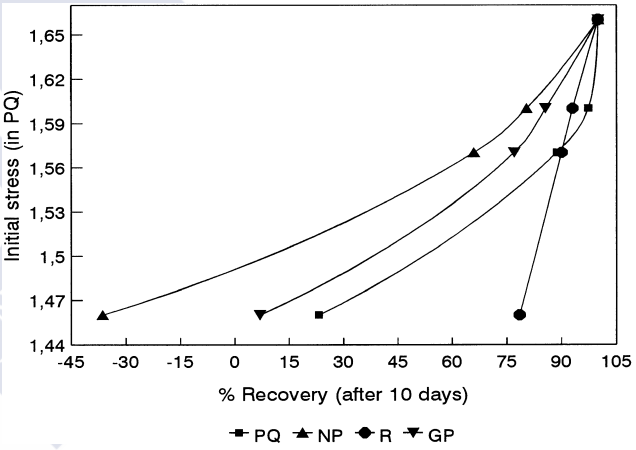

Fig. 4. Elasticity, *i.e.* rate of recovery, expressed as percentage recovery with respect to control 10 days after termination of thermal stress of *Fontinalis antipyretica*, plotted against initial stress D665/D665a ratio. PQ = D665/D665a ratio; GP = gross photosynthesis; R = respiration; NP = net photosynthesis

1985b; Azcon-Bieto *et al.* 1987; Martínez et al. 1993), similar to or lower than reported values for terrestrial bryophytes (Skre and Oechel 1981; Aro et al. 1984), and markedly lower than reported values for terrestrial phanerogams (Larcher 1977).

Respiration was strongly stimulated by increased temperature over the first 10 days of the experiment, in agreement with the findings of Sommer and Winkler (1982). At 19.8 and 24.9°C, respiration rate subsequently tended toward normal again. The apparent increase in respiration observed at 28.4 and 33.5°C between days 20 and 25 is probably attributable to decomposition, since by this stage these plants showed very low or negative net photosynthesis.

In view of the apparent lack of relationship between respiration rate and growth rate (Lambers *et al.* 1983), the results of the present study confirm that aquatic bryophytes have very low metabolic rates, probably because of physiological and structural limitations related to the hostile environmental conditions that these organisms often have to withstand.

The application for the first time of toxicological techniques (ET50, $EC50_{96h}$, $EC50_{10d}$) has allowed simple and precise characterization of the response of *F. antipyretica* to temperature in terms of resilience (elasticity and amplitude) and inertia. We consider such techniques useful for providing normalized measures of responses to temperature stress, thus facilitating comparisons between populations and between species, and between responses as manifested by different physiological variables.

Considerable research effort has been dedicated to the development of indicators of stress in plants. Pigment-based indices, such as the D665/D665a ratio used in the present study, have proved to be low-cost, rapid, and effective. However, it should be stressed that pigment indices need not necessarily show simple relationships with functional parameters (such as

Table 4. Resilience of *Fontinalis antipyretica* to thermal impacts, expressed as amplitude $EC50_{10d}$, *i.e.* the maximum degradation maximum reduction in D665/D665a ratio which permits a 50% recovery within 10 days

$EC50_{10d}$	PQ	NP	R	GP
D665/D665a ratio	1.52	1.53	1.53	1.56

PQ = D665/D665a ratio
NP = net photosynthesis
R = respiration
GP = gross photosynthesis

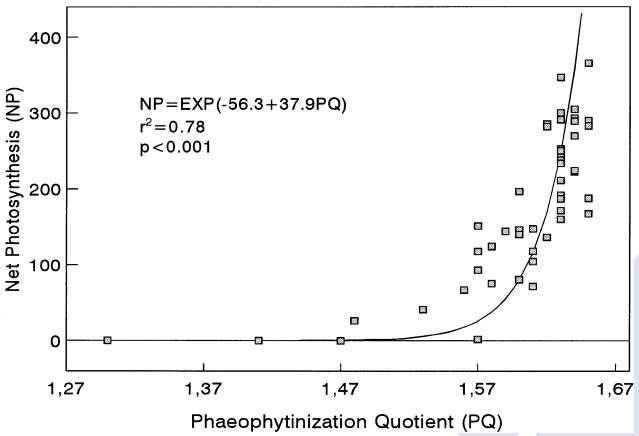

Fig. 5. Relationship between chlorophyll phaeophytinization quotient D665/D665a ratio and net photosynthesis µmol O_2/g dry weight/h in *Fontinalis antipyretica*

photosynthesis rate and growth rate). Considering the data obtained in the present study, net photosynthesis is best predicted from D665/D665a ratio by an exponential function (see Figure 5). This model suggests that small increments in D665/D665a ratio from about 1.57 onward have an increasingly marked effect on net photosynthesis, with the theoretical maximum of 425 µmol O_2/g dry weight/h being reached at about the maximum D665/D665a ratio (1.66).

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Capítulo 2

Efecto del pH en las cinéticas de carga intra- y extracelular de Al en Fontinalis antipyretica*. Cambios en los contenidos celulares de K, Mg y Ca*

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The Effect of pH on the Kinetics of Intra- and Extracellular Uptake of Al in *Fontinalis antipyretica*: Changes in the Cellular Contents of K, Mg, and Ca

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Abstract. The effect of acidity on the accumulation of Al in the aquatic bryophyte *Fontinalis antipyretica* was studied. The main interest of this study was in characterizing the relationship between the two parameters, as Al is typically mobilized in acidic environments, where it becomes extremely toxic. With this aim, a series of laboratory experiments were carried out where samples of *F. antipyretica* were held in tanks of water with different concentrations of Al (0.1–3 mg L^{-1}) and levels of acidity (pH 3–5.8). The incubation time varied between 1 and 24 h. In general, the greatest accumulations of both intra- and extracellular Al were found at a pH of 4.4. With the results obtained, the kinetics of both intra- and extracellular Al uptake was characterized, and models constructed for different combinations of Al and acidity. The study was completed by examining the relationship between the pH of the holding water and bioaccumulation of Al and the variation in concentration of cations essential to plant life, which are also indicators of changes in cellular physiology (K, Mg, Ca). In general, losses of these elements were found at intra- and extracellular locations, especially at high pH.

There exist an ever-increasing number of freshwater ecosystems that have problems associated with acidity. Acidification of surface waters may be caused in various ways. Among the causes, leaching from mine sites and industrial dumps and atmospheric deposition of acid are the most widespread. Acidity may enter a body of water directly or indirectly through the surrounding soil, vegetation, etc.

Acidic waters display a set of common characteristics. Thus, NO_3^- is usually substituted by NH_4^+ and there is an increase in the concentration of sulfate, an ion typically associated with acidification (Howells 1990). Metals, such as Al, Mn, Zn, Fe, and Co, increase in solution as they are mobilized from the soils in the catchment area and from underwater sediments (King *et al*. 1992; Lehtonen 1989; Mersch *et al*. 1993; Sager 1992). At the same time there is a change in the dissolved metals present to species that are more toxic to aquatic biota and usually more readily bioavailable (Arts *et al*. 1990). Other cations, such as Ca, Mg, and K, are also released, thus decreasing the saturation of bases in the soil exchange complex (Freedman 1995).

Acidity affects all trophic levels in aquatic ecosystems. In general it produces a decrease in biological diversity and the substitution of sensitive species with others more resistant to acidic conditions (Arts *et al*. 1990). Toxicity is due to high concentrations of H^+, which cause leaching of cations from cells or changes in the balance of ions (Bates 1993). Toxicity also increases because of an increase in the presence of such substances as Al, a key element in the acidification process that is toxic to a large number of organisms (Howells 1990; Mersch *et al*. 1993). The effects of Al on living organisms differ depending on whether it remains outside the cell in an exchangeable form (extracellular metal) or whether it enters the cells by crossing the cell wall and cell membrane (intracellular localization). It is in the latter case where the most acute damage to the cellular physiology can be caused (Satake *et al*. 1988).

For the present study, we carried out experiments simulating decreases in pH in freshwater environments with different concentrations of Al. The aim of this study was (1) to differentiate the accumulation and kinetics of Al at different concentrations and at different levels of acidity; (2) to observe, without outside interference, the effect of different levels of pH on the cells (mobilization of the essential cations: Ca, Mg, K). In addition, and with the aim of better characterizing the process, we analyzed the variations in concentration of the metals studied at intra- and extracellular locations.

Materials and Methods

Plant Material

Moss of the species *Fontinalis antipyretica* Hedw, which is often employed in studies involving biomonitoring in aquatic environments (López and Carballeira 1993a, 1993b; Carballeira *et al*. 1998), was used.

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The moss was collected from an uncontaminated stretch of the Tinto River (Galicia, northwest Spain). Plants that were submerged at a certain depth were chosen to avoid collecting those that may have suffered hydric stress at periods of low water (Wehr *et al*. 1983). The plants were rinsed, placed in plastic tanks filled with river water, and transported to the laboratory in a portable refrigerator (6 ± 2°C).

In the laboratory, samples were rinsed with distilled water to remove adhered particles. In all experiments, 2-cm-long shoot tips were used (Wells and Brown 1990). This minimized errors due to the different accumulation capacities (and thus metal concentration reached) of different parts of the plants (Wehr *et al*. 1983). The shoot tips were maintained until use in river water in a cold chamber at 16 ± 1°C, with a day:night cycle of 14:10 h, irradiation of 80 μmol photons/m^2 s, and constant aeration.

Experimental Design

Before starting the experiments, the samples (500 moss shoot tips) were preincubated for a period of 1 h in 51 of a solution of 100 mg/L $CdCl_2$ (Merck, analytical grade). This avoided interference of cations previously adhered to the moss and allowed standardization of conditions in all samples, as has previously been carried out by other authors (Wells and Brown 1990; Brown and Avalos 1991).

The moss samples were then transferred (in groups of 25 shoot tips) to a series of 18 glass recipients containing each one 1 L of a solution of Al (0, 0.1, 0.5, 1, 2, and 3 mg/L) at a given pH (3, 3.5, 4, 4.4, 5, 5.5, or 5.8). The samples were maintained in these solutions with aeration for 1, 6, or 24 h. The incubations were carried out in seven batches, each batch testing a different pH at all of the Al concentrations and all three incubation times. Al solutions were prepared using $Al(NO_3) \cdot 9H_2O$ (Merck, analytical grade) and were acidified by adding H_2SO_4 (Merck, pure grade) with a micropipette. In all cases, a pH meter was placed in the recipient so that the decrease in pH could be monitored as the acid was added. The pH meter was left in the solution until the pH had stabilized.

The concentrations of Al and pH in the water used in this work are similar to the ones that can be found in some acidified rivers as the result of mining activities (pyrite extraction) in our region (Vázquez *et al*. 2000). In some highly affected sites, the concentration of Al can be considerably higher.

The water used during the 24-h period of Al uptake, as well as in the preincubation with Cd, was spring water, the physicochemical characteristics of which are shown in Table 1. The natural pH of this water was 5.8; when we added $Al(NO_3)$ to the water the pH fell. This is the reason why we do not have data for the higher pH at the higher Al concentrations, as can be seen in the different tables and figures in the results section.

Table 1. Physicochemical characteristics of the spring water used to prepare experimental solutions

Parameter	Value
pH	5.8
CE	252 μMhos/cm
NO_2^-	<0.05 mg/L
NO_3^-	33 mg/L
NH_4^+	0.07 mg/L
Cl^-	13.9 mg/L
PO_4^{-3}	<0.05 mg/L
SO_4^{-2}	34 mg/L
Ca^{+2}	25 mg/L
Mg^{+2}	4.3 mg/L
Na^+	11.7 mg/L
K^+	3.07 mg/L
Al^{+3}	0.08 mg/L
Cd^+	ND mg/L

ND: not detected

Extraction and Analysis of Metals

Metals that concentrated at each cellular site in moss during the incubation period were extracted by sequential elution following the procedure of Brown and Buck (1978) as modified by Vázquez *et al*. (2000). The technique consisted of removal of the accumulated metals from each cellular location in a stepwise fashion. Intercellular metal was first removed and discarded by washing with distilled water, so that the first extraction was of metal joined by cationic binding to extracellular anionic exchange sites (extracellular metal). This is displaced by a cation with higher affinity for the exchange sites and/or that is found at a higher concentration than that which is to be extracted (Brown and Sidhu 1992)—in this case, Pb supplied as $Pb(NO_3)_2$ (50 mM). The intracellular metal was then extracted by drying the moss in a hot-air oven (at 60°C for 24 h) before incubating it in acid (1 M HNO_3). This metal corresponded to that present in a soluble form inside the cells (intracellular metal). Following this step, a metal residue remained that was difficult to extract and which represented a negligible proportion of the total (Vázquez *et al*. 2000).

All extractions were carried out in triplicate (eight shoot tips per replicate) in a volume of 10 ml of extractant and with constant shaking. Concentrations of Al, K, Ca, and Mg in the extracts obtained were determined by atomic absorption spectrophotometry with an air/acetylene flame (using Perkin Elmer 2100 apparatus). Aliquots of 0.1 ml of a solution of CsCl and LaCl (100.000 mg/L) were added to each sample to avoid interference when carrying out determinations (Wells and Brown 1987). Standards were made up in the corresponding displacing agent.

Data Analysis

The significance of differences in the concentrations of Al, Ca, Mg, and K in moss after 24 h of incubation in function of the Al and pH in the water were analyzed using a Kruskal-Wallis test.

The results obtained were converted into contamination factors (CFs) so that the data and models obtained could be applied to areas with different characteristics. Under normal conditions, CF can be defined as the relationship between metal concentration in a biotic or abiotic sample for a given site and a reference value obtained from a site in the stretch of water under study, situated above a point of contamination. In this case, CF was calculated as the relation between the concentration of Al measured at the end of each treatment and the initial Al concentration. The kinetics of Al uptake were fitted to different mathematical equations.

Modeling of the extracellular Al uptake process was carried out using a Michaelis-Menten equation, or its inverse where release of the element occurred:

$$[Al] = \frac{Umax \times time}{Km + time}$$

where *Umax* = maximum concentration at equilibrium, and *Km* = time necessary to reach half of *Umax*. This equation allowed calculation of the maximum equilibrium concentration reached (*Umax*), and the time required (*Teq*) to reach *Umax*. The time could

Table 2. Changes in the accumulation of extracellular aluminum (μmol/g DW) in *F. antipyretica* with varying pH and concentrations of Al (ppm) in the incubation medium

(Al) Water	Time (hs)	pH 3	SD	pH 3.5	SD	pH 4	SD	pH 4.4	DS	pH 5	SD	pH 5.5	SD	pH 5.8	SD
0	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1
	1	6.8	0.7	9.0	5.1	9.4	2.3	9.8	0.6	7.7	0.9	1.7	0.4	3.6	0.1
	6	9.8	0.6	9.7	1.2	11.5	1.1	19.4	0.9	7.4	0.9	3.0	0.5	2.3	0.5
	24	4.8	1.0	11.0	1.7	12.8	0.3	20.1	1.2	8.0	1.4	3.4	0.9	2.2	0.4
0.1	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1		
	1	23.1	1.1	42.1	8.3	73.5	3.3	70.0	0.4	26.0	2.0	20.9	2.7		
	6	22.6	2.8	38.6	3.6	74.3	1.6	79.4	2.1	33.9	3.6	32.4	0.9		
	24	18.7	0.8	41.9	3.1	76.6	6.4	77.9	3.2	35.4	2.9	32.7	5.2		
0.5	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1						
	1	28.3	1.9	40.9	4.8	80.8	5.4	89.2	7.7						
	6	31.0	4.6	48.1	9.4	78.5	4.8	91.8	3.5						
	24	28.1	1.6	55.5	2.9	76.1	9.9	90.9	2.8						
1	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1						
	1	36.8	2.4	39.6	1.8	80.7	8.0	90.7	5.2						
	6	30.8	3.2	58.2	1.2	84.5	7.7	93.7	8.9						
	24	34.2	1.8	61.4	2.9	85.1	8.5	92.4	6.8						
2	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1						
	1	37.6	1.1	47.2	1.9	82.8	7.6	87.2	4.6						
	6	32.4	1.4	58.0	4.4	83.4	3.4	93.0	8.8						
	24	34.4	0.8	66.4	2.6	80.4	9.5	88.5	3.8						
3	0	6.2	1.1	6.2	1.1	6.2	1.1	6.2	1.1						
	1	43.3	2.2	46.5	6.3	85.5	3.8	88.8	12.8						
	6	35.9	2.3	67.3	5.0	82.1	3.9	85.9	5.4						
	24	35.5	2.0	85.6	3.8	79.9	8.5	88.0	9.8						

Table 3. Variation in factors (*Umax*, *Km*) from the Michaelis-Menten equation (and its inverse) (used in modeling the kinetics of extracellular Al uptake in *F. antipyretica*) with concentration of Al and pH of the medium (r^2 = coefficient of correlation)

		(Al) Water (mg/L)					
pH	Parameter	0	0.1	0.5	1	2	3
3	*Umax*		0.54	0.78	0.86	0.88	0.93
	Km		−0.13	0.31	−0.12	−0.12	−0.19
	r^2		0.75	0.89	0.92	0.93	0.95
3.5	*Umax*	0.28	1.07	1.43	1.69	1.73	2.24
	Km	0.19	−0.04	0.34	0.6	0.4	0.9
	r^2	—	0.95	0.96	0.98	0.97	0.97
4	*Umax*	0.34	2.02	2.04	2.27	2.17	2.14
	Km	0.36	0.0013	−0.05	0.06	−0.02	−0.06
	r^2	—	0.99	0.99	0.99	0.99	0.99
4.4	*Umax*	0.58	2.12	2.43	2.48	2.42	2.31
	Km	1.13	0.13	0.02	0.02	0.04	−0.02
	r^2	0.72	0.99	0.99	0.99	0.99	0.99
5	*Umax*	0.2	0.96				
	Km	−0.04	0.39				
	r^2	—	0.93				
5.5	*Umax*	*13.95	0.92				
	Km	1.3 e-5	0.62				
	r^2	0.85	0.91				
5.8	*Umax*	*13.8					
	Km	1.3 e-5					
	r^2	0.99					

* Fitted to the inverse of the Michaelis-Menten equation

not be calculated directly from the equation, as it is asymptotic. Thus an approximation was made of the maximum uptake of Al in the moss at each pH tested and for each concentration of Al as being the concentration of Al in moss at which the daily increase in concentration is less than 1% of the previous day. The corresponding time, or *Teq*, was then calculated. If the reciprocals of both parts of the Michaelis-Menten equation are taken, a linear function whose slope is *Km*/*Umax* is obtained. The slope of this straight line

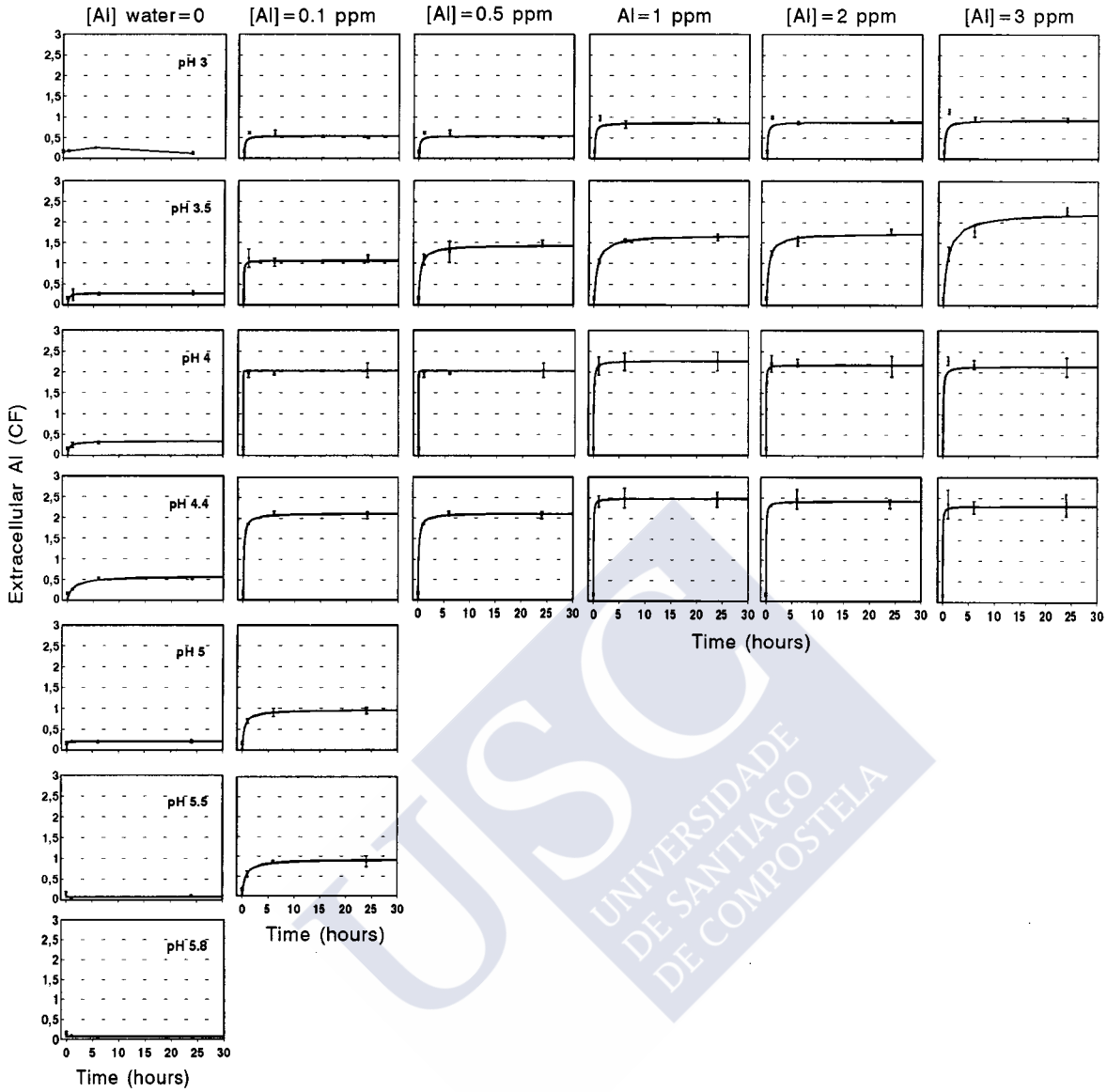

Fig. 1. Kinetics of extracellular Al uptake in *F. antipyretica* as a function of pH and concentration of Al in the incubation medium (concentration of Al in CF = contamination factors; fitted to the Michaelis-Menten equation)

can resemble the speed of extracellular metal uptake (inverted), in such a way that when the *Km*/*Umax* ratio increases its value, the speed of uptake decreases.

For the intracellular Al uptake process, the best fits were found with linear equations and logarithms:

$$[Al] = a + b \times time$$

and

$$[Al] = e^{(a+b\times time)}$$

and the speed of uptake can be obtained from slope (b).

From the fits made to the equations, models for Al uptake were constructed. Michaelis-Menten equations were used for extracellular uptake and linear functions for intracellular uptake modeling. These models were made using the mean values of extra- and intracellular Al accumulated by the moss at the different concentrations of Al tested at each pH.

Results and Discussion

Kinetics of Extracellular Al Uptake: Modeling

A base level of 6.2 μmol/g DW of extracellular Al was found after preincubation of *F. antipyretica* with Cd (100 mg/L) for 1 h (Table 2). Subsequent incubation of the moss in solutions of different pH and Al concentrations produced an accumulation of Al at extracellular binding sites. This was also observed in some cases where Al was not added to the water (at a pH of between 3 and 5), due to the fact that

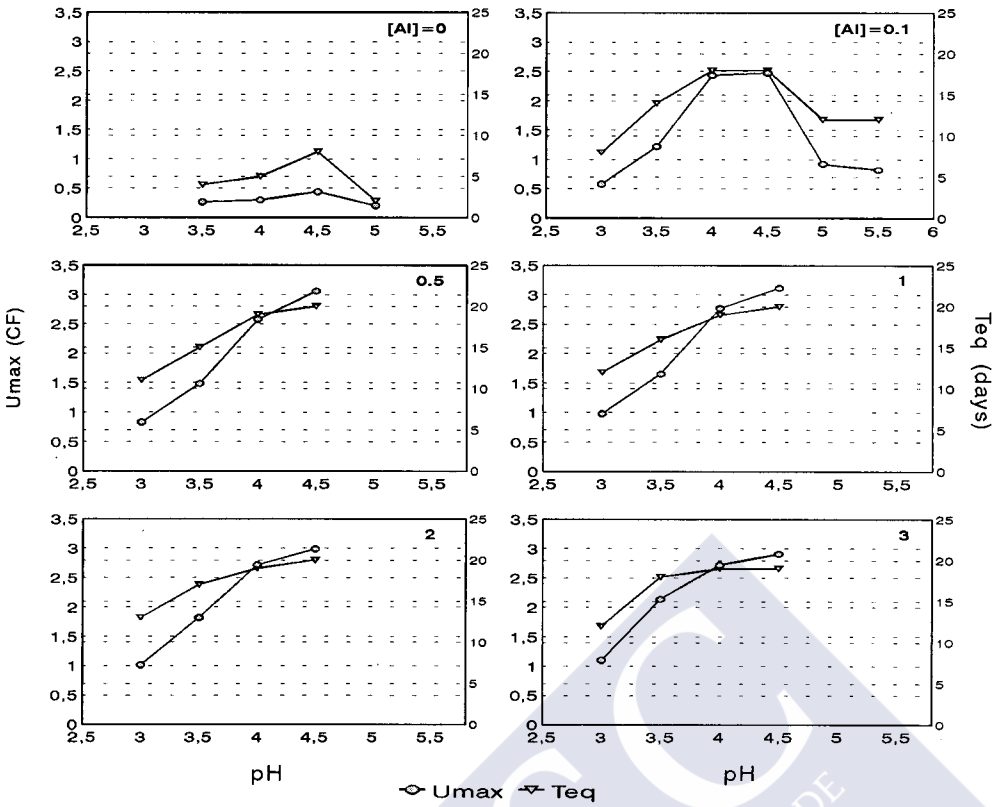

Fig. 2. Kinetics of extracellular Al uptake in *F. antipyretica*: maximum corporal concentration (*Umax*) of Al and time taken to reach equilibrium (*Teq*) as a function of pH and concentration of Al in the incubation medium (*Umax* of Al is expressed as CF = contamination factors)

the water contained a small amount of this metal, which is very soluble at low pH (Arts *et al*. 1990; King *et al*. 1992; Lehtonen 1989; Mersch *et al*. 1993; Satake and Nishikawa 1990).

Extracellular Al was accumulated mainly during the first hour of incubation. In general, it was found that the higher the concentration of Al in the medium, the greater the amount accumulated by the moss, the differences being more marked between 0–0.1 mg/L of Al in the medium. The differences between Al concentrations were highly significant ($p < 0.001$) at pH 3 and 3.5 and significant ($p < 0.05$) from 4.4 to 5.5. At pH 4 differences only were significant ($p < 0.05$) between 0 and the other concentrations of Al in water.

The pH of the medium clearly influenced Al uptake in the moss (Table 2), and the largest accumulations were produced at pH 4.4, followed closely by those at pH 4. Progressively lower uptake of Al was seen in media that were more or less acidic than this. The effect of the pH on the bioaccumulation of Al is highly significant ($p < 0.001$) for the lower Al concentrations in water (0 and 0.1 mg/L), significant ($p < 0.05$) for concentrations between 0.5 and 2 mg/L, and not significant for 3 mg/L of Al in the water. This may be related to the influence of pH on the speciation of Al, as at pH close to 4.4, there exist a mixture of forms of differing toxicity that are, in general, bioavailable (Martin 1988). At pH less than 4.4, the very toxic Al^{3+} forms predominate, and at higher pH the solubility of Al decreases; furthermore the predominant species are slightly toxic and increasingly less bioavailable (hydroxide compounds or compounds with organic ligands).

The data shows the influence of pH on Al uptake by modifying its speciation and solubility. This is in agreement with the results of Engleman and McDiffett (1996), who concluded that the accumulation of Al by bryophytes appears to be more dependent on pH than on the metal content of the medium, as the relationship is nonlinear. These authors found the maximum uptake occurred at around pH 5, slightly higher than found in the present study. Similar results were obtained in other studies (Vázquez *et al*. 2000) transplanting moss to an acidified river. The higher Al accumulations in moss were obtained in a site with a mean pH of 4.6. However the concentration of Al in water was considerably higher at another river sites with lower pH.

Modeling of the kinetics was carried out using mathematical equations, and in general good fits were obtained with the Michaelis-Menten equation (Table 3, Figure 1). The only exceptions were found in the cases where Al was not added to the water, and at pH 3 (model fits were not achieved), and pH 5.5–5.8, which followed the function obtained when the second term of the Michaelis-Menten equation is inverted.

The model allowed the parameters that characterize the kinetics of uptake to be calculated.

Maximum Concentration. This was found directly from the value of the *Umax* parameter, and corresponded, for a given

Table 4. Kinetics of extracellular Al uptake in *F. antipyretica*: variations in the *Km*/*Umax* ratio with the pH and the concentration of Al (ppm) in the incubation medium

[Al] Water	pH 3	pH 3.5	pH 4	pH 4.4	pH 5	pH 5.5	pH 5.8
0		0.679	1.059	1.948	0.2	0.000	0.000
0.1	0.241	0.037	0.001	0.061	0.406	0.674	
0.5	0.397	0.238	0.025	0.008			
1	0.140	0.355	0.026	0.008			
2	0.136	0.231	0.009	0.017			
3	0.204	0.402	0.028	0.009			

Table 5. Accumulation of intracellular aluminium (μmol/g DW) in *F. antipyretica* with different pH and concentrations of Al (ppm) in the incubation medium

(Al) Water	Time (h)	pH 3	SD	pH 3.5	SD	pH 4	SD	pH 4.4	SD	pH 5	SD	pH 5.5	SD	pH 5.8	SD
0	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4
	1	6.7	1.2	5.0	3.2	8.0	1.3	22.0	1.9	10.2	1.0	9.7	1.7	12.9	1.2
	6	5.9	0.8	7.8	1.5	11.2	1.6	19.6	2.3	15.3	0.7	11.4	0.4	11.6	1.6
	24	5.0	1.2	16.6	2.7	11.2	1.1	19.0	0.9	16.9	0.4	15.4	1.9	13.3	1.8
0.1	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4		
	1	11.5	0.7	5.7	4.3	30.8	6.1	52.3	5.7	21.1	0.7	18.2	0.9		
	6	10.5	1.3	9.1	2.3	60.1	2.4	75.6	5.4	31.9	3.2	30.6	1.8		
	24	10.7	0.7	28.5	1.7	77.7	3.8	73.3	6.2	56.0	4.0	49.3	1.4		
0.5	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4						
	1	13.6	3.7	8.3	5.7	41.0	4.8	80.2	4.6						
	6	16.7	1.5	12.5	0.5	70.1	6.7	85.8	12.7						
	24	13.4	0.6	41.7	2.8	82.4	10.0	84.5	3.7						
1	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4						
	1	17.4	2.4	13.5	3.1	39.6	8.9	86.3	3.2						
	6	17.8	0.5	19.8	2.7	88.2	4.5	92.5	4.1						
	24	15.3	1.7	42.9	3.8	104.2	5.6	81.7	8.8						
2	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4						
	1	16.0	4.2	14.2	1.2	49.1	1.9	78.5	5.1						
	6	17.9	1.3	18.3	2.0	91.9	2.5	96.7	2.4						
	24	16.0	0.3	47.7	7.9	104.2	6.0	93.4	5.8						
3	0	11.1	0.4	11.1	0.4	11.1	0.4	11.1	0.4						
	1	14.5	1.7	15.9	1.9	48.7	2.1	71.7	8.3						
	6	17.8	0.4	26.2	5.5	75.9	3.0	96.6	6.8						
	24	15.6	1.5	53.7	3.0	108.1	5.1	93.1	7.7						

element, to the stable or equilibrium concentration reached between the moss and the medium. The approximate value of *Umax*, calculated as explained in Materials and Methods, is shown in Figure 2. In general, for any concentration of Al in solution considered, *Umax* increased in value with increasing pH, up to pH 4.4, and decreased at higher pH. This seems to indicate an inverse relation between *Umax* and the concentration of Al^{3+}, which is the most abundant species at pH 3 but is replaced, at higher pH, by hydroxide forms, which are much less bioavailable and much less toxic to living organisms. Regarding the concentration of Al in the medium, there was an increase in the value of *Umax* at between 0 and 0.1 mg/L, while at higher concentrations the influence was negligible.

Equilibration Time. The time required to reach the maximum metal concentration in moss or the equilibration time (*Teq*) was calculated next. This parameter showed changes similar to those seen for *Umax* (Figure 2), so that the time required to reach a stable concentration became longer as pH increased, until around pH 4.4. The time required then became shorter. Thus, the highest *Umax* corresponded to the longest *Teq*. It must be pointed out that the *Umax* value that appears in this graph (Figure 2) is not that obtained from the Michaelis-Menten equation but an approximated value, calculated as explained in Materials and Methods.

Average Rate of Uptake. As mentioned previously, the *Km*/*Umax* ratio can be taken as the inverse of the average rate of uptake of Al during the time of incubation. Although the trends are not clear-cut (Table 4), the lowest values (which indicate a high rate of uptake) generally occurred at pH 4.4 (at which the maximum concentrations are high). In the same way, at the pH where there is little accumulation of Al, the rate of uptake was slow, the results obtained for these three parameters being consistent. Where Al was not added to the medium, the speed of binding of Al to the cell also tended to be low.

Table 6. Variation in the factors from logarithmic equations ([Al] = e∧ (*a* + *b* time)) and from linear equations ([Al] = *a* + *b* time) used in modeling the kinetics of intracellular Al uptake in *F. antipyretica*, with pH and concentration of Al in the incubation medium

		(Al) Water (mg/L)					
pH	Parameter	0	0.1	0.5	1	2	3
3	*a*	−1.73[a]	0.31	−0.93[a]	−0.8[a]	−0.81[a]	−0.85[a]
	b	−0.05	−0.67	0.02	0.03	0.03	0.03
	r^2	97.04*	35.93$^-$	61.63$^-$	79.33$^-$	90.28*	84.82$^-$
3.5	*a*	0.23	0.19	0.22	0.33	0.31	0.38
	b	9.26	0.02	0.04	0.04	0.04	0.05
	r^2	70.8$^-$	89.7*	96.58*	99.9***	99.31**	98.84**
4	*a*		0.21[a]	0.35	0.48[a]	0.55[a]	0.5[a]
	b		0.12	0.13	0.15	0.15	0.15
	r^2		92.57*	97.75**	99.09*	97.76**	98.12**
4.4	*a*	−0.65[a]	0.4[a]	0.59[a]	0.63[a]	0.65[a]	0.63[a]
	b	0.042	0.13	0.15	0.15	0.15	0.15
	r^2	84.8*	99.03**	97.02**	95.3*	98.25**	98.79**
5	*a*	0.32	0.48				
	b	6.96	0.05				
	r^2	74.2$^-$	94.15*				
5.5	*a*	0.29	0.45				
	b	6.01	0.04				
	r^2	92.29*	92.69*				
5.8	*a*	−1.05[a]					
	b	9.30E + 01					
	r^2	55.13$^-$					

[a] Fitted to a logarithmic equation
Significance levels: * $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$; $^-$ $p > 0.05$

Kinetics of Intracellular Al Uptake: Modeling

The concentration of intracellular Al following preincubation with Cd was 11.1 μmol/g DW (Table 5), almost double that at extracellular locations. This is because most of the Al at the latter site was washed away during the preincubation with Cd, as normally metals are present in greater proportions at exterior sites (Taylor 1988). During incubation in solutions of Al, the concentration of this metal inside the cell increased, although to a lesser degree than at extracellular sites.

The maximum uptake of Al in the moss occurred at pH 4.4. As with at the extracellular sites, this tendency can be related to the change in speciation of Al with acidity (Martin 1988; Kloppel *et al*. 1990). The influence of water pH on the accumulation of Al in moss was highly significant ($p < 0.001$) for 0 and 0.1 mg/L and significant ($p < 0.05$) for 0.5, 1 and 2 mg/L of Al in the water. Results concerning the effect of concentration of Al in the medium showed clear differences in intracellular Al uptake from 0–0.1 mg/L and up to 0.5 mg/L of Al in the medium (Table 5). From then onward, the differences were less, which may indicate saturation of Al carriers. These differences are highly significant ($p < 0.001$) for the pH 3 and 3.5 and significant ($p < 0.05$) for the other pH.

The kinetics of uptake—and in one case, release—of intracellular Al followed more diverse models than for extracellular locations (Table 6, Figure 3). Most of the fits were made to logarithmic equations (characterized by large accumulations during the first hour of incubation) or linear equations (with a more constant rate of uptake over the entire period of incubation). In some cases, such as where Al was not added to the medium or at pH 3, the fits to the models were not very reliable. No modeling was carried out for the kinetics corresponding to pH 4 and 0 mg/L Al.

The average speed of the process in each case is given by constant *b* (Table 6). It can be seen that the rate of uptake tended to increase with the concentration of Al in the medium and with increasing pH, in the range 3–4.4.

Biomonitoring Models

The data for Al accumulation in moss for each pH in the medium was fitted to equations to model the uptake, as outlined in Figure 4. The models obtained may assist in future determinations of the impact of acidity and metal concentration in the environment. They must first, of course, be improved with more data, as widely differing pH ranges correspond to Al concentrations in mosses that are very similar.

Changes in Cellular Contents of Ca, K, and Mg

Ca, K, and Mg are three elements that are essential to plant life. Ca is found mainly on the cell wall in an exchangeable form, K is found almost entirely at intracellular locations, and Mg is found in differing amounts at intra- and extracellular locations (Bates 1993; Brown 1982; Wells and Brown 1990). During the preincubation period in this study, Cd displaced a large amount of the Ca and Mg from the cell wall, leaving a base extracellular concentration of these elements that was lower than normal.

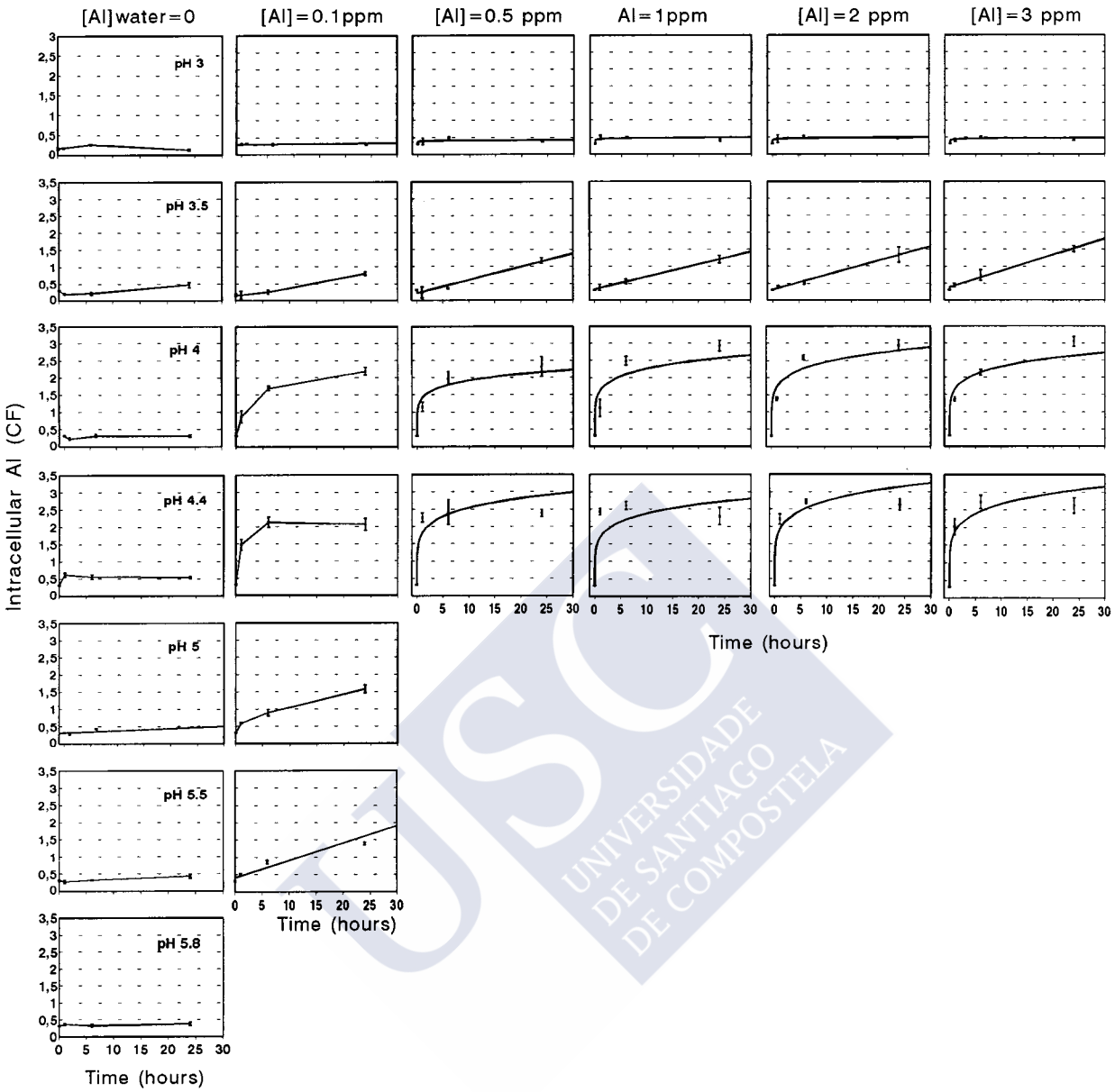

Fig. 3. Kinetics of intracellular Al uptake in *F. antipyretica* as a function of pH and of concentration of Al in the incubation medium (concentration of Al expressed as CF = contamination factors; fitted to Michaelis-Menten equation)

Extracellular Ca. The initial concentration of extracellular Ca was 60 μmol/g DW. Different results were observed after maintaining the moss in solutions of various pH and concentrations of Al. In general, Ca was released from the cell wall in very acidic conditions (pH 3–4.4) (Figure 5) and was accumulated at higher pH (pH 5–5.8). Furthermore, the higher the concentration of Al, the greater was the range of pH at which there was leaching of Ca. The effect of pH is significant ($p < 0.001$) only for the lower concentrations of Al in water (0, 0.1 mg/L). The effect of concentration of Al in the medium is significant only for pH 3 to 4.4 ($p < 0.01$). These findings can be attributed to competition between Ca^{2+}, H^+, and Al^{3+} for anionic binding sites on the cell wall, preference being given to those with higher affinity or those which are present at a higher concentration. In fact, in experiments carried out with different bryophytes, it can be seen that there is sequential affinity for the anionic extracellular sites in the order: Al > H > Ca (Wieder *et al*. 1990; Wells and Brown 1990; Sager 1992).

Thus it is easy to explain how, at pH lower than 5, the high concentrations of proton and of Al in solution, along with their high affinity for anionic extracellular sites, cause release of Ca from the cell wall by cationic exchange. At pH of 5 or higher, the decrease in concentration of H^+, along with the decrease in solubility of Al and the change in its speciation, are sufficient for the Ca present in the water to compete successfully for the ionic binding sites on the cell wall, so there is uptake of this element. Washing out of Ca in acidic conditions has been observed by other authors (Albers and Camardese 1993;

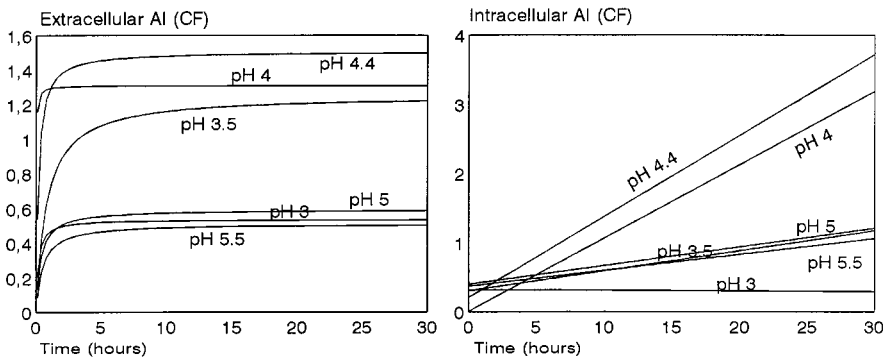

Fig. 4. Models for biomonitoring of episodic impact of acidification, as a function of intra- and extracellular Al uptake and of time of exposure of samples of *F. antipyretica* saturated with Cd (concentration of Al is expressed as CF = contamination factors)

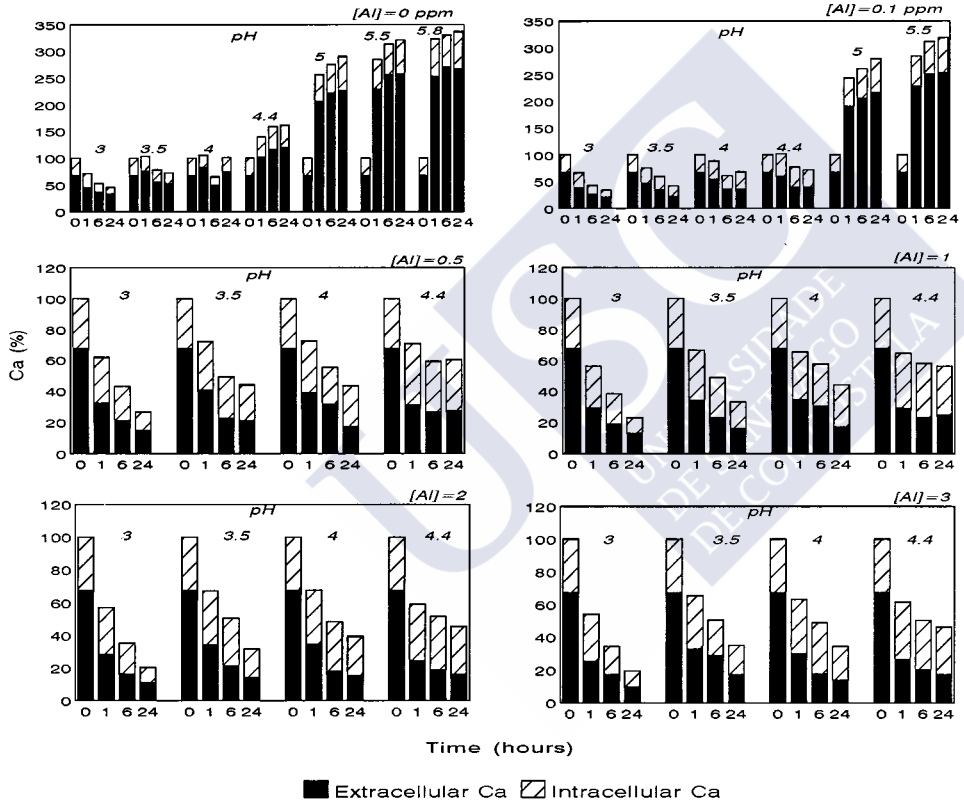

Fig. 5. Changes in the concentration of intra- and extracellular Ca in *F. antipyretica* as a function of pH and concentration of Al (ppm) in the incubation medium (expressed as percentage of initial value)

Wieder *et al*. 1990; Crist *et al*. 1994; Mersch *et al*. 1993; Sager 1992). In the same way, the greater the concentration of Al in solution, the greater the washing out of Ca (Figure 5). The release of extracellular Ca due to cationic exchange with Al is much less than that due to exchange with H^+. In fact, the greatest accumulation of extracellular Al occurs at around pH 4.4 and is not accompanied by greater release of extracellular Ca.

Intracellular Ca. The concentration of Ca inside the cells at the start of incubation in solutions of different pH and Al concentrations was approximately half that of the extracellular concentration.

Similar to what took place at extracellular sites during incubation in solutions of pH close to neutral, there was uptake of Ca by the cell, while at lower pH there was progressively greater leaching of intracellular Ca. This effect of the pH is significant ($p < 0.05$) only for low Al concentrations in the water (0, 0.1 mg/L). This is due to competition between protons and Ca to bind to carriers (Taylor 1988). Transport sites appear to consist of transmembrane proteins with many binding sites for protons, and when this competition occurs, the struc-

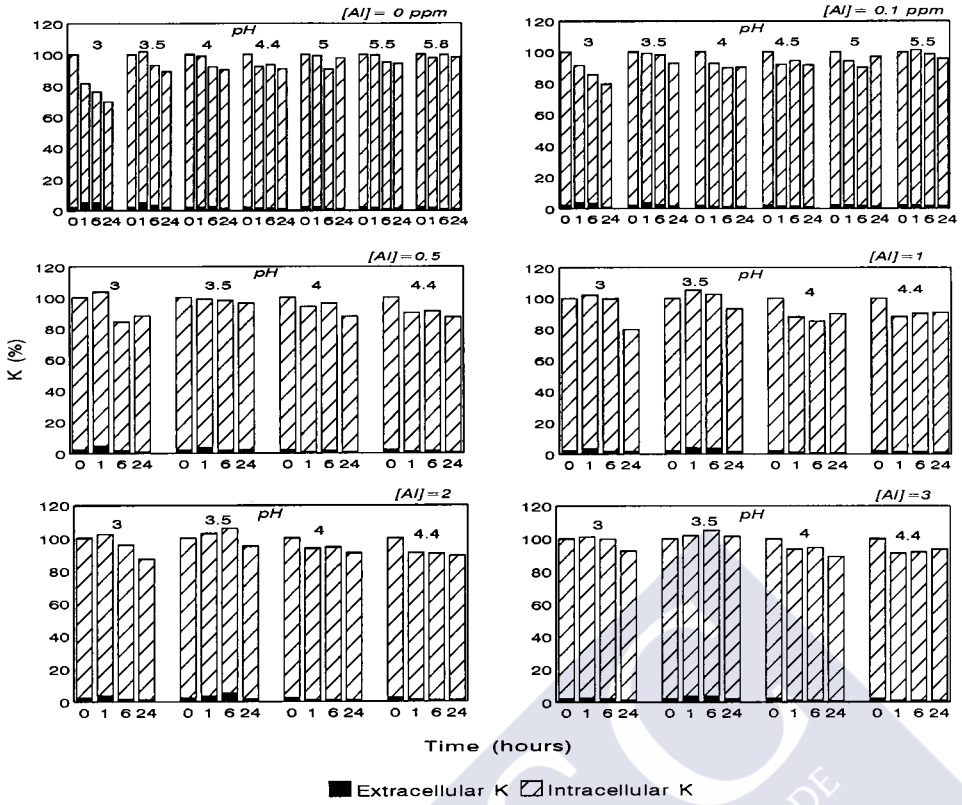

Fig. 6. Changes in the quantity of intra- and extracellular K in *F. antipyretica* as a function of pH and concentration of Al (ppm) in the incubation medium (expressed as percentage of initial value)

ture of the active transport sites changes (Wells and Brown 1990), producing a change in permeability, which leads to loss of Ca.

The concentration of Al in the medium does not appear to interfere with Ca uptake into the cell (Figure 5), the concentrations of intracellular Ca found being very similar for different concentrations of Al at this site (no significant differences were found in all cases). The ionic radius of Al^{3+} is much greater than that of Ca^{2+}, therefore they probably do not employ the same carriers to gain access to the cellular cytoplasm. In fact, Al^{3+} and Fe^{3+} have similar ionic radii, and Al is able to make use of transferrin, the protein carrier of Fe (Martin 1988).

Extracellular K. In this case, the initial concentration was only 8.2 μmol/g DW of extracellular K (Figure 6), with small (not well-understood and not significant) variations occurring throughout the study.

Intracellular K. Initial concentration 363 μmol/g DW intracellular K. There was a loss of K from the moss in all solutions tested (Figure 6), which indicates a change in membrane permeability (Taylor 1988). Greatest losses were always produced in the most acidic solutions. As with Ca, this may be due to binding with H^+, causing changes in the structure of the active transport sites (Wells and Brown 1990). The presence of Al in the medium appears to diminish slightly the leaching of K—this effect being most apparent at the lowest pH, although there was no relation between the concentrations of intracellular Al and K. The effect of the pH or the Al in water was no significant in all cases.

Extracellular Mg. In the moss used in this study the initial concentration was 8 μmol/g DW (Figure 7). The effect of pH and of concentration of Al in the medium on Mg accumulation at this site was almost identical to that registered for Ca and reported in the corresponding section. We only found significant differences ($p < 0.05$) between pH and for the lower concentrations of Al in water (0, 0.1 mg/L). As for Ca, the trends observed were presumably due to competition between cations for exchange sites.

Intracellular Mg. At this location the initial concentration of Mg was 52 μmol/g DW. As with K, the most general trend was a loss of Mg (Figure 7), which became less pronounced as pH increased. This indicates an effect of H^+ on membrane permeability.

The concentration of Al in the water has little influence, although it has been stated that this metal can cause deficiencies in mineral elements, such as Mg, by directly affecting membranes or membrane carriers (Taylor 1988). Magnesium has an ionic radius only slightly greater than that of Al and thus may be able to use the same carrier (Martin 1988). The effect of pH and Al concentration in water was no significant in all cases.

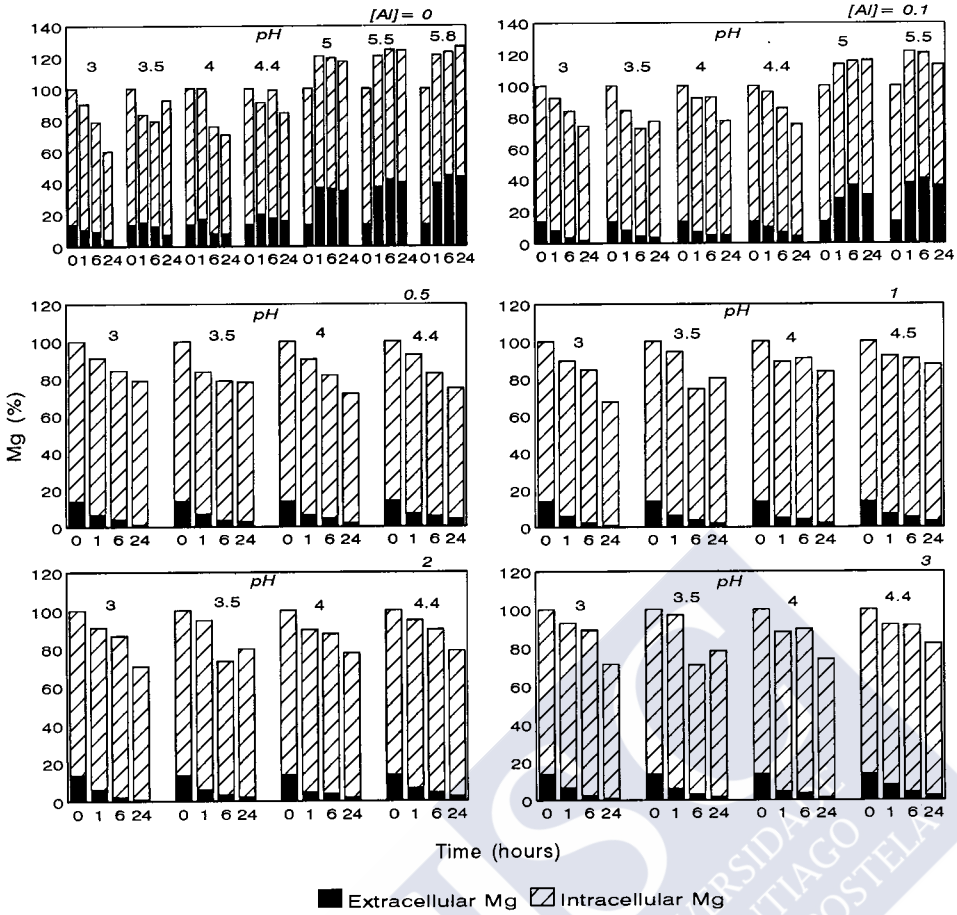

Fig. 7. Changes in the quantity of intra- and extracellular Mg in *F. antipyretica* as a function of pH and concentration of Al (ppm) in the incubation medium (expressed as percentage of initial value)

Conclusions

The most important conclusions were:

1. Accumulation of Al (both extra- and intracellular) in *F. antipyretica* is directly related to the pH of the medium, with the highest accumulation being found at around pH 4.4, and less at either higher or lower pH. The amount of Al in the medium is of secondary importance.
2. The clear-cut, rapid effect of pH on the medium on the intra- and extracellular concentration of Al in *F. antipyretica* suggests that this moss could be used to monitor acidic environments.
3. For this, the uptake models constructed may be useful, but these should be improved to give more reliable results.
4. In very acidic environments (pH less than 5) moss releases extracellular Ca and Mg, as well as intracellular Ca. The effect of Al in solution, at the range of concentrations used in this study, was of much less importance.
5. Intracellular K and Mg were released throughout the entire range of acidity and Al concentration used in the present study, the extent of the effect was directly proportional to the acidity of the medium.
6. Accumulation of Al inside the cell does not appear to cause release of intracellular essential elements (Ca, K, Mg).

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Capítulo 3

Cinéticas de carga de As, Hg, Sb y Se en el musgo acuático Fontinalis antipyretica *Hedw.*

Water Air Soil Pollut (2012) 223:3409–3423
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Uptake Kinetics of As, Hg, Sb, and Se in the Aquatic Moss *Fontinalis antipyretica* Hedw

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Abstract Laboratory experiments were carried out to study the uptake kinetics of selected metals and metalloids in the aquatic moss *Fontinalis antipyretica*. For this purpose, moss specimens from a clean site were exposed to concentrations of As, Hg, Sb, and Se ranging from 0.1 to 10,000 μg l^{-1}, for incubation times of between 1 and 22 days, and the tissue concentrations of the metals in the moss specimens were then measured. Uptake kinetics followed different patterns in relation to exposure time, although the most common was Michaelis–Menten kinetics. On the contrary, the contamination factors followed very similar patterns in relation to the exposure concentrations in all cases, with a good fit to logarithmic equations. The bioconcentration factors tended to decrease as exposure concentration increased. The bioconcentration factors for Hg were extremely high, even at the lowest concentration in water and for the shortest incubation time, which implies that *F. antipyretica* has a high capacity to magnify Hg levels in water, which is an important characteristic in a good biomonitor. According to the time to reach equilibrium, the minimum exposure time recommended for use in active biomonitoring by means of transplants is very variable, although high levels of the elements, except Sb, were found in the moss tissues within a few days. We do not recommend the use of this moss species to biomonitor low concentrations of Sb in water. The differences in maximum contamination factors and lowest bioconcentration factors suggest that As and Se were the most toxic of the elements under study.

Keywords Aquatic moss · Bryophytes · *Fontinalis antipyretica* · Metals · Metalloids · Uptake

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1 Introduction

Bryophytes are considered excellent accumulation indicators of metals and other contaminants (Zechmeister et al. 2003), and *Fontinalis antipyretica* is one of the species most commonly used to biomonitor aquatic environments (e.g., Fernández et al. 2006; Pekka et al. 2008; Rasmussen and Andersen 1999; Roeck et al. 1995). For a better understanding of biomonitoring studies, many experiments with aquatic bryophytes have been carried out to investigate the metal uptake kinetics (e.g., Cesa et al. 2008; Claveri et al. 1994; Ferreira et al. 2009; López et al. 1994; Martins and Boaventura 2002). However, very few studies have addressed the uptake kinetics of the elements considered here. With regard to Hg, Samecka-Cymerman and

Kempers (1995) studied the uptake kinetics with the liverwort *Scapania undulata*; Cesa et al. (2008) with the moss *Rhynchostegium riparioides*, and Cenci (2000) with *F. antypiretica*, the same species as used here, although the samples were only exposed to a single concentration. As far as we know, no studies on the uptake kinetics of As, Sb, and Se have been carried out with aquatic bryophytes.

Mercury, As, and Se have been widely studied as they are of environmental concern (Mandal and Suzuki 2002; Simmons and Wallschläger 2005; Von Burg and Greenwood 1991). However, Sb has received little attention (Filella et al. 2002; Maher 2009), although the number of studies on this element has increased in recent years (Filella et al. 2009). Because of the general lack of data on the uptake kinetics of the aforementioned elements in aquatic bryophytes, the main objective of this study was to obtain information about the bioconcentration patterns of As, Hg, Sb, and Se in *F. antipyretica*.

2 Materials and Methods

The moss used in the experiments was collected from a clean site in the upper stretches of the river Lérez (Galicia, NW Spain). Moss mats were manually collected at a depth that was low enough to avoid specimens that may have suffered from hydric stress due to exposure to the air (Wehr et al. 1983). Samples were rinsed in situ and transported in river water in cool boxes. Once in the laboratory, the moss samples were washed with distilled water. Only apical tips (2 cm) were used in the experiments (Wells and Brown 1990) to minimize any errors due to the different accumulation capacities of the different parts of the plant (Wehr et al. 1983).

Incubations were performed in glass beakers with 5 l of each of the different dilutions 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 containing 1,000 mg l^{-1} of the element under consideration (Merck and Panreac: As_2O_5, $Hg(NO_3)_2$, $SbCl_3$, and SeO_2, respectively) in tap water (the characteristics of which are shown in Table 1) filtered through an activated carbon filter. Incubations were carried out at 16±0.1°C, 80 μmol photons m^{-2} s^{-1} of photosynthetically active radiation and a photoperiod 12:12 of L/D. The water was aerated continuously by means of an air pump and changed every 5 days. The exposure times were 1, 2, 4, 7, 11, 16, and 22 days.

Table 1 Characteristics of the water used in the experiments

Parameter	Unit	
pH		7.1
Conductivity	μS cm^{-1}	100
As	μg l^{-1}	<2
Hg	μg l^{-1}	<0.2
Sb	μg l^{-1}	<2
Se	μg l^{-1}	<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

Source: Town Council of Santiago de Compostela

The concentrations of elements in the moss were determined in 300±5-mg tissue samples, digested with nitric acid at high temperature and high pressure, as previously described (López and Carballeira 1993; Wehr et al 1983). The concentrations of the elements were determined by atomic fluorescence spectroscopy (As, Sb and Se: PSA Excalibur, and Hg: PSA Merlin Plus). Quality control was carried out by parallel analysis of certified reference material of the aquatic moss *Platihypnidium riparioides* (BCR-61) from the Community Bureau of Reference.

The concentrations of the different elements were transformed into contamination factors (CF) for data analysis and graphic representation. The CF is the ratio between the concentration of an element in a sample from a site in a river and the reference concentration from a clean site upstream of the pollution point (André and Lascombe 1987). In the present study, the CFs were calculated by dividing the tissue concentration of each element in each incubation time by the tissue concentration in the control at the same time. This enables comparison of the data and models for the different metals and correction of the possible bioconcentration of the low levels of elements that may be present in the tap water (Vázquez et al. 2000; Fernández et al. 2006).

The kinetics of uptake were fitted with different equations. In aquatic bryophytes, the uptake kinetics often fit well to the Michaelis–Menten equation

frequently used in enzyme kinetics studies (Brown and Beckett 1985; Cenci 2000; Fernández et al. 2006; Vázquez et al. 2000). For this reason, we initially fitted a version of this equation (1) to the data, where CF represents the contamination factor reached in a given time, CF_{max} is the maximum contamination factor, K_m the time taken to reach half of the maximum contamination factor, and t is the time in days.

$$CF = \frac{CF_{max} \times t}{K_m + t} \quad (1)$$

The fits were carried out with the aid of SPSS® version 15.0, by means of nonlinear regression, and with sequential quadratic programming as the estimation method. As the minimum value of the contamination factors is always 1, the calculations were performed by subtracting 1 from all CF values so that the fit started at 0. Otherwise, the program would produce erroneous estimations of K_m, especially for low CF_{max} values. At the end of the procedure, the CFs were corrected by adding 1 to each of the values obtained.

When the fit to Eq. (1) was not highly significant (i.e., the fit was only significant at $p>0.01$), other fits were tested by means of linear regression (Eq. 2), and different regression models were used for curve estimation (logarithmic, Eq. 3; inverse, Eq. 4; exponential, Eq. 5; logistic, Eq. 6; power, Eq. 7; S, Eq. 8), also with the aid of SPSS® version 15.0. The model finally chosen in each case was the model that provided the best fit (i.e., at the highest significance level; Table 2).

$$CF = a + b \times t \quad (2)$$

$$CF = a + b \times \ln(t+1) \quad (3)$$

$$CF = a + \frac{b}{t+1} \quad (4)$$

$$CF = a \times e^{b \times t} \quad (5)$$

$$CF = \frac{1}{\frac{1}{u} + a \times b^t} \quad (6)$$

where u is the upper bound value

$$\ln CF = \ln a + b \times \ln(t+1) \quad (7)$$

$$\ln CF = a + \frac{b}{t+1} \quad (8)$$

Other parameters calculated to complete the information on uptake kinetics were the time to equilibrium (T_{eq}) calculated in the regression equation used in each case, as the exposure time beyond which the daily rate of increase in CF was less than 1%. The equilibrium contamination factor (CF_{eq}) was estimated as the predicted value of CF at T_{eq} (López et al. 1994). The mean velocity of uptake (V_c) was calculated by dividing CF_{eq} by T_{eq}.

3 Results and Discussion

The initial concentrations of As, Hg, Sb, and Se in the moss collected for the experiments (Table 3) were similar to or lower than those reported for aquatic mosses from clean sites or concentrations cited as background levels (Carter and Porter 1997; Cesa et al. 2010; Culioli et al. 2009; Gapeeva et al. 2010; Nimis et al. 2002; Pekka et al. 2008; Roeck et al. 1995; SEPA 2000).

As regards the uptake kinetics, the fit to the Michaelis–Menten equation (Eq. 1) was adequate in many cases (Table 4, Figs. 1, 2, 3, and 4). Of the different models tested for the data for which this equation did not provide a good fit, in four cases a good linear fit was found (Eq. 2); in two cases, the most appropriate model was a logarithmic equation (Eq. 3; Table 5).

In the six remaining cases, none of the models tested was suitable because of a large decrease in the contamination factors after the first days of incubation; for this reason, only the initial points were fitted. In three instances, a linear equation fitted well to these points, and in the other three, an inverse equation was adequate (Eq. 4).

In general, bioconcentration was rapid in the first 2 to 4 days of exposure. Arsenic and Se followed Michaelis–Menten kinetics most closely, although this model did not provide a good fit for the highest exposure concentration because of a decrease in the CF values after an initial increase. The concentration of As increased in moss until a maximum CF of almost 300 was reached on the second day of exposure, whereas the maximum CF for Se, 631, was reached on the first day of exposure. In both cases, the maximum values were followed by a decrease in the CF over time. Fernández et al. (2006) found that uptake of large quantities of metals by *F. antipyretica*

Table 2 Regression models tested in those cases in which the Michaelis–Menten did not provide a satisfactory fit to the uptake kinetics data

Element	Concentration (μg l^{-1})	Model	R^2	F	df 1	df 2	Significance
As	10,000	Linear	0.859	6.111	1	1	0.2447
		Exponential	0.771	3.373	1	1	0.3174
		Logistic	0.823	4.640	1	1	0.2767
		Logarithmic	0.946	17.55	1	1	0.1492
		Inverse	0.989	92.94	1	1	0.0658
		Power	0.883	7.572	1	1	0.2219
		S	0.953	20.46	1	1	0.1385
Hg	1	Linear	0.872	40.78	1	6	0.0007
		Exponential	0.369	3.503	1	6	0.1104
		Logistic	0.567	7.848	1	6	0.0311
		Logarithmic	0.639	10.60	1	6	0.0173
		Inverse	0.351	3.239	1	6	0.1220
		Power	0.639	10.61	1	6	0.0173
		S	0.865	38.65	1	6	0.0008
	10	Linear	0.920	69.31	1	6	0.0002
		Exponential	0.264	2.151	1	6	0.1928
		Logistic	0.454	4.989	1	6	0.0669
		Logarithmic	0.971	202.8	1	6	0.0000
		Inverse	0.758	18.75	1	6	0.0049
		Power	0.545	7.193	1	6	0.0364
		S	0.864	38.16	1	6	0.0008
	100	Linear	0.816	26.64	1	6	0.0021
		Exponential	0.339	3.084	1	6	0.1296
		Logistic	0.466	5.226	1	6	0.0623
		Logarithmic	0.864	38.15	1	6	0.0008
		Inverse	0.606	9.216	1	6	0.0229
		Power	0.655	11.42	1	6	0.0149
		S	0.860	36.96	1	6	0.0009
	1,000	Linear	0.952	120.1	1	6	0.0000
		Exponential	0.295	2.515	1	6	0.1639
		Logistic	0.441	4.738	1	6	0.0724
		Logarithmic	0.896	51.52	1	6	0.0004
		Inverse	0.607	9.273	1	6	0.0227
		Power	0.597	8.881	1	6	0.0246
		S	0.910	60.70	1	6	0.0002
	10,000	Linear	0.817	8.938	1	2	0.0960
		Exponential	0.495	1.963	1	2	0.2962
		Logistic	0.564	2.590	1	2	0.2488
		Logarithmic	0.960	48.31	1	2	0.0201
		Inverse	0.987	153.3	1	2	0.0065
		Power	0.723	5.227	1	2	0.1495
		S	0.897	17.43	1	2	0.0529
Sb	0.1	Linear	1.000	–	1	0	–
	1	Linear	0.780	3.554	1	1	0.3105

Table 2 (continued)

Element	Concentration (μg l^{-1})	Model	R^2	F	df 1	df 2	Significance
		Exponential	0.766	3.268	1	2	0.3217
		Logistic	0.832	4.963	1	1	0.2686
		Logarithmic	0.890	8.111	1	1	0.2150
		Inverse	0.958	22.74	1	1	0.1316
		Power	0.879	7.267	1	1	0.2261
		S	0.951	19.23	1	1	0.1428
	10	Linear	0.986	140.3	1	2	0.0071
		Exponential	0.871	13.55	1	2	0.0665
		Logistic	0.980	97.91	1	2	0.0101
		Logarithmic	0.890	16.12	1	2	0.0568
		Inverse	0.720	5.135	1	2	0.1517
		Power	0.979	91.88	1	2	0.0107
		S	0.981	104.1	1	2	0.0095
	100	Linear	0.946	105.8	1	6	0.0000
		Exponential	0.655	11.40	1	6	0.0149
		Logistic	0.916	65.67	1	6	0.0002
		Logarithmic	0.910	60.72	1	6	0.0002
		Inverse	0.593	8.745	1	6	0.0254
		Power	0.926	74.97	1	6	0.0001
		S	0.927	77.32	1	6	0.0001
	10,000	Linear	0.609	9.336	1	6	0.0224
		Exponential	0.295	2.508	1	6	0.1644
		Logistic	0.385	3.762	1	6	0.1005
		Logarithmic	0.532	6.827	1	6	0.0400
		Inverse	0.341	3.098	1	6	0.1289
		Power	0.551	7.374	1	6	0.0349
		S	0.606	9.222	1	6	0.0229
Se	10,000	Linear	1.000	–	1	0	–

may lead to a posterior release of the metals. However, another possible explanation for this behavior is that, given the high concentration of exposure at which this occurred (10,000 μg l^{-1}), it is possible that the initial increase had taken place in dead moss (Pickering and Puia (1969) reported an increase in Zn uptake in

Table 3 Values of selected variables for incubations of *F. antipyretica*

		As	Hg	Sb	Se
Tissue concentration	Initial (ng g^{-1})	1,680	0.978	199	483
	Maximum (μg g^{-1})	399	57,466	31,888	497
CF	Maximum	293	134×10^5	46,661	631
BCF	Minimum	6.15	311	16.33	28.65
	Maximum	12,056	610,437	17,887	6,393

CF contamination factor, *BCF* bioconcentration factor

Table 4 Uptake kinetics of the different elements accumulated in samples of *F. antipyretica* exposed to different concentrations of the elements in water

Element		Concentration in water (μg l^{-1})					
		0.1	1	10	100	1,000	10,000
As	CF_{max}	2.15	2.58	13.49	76.77	277.9	
	K_m	5.57	1.62	1.89	1.05	0.482	
	K_m/CF_{max}	2.59	0.628	0.140	0.014	0.002	
	r^2	0.881**	0.854*	0.950**	0.932**	0.978**	
Hg	CF_{max}	12039					
	K_m	2.61					
	K_m/CF_{max}	0.0002					
	r^2	0.932**					
Sb	CF_{max}					3,245	
	K_m					0.318	
	K_m/CF_{max}					0.0001	
	r^2					0.795*	
Se	CF_{max}	1.67	2.46	7.48	55.8	424.5	
	K_m	3.92	0.807	1.27	0.657	1.33	
	K_m/CF_{max}	2.35	0.328	0.170	0.012	0.003	
	r^2	0.829*	0.952**	0.949**	0.956**	0.862**	

The fits correspond to a Michaelis–Menten type equation: $CF=(CF_{max}\times t)/(K_m+t)$

CF contamination factor reached in a given time, CF_{max} maximum contamination factor, K_m time to reach half of the value of CF_{max}, *t* time (in days)

*$p<0.01$; **$p<0.001$

freshly killed *F. antipyretica*) and that the subsequent decrease was due to the gradual deterioration of the dead moss. In fact, the samples exposed to the highest concentrations appeared very damaged by the end of the study period.

For Hg, the fit to the Michaelis–Menten equation was only adequate for the lowest exposure concentration; good fits were obtained with linear and logarithmic equations for the remaining exposure concentrations (Table 5), and therefore, no clear trend was observed. For the highest exposure concentration, there was a decrease in the CFs, as was the case with As and Se although not as pronounced for the CFs for the longest incubation times (Fig. 2). Although the uptake was very variable in relation to the different exposure concentrations, for Hg and all exposure concentrations, the CFs reached from the first exposure time were the highest of all the elements studied, which is an important characteristic in a good biomonitor.

The patterns observed for Sb were the most erratic. For the lowest exposure concentrations, unlike the other elements, the CFs decreased over time after an initial increase, which suggests that *F. antypiretica* may not be suitable as a biomonitor for low concentrations of Sb in water. For the highest exposure concentrations, it was possible to fit the whole data set, and a good linear fit was obtained in two cases, and a Michaelis–Menten one in the other case.

In the modified Michaelis–Menten equation, K_m represents the time taken to reach half of the maximum contamination factor. For As, it almost always tended to decrease with increasing exposure concentration (Table 4), which implies a steeper slope in the initial part of the curve, and therefore less time to reach equilibrium. Consequently, in a hypothetical biomonitoring study with transplanted moss samples in a river strongly contaminated with As, the exposure time could be very short (e.g., a value equal to half of the CF_{max} is reached in approximately 1 day for an exposure concentration of 100 μg l^{-1}). For Se, K_m was clearly higher for the lowest exposure concentration, after which there was no progressive decrease as the

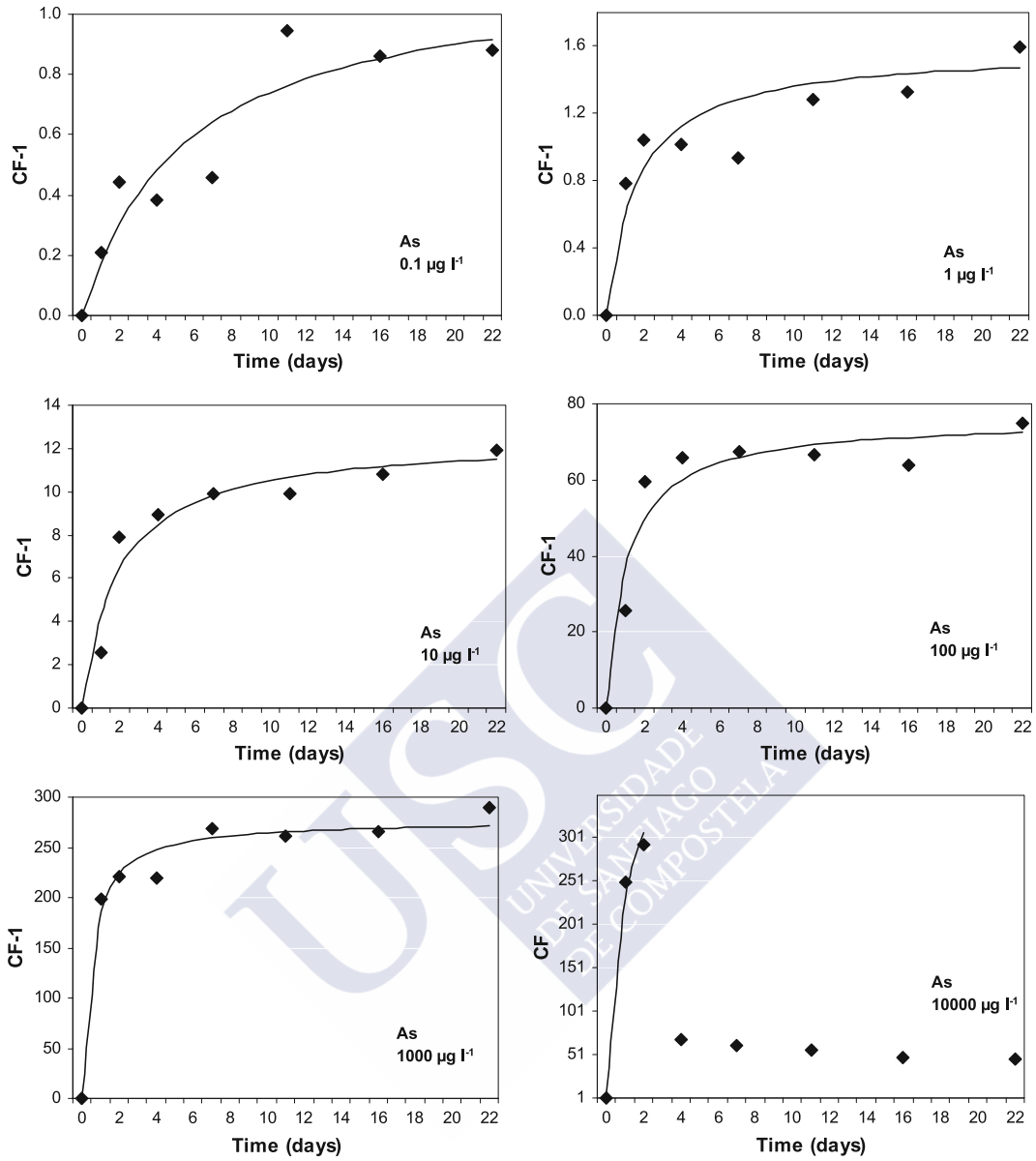

Fig. 1 Kinetics of As uptake in *F. antipyretica* at different concentrations of As in water (*CF* contamination factor)

exposure concentration increased. For the other two elements, the Michaelis–Menten equation only provided an adequate fit to the data corresponding to one exposure concentration.

Taking the reciprocal of both sides of the Michaelis–Menten equation, a linear function whose slope is K_m/CF_{max} was obtained; the slope may resemble the rate of uptake (inverted). For As and Se, the values of this ratio decreased gradually (Table 4), which implies a higher rate of uptake as the exposure concentration increased. This ratio was similar for both elements for each exposure concentration. For the first concentration, for which there was also a fit with Hg, a much lower value for this element was observed, thus implying a high affinity of the moss for Hg. For Hg and Sb, for which other equations provided better fits, the value of the constant "*b*," which represents the slope of the fit, and thus the uptake rate, tended to increase gradually as the exposure concentration

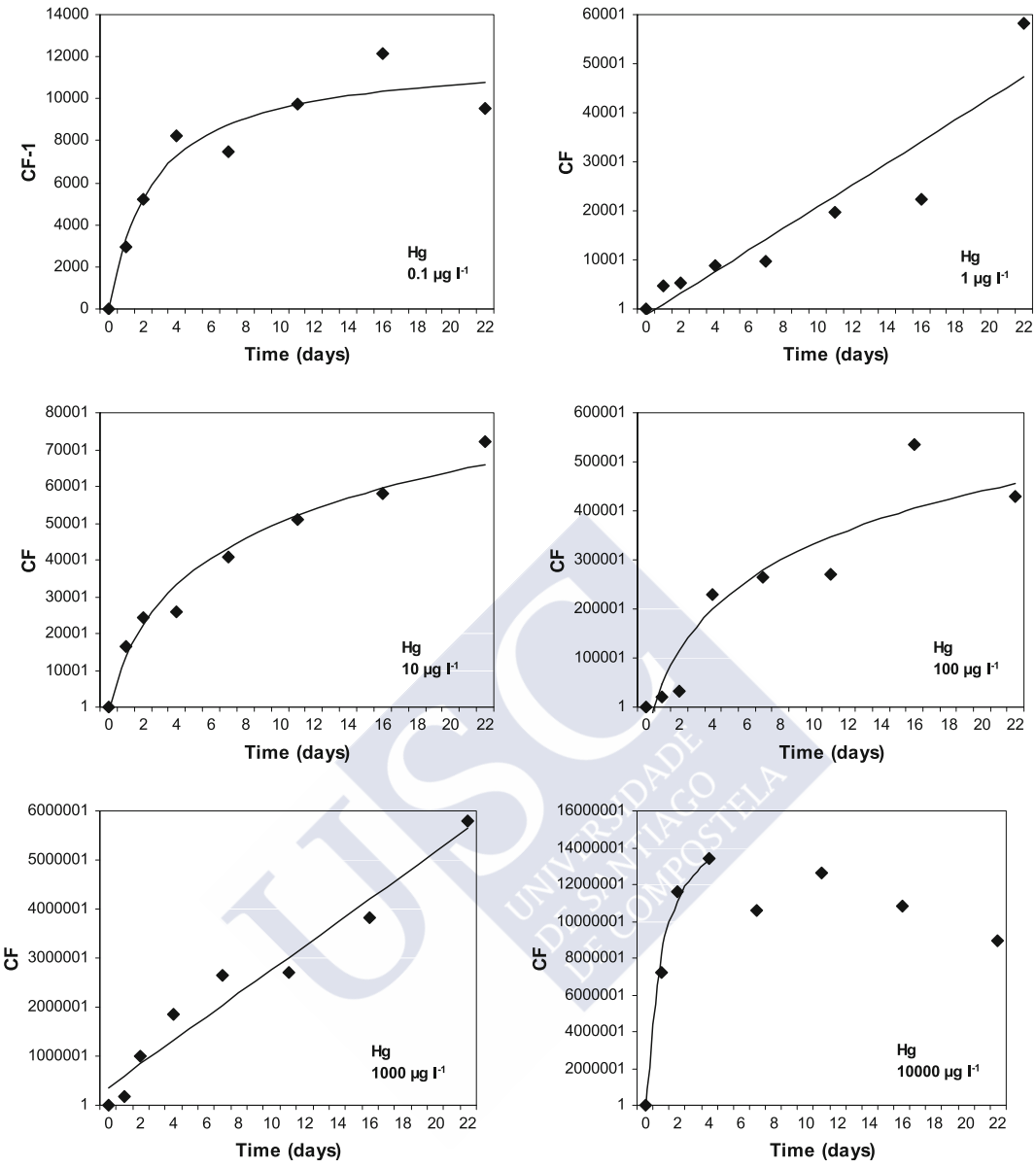

Fig. 2 Kinetics of Hg uptake in *F. antipyretica* at different concentrations of Hg in water (*CF* contamination factor)

increased (in inverse equations with negative sign; Table 5). In these cases, the values of *b* for Hg were clearly higher than for Sb. In summary, the affinity of *F. antipyretica* for the studied elements was: Hg>Sb>As≈Se.

The times to equilibrium (T_{eq}) follow a similar pattern to K_m, but also enable comparison of cases fitted by models other than the Michaelis–Menten model, except for the linear fit. For Se and particularly As, this value tended to decrease gradually as the exposure concentration increased (Table 6). Mercury did not follow a clear pattern in this aspect, whereas for Sb, T_{eq} appeared to increase as the exposure concentration increased, the lowest values of this parameter corresponded to this element. There were large differences depending on the element and the exposure concentration, so that in a transplant experiment, the recommended exposure time would range from a few days to 1 month. The equilibrium contamination factor (CF_{eq}) always increased as the exposure

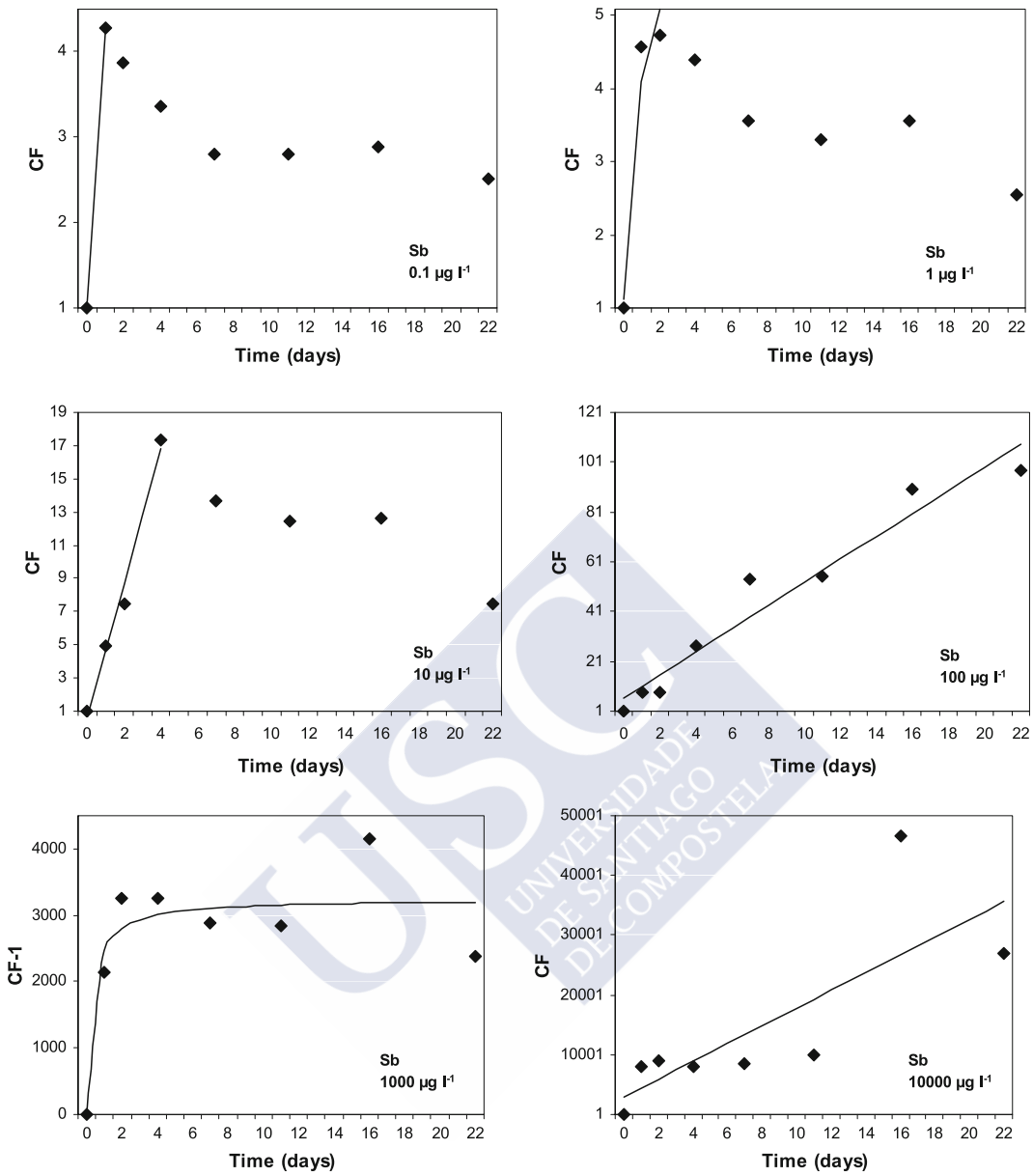

Fig. 3 Kinetics of Sb uptake in *F. antipyretica* at different concentrations of Sb in water (*CF* contamination factor)

concentration increased, whereas the mean uptake rate (V_c) followed the same pattern, except in one case.

For each incubation time, the CFs reached as a function of the concentration of each element in water are shown in Fig. 5. Because of the logarithmic scale of the concentrations used in the experiments, for accurate interpretation of the results, the data on the *x*-axis should be plotted on the same scale. The large differences in the exposure concentrations used also led to large differences in the CFs reached, so that the *y*-axis was also plotted on a logarithmic scale. A logarithmic fit according to Eq. (9) was adequate for these data. In the case of As, the points corresponding to the highest exposure concentrations and to incubation times of between 4 and 22 days decreased clearly with respect to the increasing trend in the previous values, and were therefore excluded from the regression analysis.

$$\log_{10}\mathrm{CF} = a + b \times \log_{10}\text{ exposure concentration} \quad (9)$$

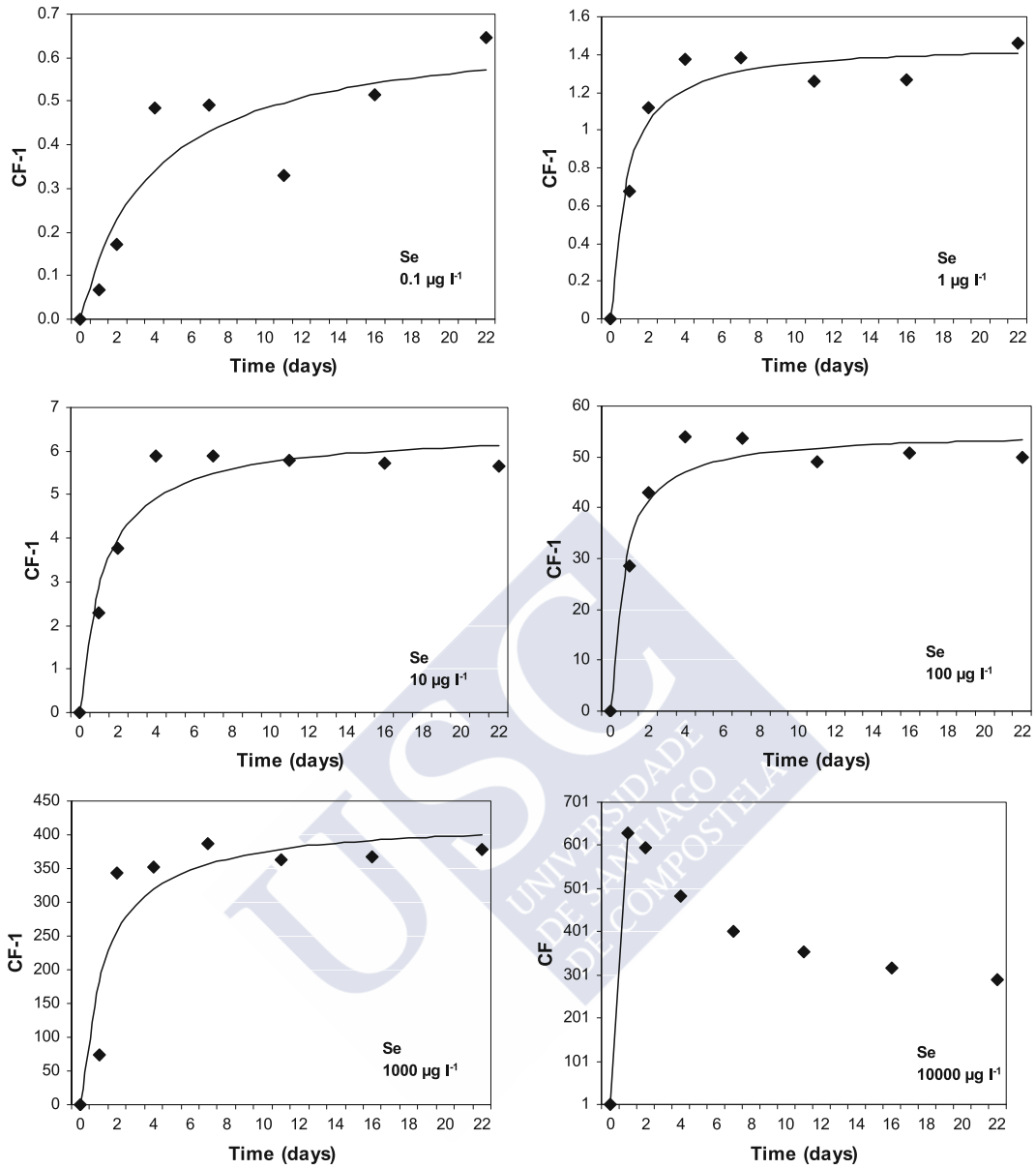

Fig. 4 Kinetics of Se uptake in *F. antipyretica* at different concentrations of Se in water (*CF* contamination factor)

This equation provided significant fits for all elements and for all incubation times. The values of the slope (constant *b* in Table 7) were very similar for all concentrations of each element. For As, they ranged from a minimum of 0.528 and a maximum of 0.608; for Hg, they ranged from 0.618 and 0.685; 0.703 and 0.889 for Sb; and 0.534 and 0.603 for Se. Therefore, the same type of equation provided a satisfactory fit to the points in all cases, in clear contrast with what we have discussed in regard to the curves shown in Figs. 1, 2, 3, and 4, in which depending on the element or the exposure concentration, the changes in the CFs throughout the incubation time were often very different.

The previously mentioned different affinities of the moss for the four elements (as observed by the different uptake rates) (Hg>Sb>As≈Se) may be partly due to different degrees of toxicity of these elements to the moss. The maximum CFs were lower for As (Table 3), which was the element for which the decrease in the

Table 5 Uptake kinetics of the different elements accumulated in *F. antipyretica* exposed in laboratory experiments to different concentrations in water

Element		Concentration in water (μg l^{-1})					
		0.1	1	10	100	1,000	10,000
As	*a*						458[c,d]
	b						−452
	r^2						0.989ns
Hg	*a*		−1,259[a]	−1,252[b]	−68,850[b]	362,000[a]	167×10^5 [c,d]
	b		2,209	21,420	167,200	240,200	-169×10^5
	r^2		0.872***	0.971***	0.864***	0.952***	0.987**
Sb	*a*	1.000[a,d]	7.07[c,d]	0.602[a,d]	6.38[a]		2,973[a]
	b	3.28	−5.95	4.04	4.63		1,482
	r^2	1.000	0.958ns	0.986**	0.946***		0.609*
Se	*a*						1.000[a,d]
	b						630
	r^2						1.000

The fits correspond either to a linear: $CF=a+b\times t$; logarithmic: $CF=a+b\times\ln(t+1)$, or to a inverse equation: $CF=a+b/(t+1)$

CF contamination factor reached in a given time; *a* and *b* constants, *t* time (in days), *ns* not significant

*$p<0.05$; **$p<0.01$; ***$p<0.001$

[a] Fitted to a linear equation

[b] Fitted to a logarithmic equation

[c] Fitted to a inverse equation

[d] Only the initial points were fitted

Table 6 Time to reach equilibrium (T_{eq}, in days), equilibrium concentrations (CF_{eq}, in contamination factors), and mean uptake rate (V_c, in contamination factors/day) for different elements accumulated in samples of *F. antipyretica* exposed in laboratory experiments to different concentrations of the elements in water

Element		Concentration in water (μg l^{-1})					
		0.1	1	10	100	1,000	10,000
As	T_{eq}	21	12	13	10	7	2[a]
	CF_{eq}	1.70	2.27	11.78	69.5	260	307
	V_c	0.081	0.189	0.906	6.95	35.2	154
Hg	T_{eq}	15		29	31		4[a]
	CF_{eq}	10,250	–	71,600	511×10^3	–	133×10^5
	V_c	684		2,470	16,480		333×10^4
Sb	T_{eq}	1[a]	2[a]	4[a]		5	
	CF_{eq}	4.28	5.08	16.78	–	3,051	–
	V_c	4.28	2.54	4.19		610	
Se	T_{eq}	18	9	11	8	11	1[a]
	CF_{eq}	1.37	2.26	6.70	51.6	379	631
	V_c	0.076	0.251	0.609	6.45	34.5	631

These parameters were not calculated in the cases of a linear fit

[a] Only initial points were fitted

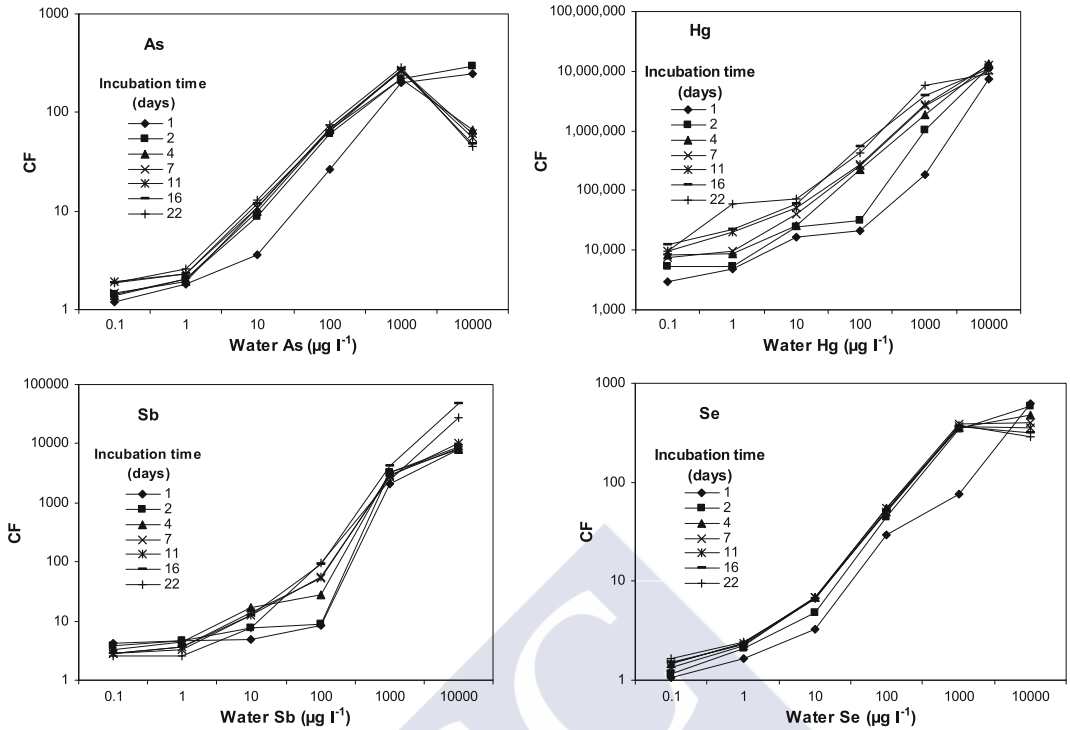

Fig. 5 Contamination factors (*CF*) obtained as function of the exposure concentration of each element

CF values for the highest exposure concentration was sharpest after the initial uptake; the decrease was slightly greater at longer incubation times (Figs.1 and 5). This was also the element for which exposure at the highest concentrations caused the most deteriorated appearance of the moss at the end of the study period.

Table 7 Constants obtained in fitting a logarithmic equation ($\log_{10}CF = a + b \log_{10}$ water concentration) to the plots of contamination factors vs. exposure concentrations (Fig. 5)

Element		Incubation time (days)						
		1	2	4	7	11	16	22
As	*a*	0.372	0.544	0.530	0.543	0.614	0.614	0.644
	b	0.531	0.528	0.593	0.608	0.573	0.576	0.585
	r^2	0.940**	0.960***	0.967**	0.963**	0.953**	0.960**	0.966**
Hg	*a*	3.70	3.89	4.14	4.20	4.35	4.46	4.56
	b	0.624	0.677	0.685	0.682	0.648	0.640	0.618
	r^2	0.863**	0.880**	0.942**	0.963***	0.966***	0.968***	0.957***
Sb	*a*	0.637	0.669	0.775	0.731	0.710	0.725	0.619
	b	0.703	0.726	0.735	0.764	0.778	0.889	0.862
	r^2	0.762*	0.776*	0.879**	0.921**	0.919**	0.921**	0.921**
Se	*a*	0.305	0.433	0.536	0.543	0.512	0.538	0.563
	b	0.565	0.603	0.571	0.562	0.560	0.546	0.534
	r^2	0.951***	0.957***	0.958***	0.948**	0.949***	0.938**	0.930**

*$p<0.05$; **$p<0.01$; ***$p<0.001$

Selenium was the next with regard to minimum CFs, in this case there was also a decrease, although not as drastic for the longest incubation times for a concentration of 10,000 μg l^{-1} of Se in water (Figs. 4 and 5). For this concentration, as was the case with As, but in this case more clearly, the CFs decreased with increasing incubation time. The highest CFs for Sb, and above all for Hg, together with the fact that there was no large decrease in these values at higher exposure concentrations, suggests lower toxicity of both elements to *F. antipyretica*. The same order as for the CFs (As<Se<Sb<Hg) also occurred for the maximum tissue concentrations and for the maximum bioconcentration factors (Table 3). It is possible that the elements for which the moss showed the highest uptake capacity were preferentially accumulated in extracellular compartments and thus not in direct contact with the cytoplasm. These elements would therefore not have an immediate effect on the moss metabolism (Sidhu and Brown 1996).

The bioconcentration factor (BCF) can be defined as the ratio between the concentration of a contaminant in an organism and its concentration in the ambient medium (Walker et al. 2006). In a study of uptake kinetics in *F. antipyretica*, Gonçalves and Boaventura (1998) subtracted the initial concentration (prior to exposure) from the final concentration reached in the moss samples because even when samples are growing in uncontaminated locations, there will always be a background concentration of elements that should be taken into consideration. In the present study, as the concentration of the elements under study sometimes increased slightly in the control samples throughout the exposure period, this was corrected by application of Eq. (10), based on that used by Gonçalves and Boaventura (1998) to calculate the BCF, where C is the concentration of the element in a sample after a certain time of exposure to a given concentration in water; C_c is the concentration of that element in the control after the same time of incubation, and C_w is the concentration of the element in the water.

$$\mathrm{BCF} = \frac{C - C_c}{C_w} \tag{10}$$

The BCFs tended to decrease with increasing concentration at which mosses were exposed (Fig. 6). This has also been observed for *F. antipyretica* by Gonçalves and Boaventura (1998) and by Martins

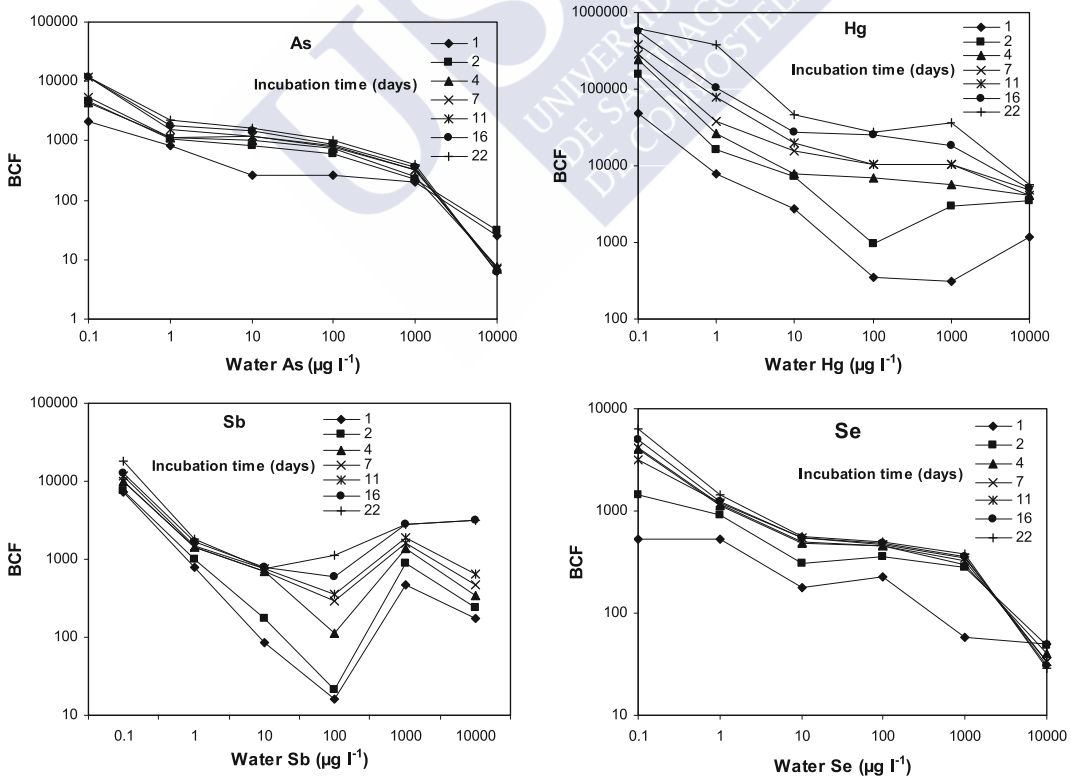

Fig. 6 Bioconcentration factors (*BCF*) obtained as function of the exposure concentration of each element

and Boaventura (2002) for Cu and Zn, respectively, and for *R. riparioides* by Cesa et al. (2008) for different trace elements, including As and Hg. This trend was clearer for As, Hg, and Se; Sb differed slightly, with the lowest values for almost all incubation times at 100 μg l^{-1}, with a subsequent increase, then a further decrease at the highest exposure concentration. The gradual decrease in the BCFs implies a lower sensitivity of *F. antipyretica* as regards detecting higher levels of pollution in water. However, it should be noted that the highest concentrations to which the mosses were exposed in this study would hardly ever be reached in rivers, except in cases of extreme contamination. The data reported by Koch et al. (1999) enabled us to calculate a BCF of 823 for As in the moss *Funaria hygrometrica* growing in hot springs with water concentrations of 288 μg l^{-1} of this metalloid. This is a very similar value to the BCFs obtained in the present study for the closest exposure concentration used (100 μg l^{-1}).

The maximum BCFs for Hg were remarkable and clearly higher (both minimum and maximum) than the other elements studied (Table 3). This demonstrates the high capacity of *F. antipyretica* to magnify the Hg levels in water, which is an important trait for a species to be a good biomonitor. For example, after only 1 day of exposure to the lowest water concentration (0.1 μg l^{-1}), the concentration in moss increased from 0.978 to 4,860 ng g^{-1}, while the control for the same time period reached 1.65 ng g^{-1}. Cesa et al. (2009) reported BCFs for Hg in *R. riparioides* of the same order of magnitude as those found here. The lowest BCFs for As and Se were found for the maximum incubation time, which supports the previously expressed idea that these elements appear to be the most toxic to the moss. For Sb and Hg, the lowest BCFs were found after the lowest incubation time, which is reasonable to expect in elements with a supposedly lower toxicity. Although toxicity cannot be assessed properly from a kinetic uptake study, these results are consistent with those of Díaz et al. (submitted), which reported a lower toxicity (based on net photosynthesis) of tissue Sb and Hg with respect to As and Se for *F. antipyretica*.

4 Conclusions

The uptake kinetics followed different patterns depending on the element and the exposure concentration, although in half of the cases they followed a Michaelis–Menten-type pattern. Arsenic and Se displayed the most homogeneous loading patterns, whereas Sb displayed very irregular behavior. On the contrary, the plots of the contamination factors against the exposure concentrations followed the same pattern, and a logarithmic equation provided a good fit to the data. The bioconcentration factors also followed similar patterns and tended to decrease as the exposure concentration increased.

Despite the different responses in the bioconcentration and the different equilibrium times, high bioconcentration factor values were generally reached in the mosses within a few days of exposure, particularly with Hg, which implies that *F. antipyretica* has a high capacity to magnify low levels of these elements in water, thus facilitating their detection.

Because of the different equilibrium times depending on the element and the exposure concentration, the minimum time recommended for use in active biomonitoring studies by means of transplants will be very variable, although high tissue levels of these elements, except Sb, are found within a few days. Because of this and the irregular bioconcentration patterns found for this element, we do not recommend the use of *F. antipyretica* for biomonitoring low levels of Sb in water. Of the elements studied, As and Se appear to be the most toxic to *F. antipyretica*.

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Capítulo 4

Arsénico y mercurio en briófitos acuáticos nativos: diferencias entre especies

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Arsenic and Mercury in Native Aquatic Bryophytes: Differences Among Species

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Abstract This study investigated the capacities of five species of aquatic bryophytes to accumulate As and Hg from their natural habitats in rivers in Galicia (NW Spain). The distributions of the concentrations of both elements in all species were skewed to the right, with a higher incidence of extreme values in the As data, which may indicate a greater degree of contamination by this metalloid. There were no significant differences in the accumulation of either of the elements between the different species studied, which justifies their combined use as biomonitors of As and Hg, at least in the study area.

Keywords Biomonitoring · Interspecies calibration · Rivers · Galicia

Aquatic bryophytes are considered to be excellent indicators of the presence of a wide range of contaminants (Zechmeister et al. 2003). These organisms can accumulate much higher concentrations of elements in their tissues than the concentrations present in the water where they develop (Díaz et al. 2012), thus facilitating detection of such elements. Although contaminant levels in water may undergo large temporal variations, bryophytes integrate the levels of contaminants in water over time (Cesa et al. 2006), and therefore less sampling effort is required. Moreover, bryophytes only assimilate the bioavailable fraction from water, which cannot be measured by simple chemical analysis of water samples. Selection of which species to use is a key factor in biomonitoring studies. When single species of native biomonitors are used, selection of the sampling sites may be greatly restricted by the presence or absence of the species. To overcome this problem, several species may be used, although intercalibration of the responses of the species to different levels of contaminants must first be carried out (López and Carballeira 1993; Vuori and Helisten 2010). While intercalibration studies are relatively common in biomonitoring of atmospheric contamination with terrestrial mosses (e.g. Fernández et al. 2000; Carballeira et al. 2008), the same is not true for aquatic bryophytes.

The present study investigated the different capacities of four species of aquatic moss and one species of aquatic liverwort to accumulate As and Hg from their natural habitats. For this purpose, data from an extensive sampling survey of rivers in NW Spain were analysed, exploiting the presence of more than one species at many of the sampling sites.

Materials and Methods

The moss species *Fontinalis antipyretica* Hedw., *Platyhypnidium riparioides* (Hedw.) Dixon, *Brachythecium rivulare* Schimp., *Fissidens polyphyllus* Wilson ex Bruch

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& Schimp., and the liverwort *Scapania undulata* (L.) Dum were collected from 218 sampling sites (100 m stretches) distributed along rivers throughout Galicia (NW Spain). The total number of samples collected was 424. Sampling was carried out in summer, and the plants were collected from depths that ensured that they had not suffered hydric stress through exposure to the air (Wehr et al. 1983). The samples were washed in situ with the river water and were transported to the laboratory in cool boxes. In the laboratory, apical segments (2 cm long) were separated from the plants for analysis (Wells and Brown 1990), to minimize any possible error due to different accumulation capacities of different parts of the plants (Wehr et al. 1983). The apical segments were washed thoroughly with distilled water prior to analysis. The element concentrations were determined in extracts obtained from aliquots (300 mg dry weight) of sample digested with nitric acid in Teflon flasks, at high pressure and temperature, according to the procedure described by Wehr et al. (1983) and López and Carballeira (1993). The concentrations of the elements were determined by atomic fluorescence spectroscopy (PSA Excalibur for As, and PSA Merlin Plus for Hg). The limits of detection were 0.16 ng g^{-1} and 0.10 ng g^{-1} for As and Hg, respectively. The analytical quality of the results was determined by parallel analysis of certified reference material BCR-61 (*P. riparioides*) from the Community Bureau of Reference. The percentages of recovery were 87 % and 84 % for As and Hg, respectively.

Differences in the capacities of the five species to bioaccumulate the studied elements were determined by regression analysis. The hypothesis tested (F-test) was that different pairs of species had the same capacity for bioconcentration, i.e. that the value of the slope of the regression line was 1. The data were transformed (the As data by logarithmic transformation and the Hg data by square root transformation) prior to regression analysis, to satisfy the requirement for normally distributed data. The regression equations were only calculated when there were at least 10 pairs of points. As regressions thus established do not include any independent variables, the regression models were of type II, and the major axis regression method was applied (Sokal and Rohlf 1995; Warton et al. 2006). The calculations were carried out with SMATR software (Warton et al. 2006; Falster et al. 2006).

Results and Discussion

The most abundant species in the study area were the mosses *F. antipyretica* and *P. riparioides*, followed by the liverwort *S. undulata*; the mosses *B. rivulare* and *F. polyphyllus* were relatively scarce.

The mean concentrations of As in the bryophytes were approximately 2 orders of magnitude greater than those of Hg (Table 1), which may be expected given that As is usually found at higher natural concentration in fluvial waters (Reiman and de Caritat 1998), although aquatic bryophytes display a higher capacity to bioconcentrate Hg than to bioconcentrate As (Díaz et al. 2012). The concentrations of As were also more variable, and the overall and individual coefficients of variation for each species were always higher for As than for Hg (Table 1). The two most abundant species (*F. antipyretica* and *P. riparioides*) were those for which the coefficients of variation were highest.

No relationship was observed between the type of lithological substrates in the different river basins and the

Table 1 Descriptive statistics for As (concentrations in μg g^{-1}) and Hg (concentrations in ng g^{-1}) in different species of aquatic bryophytes in rivers in Galicia (NW Spain)

	n	Mean	Median	Mode	S.D.	C.V.	Max/mode
As							
F. antipyretica	171	12.43	5.73	3.31	18.13	145.9	38.67
P. riparioides	138	14.46	6.16	3.78	22.94	158.6	52.30
B. rivulare	23	12.08	6.74	4.13	14.98	124.1	11.30
F. polyphyllus	23	8.37	6.17	3.29	10.70	127.8	12.74
S. undulata	62	11.54	6.87	4.79	13.83	119.8	15.80
Pooled data	417	12.73	6.03	3.35	18.87	148.2	59.01
Hg							
F. antipyretica	174	147.2	101.0	61.4	150.6	102.3	11.91
P. riparioides	139	128.7	89.05	73.9	126.9	98.61	9.23
B. rivulare	22	119.0	80.19	61.6	105.8	88.86	5.97
F. polyphyllus	22	225.1	163.0	107	182.6	81.13	5.37
S. undulata	67	139.4	105.7	78.1	120.7	86.54	7.11
Pooled data	424	142.5	96.79	65.9	139.5	97.92	11.09

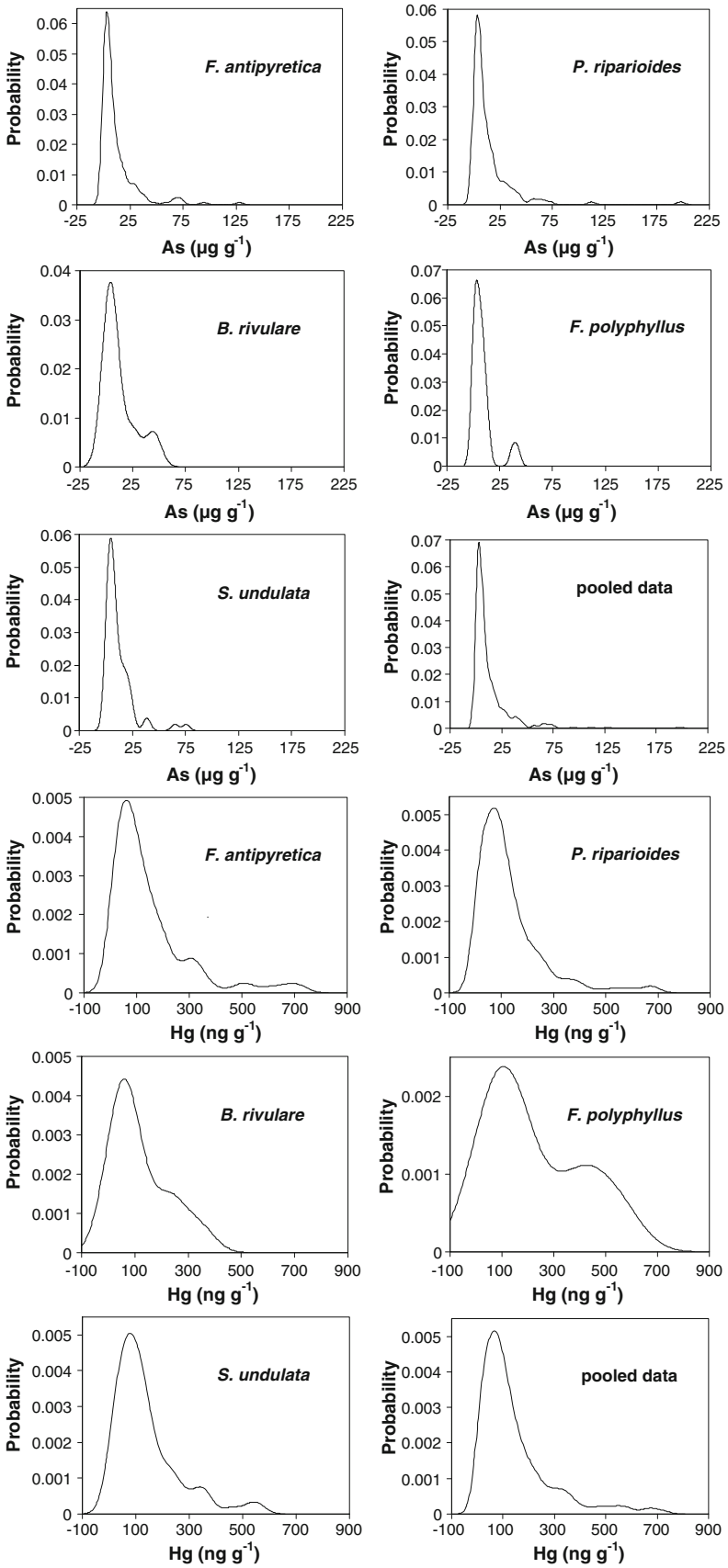

Fig. 1 Kernel smoothing distributions of the concentrations of As and Hg measured in aquatic bryophytes growing in rivers in Galicia (NW Spain)

Table 2 Classification of the sampling sites in relation to the contamination factor (CF) for each element, according to the classification proposed by Mouvet (1986)

		As		Hg	
		n	%	n	%
Extreme contamination	54 < CF	1	0.24	0	0.00
Serious contamination	18 < CF < 54	13	3.12	0	0.00
Moderate contamination	6 < CF < 18	60	14.39	27	6.37
Suspected contamination	2 < CF < 6	122	29.26	126	29.72
No contamination	CF < 2	221	53.00	271	63.92

concentrations of the elements studied, in contrast to previous reports (Carballeira and López 1997; Samecka-Cymerman and Kempers 1999; Samecka-Cymerman et al. 2007; Vuori and Helisten 2010).

The kernel smoothing technique provides a better picture of the distribution of the combined data than classical frequency histograms, as in the latter the distribution is very sensitive to the number of classes established and to the position of the classes along the variable axis (Aboal et al. 2006). The kernel-smoothing distributions (Fig. 1) were similar in all cases, and the distributions were skewed to the right. The modal values observed in these distributions (Table 1) were similar to the concentrations of As in *F. antipyretica* from clean sites, reported by other studies (Nimis et al. 2002; Culioli et al. 2009; Martínez and Shu-Nyamboli, 2011), and similar or lower to the concentrations of Hg in different species of aquatic bryophytes, also from clean sites (Samecka-Cymerman and Kempers 1998; Nimis et al. 2002; Cesa et al. 2009). Therefore, we decided to use the modal values as the reference levels for establishing the background concentrations of each element. The concentrations of the elements in the most abundant species were also widely dispersed in the kernel-smoothing distributions, as reflected by the coefficient of variation. The distribution of the concentrations of As was skewed further to the right than the distribution of the concentrations of Hg, indicating that the extreme concentrations were further from the modal value. The ratio between the maximum and modal value (Table 1) was higher for As than for Hg in all species. These findings may indicate greater contamination by As than by Hg.

The Contamination Factor (CF) can be defined as the concentration of an element in a sample divided by the background concentration of the element (Carballeira and López 1997). As already mentioned, the modal value from the kernel-smoothing distributions was used as the background level. To characterize the sampling points in relation to the CF, the classification proposed by Mouvet (1986) was applied (Table 2). For As, the CF was below 6 at 82 % of the sampling points, which were therefore included in the *no contamination* or *suspected contamination* categories; one of the other sites was classified as extremely contaminated. For Hg, 94 % of the sampling sites fell within the *no contamination* and *suspected contamination* classes and none of the sampling sites were within the two higher classes.

The regressions between pairs of species were highly significant (almost always at $p < 0.001$) for both elements, except for Hg in *B. rivulare* and *F. antipyretica*, which were not significantly related (Fig. 2). Significant differences from a line of slope 1 were not observed in any of the cases (Table 3), indicating that there are no differences in bioconcentration of As and Hg by the species considered. Despite the differences in the accumulation capacities of different aquatic bryophyte species for different metals reported in other studies (López and Carballeira 1993; Vuori and Helisten 2010), the combined use of the species studied appears valid, at least in the study area and for the elements considered.

These results may be extrapolated to sites with similar characteristics to those of the study area. The water in Galician rivers is characterized by a pH close to neutral, low conductivity and a low level of hardness, as limestone rocks are scarce (Antelo and Arce 1996). However, different results may be expected in rivers with different characteristics.

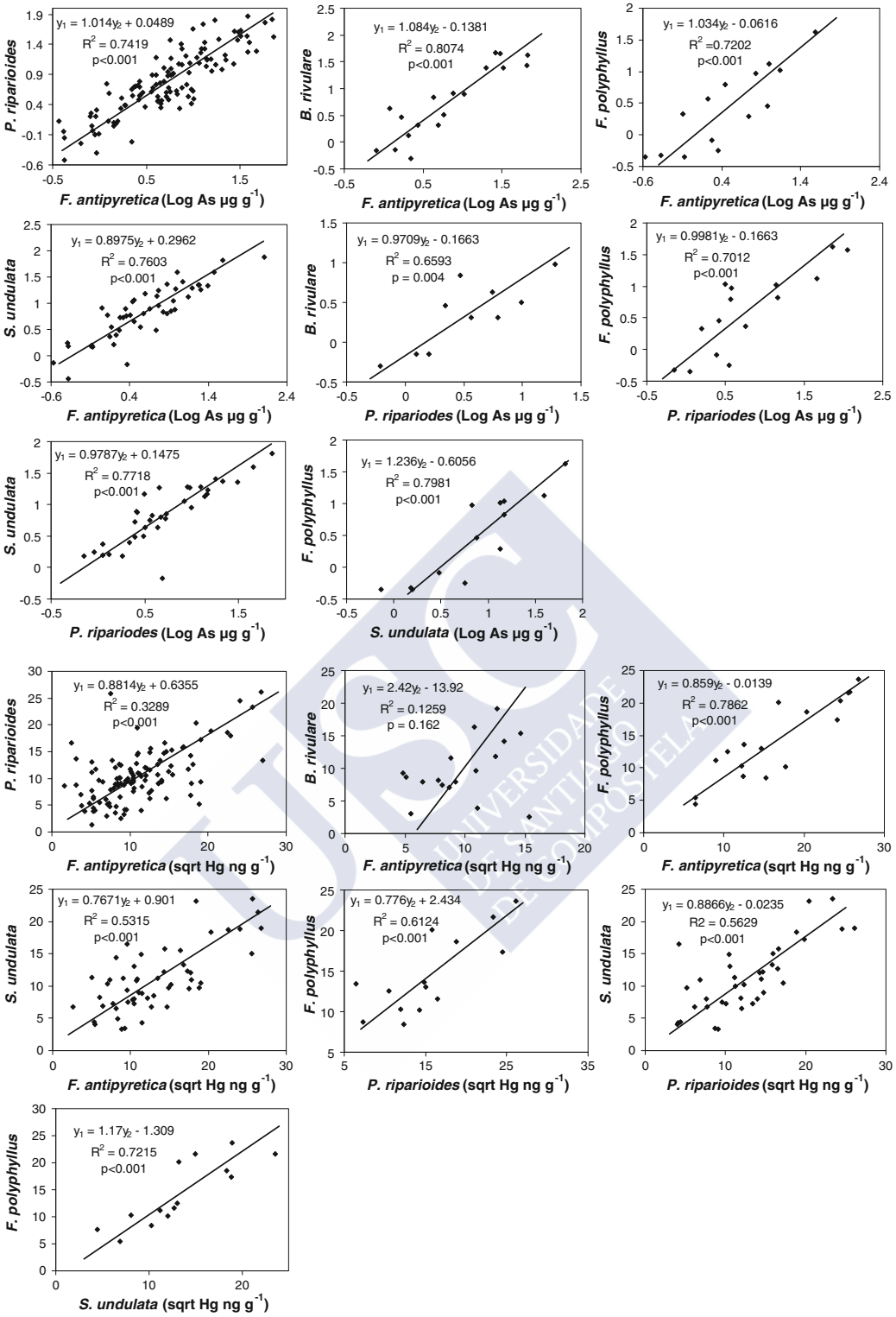

Fig. 2 Regressions (model II) established between different species of aquatic bryophytes for tissue concentrations of As and Hg. The same units are used in the *x* and *y axes* (concentrations of As subjected to $\log_{10}$ transformation and concentrations of Hg subjected to square root transformation)

Table 3 F-values and the associated significance levels for the differences between the pairs of species and a line of slope 1 (see Fig. 2)

	n	F	*p*
As			
F. antipyretica–P. riparioides	116	0.060	0.806
F. antipyretica–B. rivulare	18	0.433	0.520
F. antipyretica–F. polyphyllus	14	0.035	0.854
F. antipyretica–S. undulata	49	1.751	0.192
P. riparioides–B. rivulare	10	0.013	0.910
P. riparioides–F. polyphyllus	15	0.004	0.948
P. riparioides–S. undulata	36	0.053	0.819
S. undulata–F. polyphyllus	13	1.985	0.187
Hg			
F. antipyretica–P. riparioides	115	0.887	0.348
F. antipyretica–B. rivulare	17	2.175	0.161
F. antipyretica–F. polyphyllus	17	1.284	0.275
F. antipyretica–S. undulata	53	3.873	0.055
P. riparioides–F. polyphyllus	14	1.245	0.286
P. riparioides–S. undulata	37	0.656	0.423
S. undulata–F. polyphyllus	14	0.769	0.398

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Capítulo 5

Niveles de fondo de mercurio y arsénico en briófitos de los ríos gallegos

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Niveles de fondo de mercurio y arsénico en briófitos de los ríos gallegos

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Resumen

En el estudio de contaminantes que pueden estar presentes de forma natural en el ambiente es conveniente intentar establecer los llamados niveles de fondo, es decir, sus niveles naturales, para poder evaluar adecuadamente sus concentraciones. Los briófitos acuáticos son organismos frecuentemente empleados como biomonitores de contaminación, tanto acuática como terrestre, debido a una serie de características, como pueden ser la capacidad de acumular contaminantes en concentraciones mucho más altas a las existentes en el medio físico, la de integrar en el tiempo esas concentraciones o al hecho de que el análisis de un contaminante en un organismo nos esté reflejando biodisponibilidad.

En este trabajo se muestran los niveles de fondo calculados mediante métodos estadísticos de arsénico y mercurio en cuatro especies de musgos acuáticos y una hepática de ríos gallegos.

Palabras clave: briófitos, bioindicadores, Galicia, contaminación.

Introducción

Numerosos contaminantes se encuentran frecuentemente en aguas fluviales en concentraciones que se pueden considerar muy bajas desde un punto de vista analítico (lo cual no quiere decir que biológicamente sean concentraciones irrelevantes). Estas bajas concentraciones originan dificultades analíticas, como la necesidad de disponer de equipamiento de laboratorio costoso, o el aumento de la probabilidad de contaminación de las muestras durante su procesado. Además la variabilidad temporal de estos contaminantes suele ser muy elevada, lo que hace necesario un elevado esfuerzo de muestreo para poder dar así una imagen real de la situación. Por otro lado, un simple análisis químico de un contaminante en agua no da una idea clara de su disponibilidad para los seres vivos. Los briófitos acuáticos son empleados frecuentemente como bioindicadores acumulativos de contaminantes (Zechmeister et al. 2003) porque pueden cargar en sus tejidos concentraciones mucho más elevadas que las existentes en el agua en la que se desarrollan (Díaz et al. en prensa), son integradores en el tiempo de los niveles de contaminantes (Cesa et al. 2006), y sólo asimilarán, por definición, lo que está biodisponible.

Mercurio y arsénico son dos importantes contaminantes en aguas fluviales. Sin embargo, al ser constituyentes de la propia corteza terrestre, su presencia en bajas concentraciones no tiene porque implicar necesariamente una contaminación de origen humano. Por este motivo es interesante intentar establecer sus niveles de fondo, es decir, sus niveles naturales, para poder evaluar adecuadamente sus concentraciones. Frecuentemente los niveles de fondo en briófitos acuáticos se establecen en función de las concentraciones encontradas en zonas no afectadas por actividades antrópicas (Samecka-Cymerman et al. 1991, Samecka-Cymerman y Kempers 1998, 1999, Cesa et al. 2009, Vuori y Helisten 2010). Sin embargo, es difícil saber si una zona está realmente limpia, además es muy probable que no exista ningún lugar en el planeta con unos niveles de contaminación que pudiéramos considerar como preindustriales, dada la enorme facilidad de dispersión que tienen ciertos contaminantes. En el presente estudio nos referimos a nivel de fondo como la concentración natural de un elemento representativa de una zona de estudio, que a pesar de estar influenciada por la actividad humana, está bien

conservada. El nivel de fondo debe ser establecido a una escala regional, dada la gran variabilidad natural existente en las concentraciones de los diferentes elementos (Carballeira y López 1997, Reimann y Garrett 2005, Vuori y Helisten 2010).

En este trabajo se presentan los niveles de fondo de mercurio y arsénico en diferentes especies de briófitos acuáticos de ríos de Galicia calculados mediante diferentes aproximaciones estadísticas.

Material y métodos

Se recogieron las siguientes especies de musgos: *Fontinalis antipyretica* Hedw., *Platyhypnidium riparioides* (Hedw.) Dixon, *Brachythecium rivulare* Schimp., *Fissidens Polyphyllus* Wilson ex Bruch & Schimp., así como la hepática *Scapania undulata* (L.) Dum; procedentes de un total de 218 estaciones de muestreo (tramos de 100 m) repartidas por ríos de toda Galicia. El muestreo tuvo lugar en los meses de verano, las plantas colectadas estaban a una profundidad suficiente que asegurara que en ningún momento hubieran sufrido estrés hídrico por exposición al aire (Wehr et al. 1983). Se lavaban in situ con agua del propio río y se transportaban en neveras portátiles. Una vez en el laboratorio se separaban los 2 cm apicales (Wells and Brown 1990) para minimizar cualquier error debido a las diferentes capacidades de acumulación de las distintas partes de la planta (Wehr et al. 1983). Los ápices se lavaban minuciosamente con agua destilada. Las concentraciones elementales se determinaban en extractos obtenidos a partir de 300 mg (peso seco) de muestra digerida con ácido nítrico en bombas de teflón a alta presión y temperatura según el procedimiento descrito por López y Carballeira (1993) y Wehr et al. (1983). La lectura se llevó a cabo mediante espectroscopía de fluorescencia atómica: As con un aparato PSA Excalibur y Hg con un aparato PSA Merlin Plus. La calidad analítica de los resultados fue contrastada mediante el análisis paralelo de material de referencia certificado BCR-61 (*Platihypnidium riparioides*) del Community Bureau of Reference.

Para la determinación de los niveles de fondo se emplearon los siguientes métodos:

Método 1

Un método propuesto hace ya 50 años pero todavía utilizado en estudios geoquímicos es emplear en estudios extensivos la media ± 2 desviaciones típicas de las concentraciones encontradas como rango de los niveles de fondo. Este método ha sido criticado por Reimann et al. (2005), quienes señalan que tanto medias como desviaciones típicas son los mejores estimadores de centralización y dispersión de datos que siguen una distribución normal, pero no son adecuados para datos geoquímicos o ambientales, ya que suelen presentar un fuerte sesgo debido a que suelen representar más de una población/proceso. Estos autores indican que en la búsqueda de niveles de fondo nos interesan datos atípicos ("outliers") originados de diferentes distribuciones (a menudo superpuestas) asociadas a procesos que son raros en el ambiente (como un foco de contaminación o la presencia de una mineralización), y no los valores extremos de una distribución normal (o lognormal). Es decir, los datos atípicos que intentamos separar no vienen a ser la cola de la distribución de la población de fondo, sino que pertenecen a otra distribución. Por todo ello estos autores proponen como equivalente a este método emplear la mediana ± 2 desviaciones absolutas medianas. Siendo la desviación absoluta mediana la mediana de las desviaciones absolutas de los datos respecto a la mediana muestral. Será este último método el que seguiremos en el presente trabajo, tomando como nivel de fondo el valor superior de este rango.

Método 2

Otro sistema consiste en eliminar las concentraciones más elevadas hasta alcanzar un coeficiente de variación del conjunto de las muestras restantes lo más próximo a 60%, que corresponderían a estaciones limpias. De esta manera se intenta eliminar la cola de una distribución sesgada a la derecha, que es lo que se encuentra frecuentemente en estudios de contaminación. Este método empleado inicialmente para determinar niveles de fondo de metales en suelos (Bonnefoy y Bourg, 1984), ha sido aplicado por Mouvet (1986), Carballeira y López (1997) y por Carballeira et al. (2002), para determinar niveles de fondo en briófitos. El nivel de fondo finalmente es estimado como el límite superior del intervalo de confianza al 95% de la media de la concentración del elemento en el briófito en esas estaciones supuestamente limpias. De esta manera en el

cálculo del nivel de fondo se tienen en cuenta las diferencias entre elementos en cuanto a su variabilidad natural, de manera que a los más variables se les atribuye un valor mayor (Carballeira y López, 1997).

Método 3

Cesa et al. (2010) encuentran una distribución en la concentraciones de elementos traza en muestras de *P. riparioides* sesgada la derecha, lo que implicaría la existencia de dos componentes, las concentraciones más bajas indicarían valores de fondo y las altas serían debidas a contaminación (Nimis et al. 2001). Por ello eliminan las concentraciones más elevadas gradualmente hasta que los datos restantes alcancen la normalidad. Ésta es evaluada con los tests de Shapiro-Wilks y Lilliefors ($p<0.01$). El nivel de fondo correspondería a la mediana de los datos finales.

Método 4

Otro método propuesto por Reimann et al. (2005) es utilizar los clásicos diagramas de cajas (Figura 1). El umbral superior del nivel de fondo quedaría establecido como la valla interna superior de un diagrama de cajas en el que se represente el conjunto de datos estudiados. Ésta se encuentra situada a 1.5 veces el recorrido intercuartílico por encima del tercer cuartil.

Método 5

El empleo de gráficas de frecuencias acumuladas para la determinación de niveles de fondo geoquímicos propuesto por Sinclair (1974) es todavía muy utilizado en este tipo de estudios (por ejemplo Martínez et al. 2007, Zhao et al. 2007). En estas gráficas se representa en un eje la concentración del elemento y en otro eje el porcentaje de frecuencias acumuladas en una escala tal que hace que una distribución normal (gaussiana) quede representada por una línea recta (Figura 2). Mediante este método podemos separar diferentes subpoblaciones, cada una de ellas viene a ser el conjunto de datos que forman un tramo recto en esa representación. Este método también ha sido adaptado para determinar el nivel de fondo de metales en briófitos tanto acuáticos (Carballeira y López, 1997) como terrestres (Carballeira et al. 2002), asumiendo que el primer tramo recto corresponde a muestras limpias. El nivel de fondo

finalmente es estimado, de manera análoga al método 2, como el límite superior del intervalo de confiaza al 95% de la media de la concentración del elemento en el briófito en esas estaciones supuestamente limpias.

Método 6

Mediante una técnica conocida como suavizado por núcleo (*kernel smoothing*) se puede apreciar mejor la distribución de un conjunto de datos que mediante el clásico histograma de frecuencias, dado que éste es muy sensible al número de clases establecidas y a la posición de las clases en el eje, lo cual no sucede con el suavizado por núcleo (Aboal et al. 2006). Nosotros empleamos la citada técnica para definir el nivel de fondo como la concentración correspondiente a la primera moda (Figura 3).

Resultados y discusión

Los musgos *F. antipyretica* seguido de *P. riparioides* fueron las especies más abundantes en la zona de estudio, ambas especies muestran requerimientos ambientales muy similares (López et al., 1997). La siguiente más abundante fue la hepática *S. undulata*, siendo bastante escasos los musgos *B. rivulare* y *F. polyphyllus*.

El As presentó una mayor variabilidad en sus concentraciones, el coeficiente de variación total y el de cada especie fue siempre mayor al del Hg (Cuadro 1). Las dos especies con mayor número de muestras (*F. antipyretica* y *P. riparioides*) fueron las que mostraron unos coeficientes de variación mayores. También fueron las que presentaron un rango mayor de concentraciones.

Mediante análisis de la varianza se buscaron diferencias en las concentraciones medias entre especies en aquellos puntos en los que coexistían. Únicamente en dos casos se encontraron diferencias significativas: *S. undulata* presentó una concentración media de As mayor que *F. antipyretica* ($p=0.016$) y *F. antipyretica* mostró una concentración media de Hg superior a la de *S. undulata* ($p=0.044$).

En el Cuadro 2 se pueden observar las diferencias existentes entre los niveles de fondo calculados mediante los diferentes métodos. Es necesario señalar que el escaso número de muestras existente para *B. rivulare* y *F. polyphyllus* hace que los valores aquí presentados los consideremos como meramente

orientativos. El método 4 proporcionó unos niveles de fondo claramente más elevados en todos los casos. Las concentraciones calculadas por el método 1 fueron las siguientes más elevadas para el As y para algunas especies en el caso del Hg. El resto de métodos proporciona unos valores bastante más parecidos dentro de cada especie, por lo que parecen una mejor aproximación al nivel de fondo. De estos métodos, el número 5 (el empleo de curvas de frecuencias acumuladas) presenta el inconveniente de ser más subjetivo, por la necesidad de determinar los posibles puntos de inflexión, que además pueden no estar muy bien definidos (Figura 2). De los métodos restantes, el método 2 lo consideramos un tanto arbitrario en su definición del punto de corte del coeficiente de variación del 60%. El método 3 asume que el conjunto de muestras procedentes de lugares limpios debería de seguir una distribución normal, lo que no tiene porque ser siempre así. Por tanto consideramos el método 6, es decir, establecer como nivel de fondo a la concentración correspondiente a la primera moda de la distribución de datos con suavizado por núcleo el más adecuado.

Atendiendo a las tres especies más abundantes, el nivel de fondo establecido mediante el método 6 fue siempre mayor para ambos elementos en *S. undulata*, seguido *de P. riparioides* y *F. antipyretica*.

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Cuadro 1. Estadística descriptiva de As (concentraciones en µg g^{-1}) y Hg (concentraciones en ng g^{-1}) en diferentes especies de briófitos acuáticos de ríos gallegos.

		n	Media	Mediana	Mínimo	Máximo	S.D.	C.V.
As	*F. antipyretica*	171	12.43	5.73	0.01	128.0	18.13	145.9
	P. riparioides	138	14.46	6.16	0.30	197.7	22.94	158.6
	B. rivulare	23	12.08	6.74	0.50	46.67	14.98	124.1
	F. polyphyllus	23	8.37	6.17	0.44	41.93	10.70	127.8
	S. undulata	62	11.54	6.87	0.36	75.67	13.83	119.8
	Total	417	12.73	6.03	0.01	197.7	18.87	148.2
		n	Media	Mediana	Mínimo	Máximo	S.D.	C.V.
Hg	*F. antipyretica*	174	147.2	101.0	2.71	731	150.6	102.3
	P. riparioides	139	128.7	89.05	1.68	682	126.9	98.61
	B. rivulare	22	119.0	80.19	1.02	368	105.8	88.86
	F. polyphyllus	22	225.1	163.0	6.31	575	182.6	81.13
	S. undulata	67	139.4	105.7	11.19	555	120.7	86.54
	Total	424	142.5	96.79	1.02	731	139.5	97.92

Cuadro 2. Niveles de fondo de As y Hg calculados por diferentes métodos (ver Material y Métodos) en diferentes especies de briófitos acuáticos de ríos gallegos.

		F. antipyretica	*P. riparioides*	*B. rivulare*	*F. polyphyllus*	*S. undulata*
As (µg g^{-1})	método 1	14.3	15.6	16.1	14.6	16.0
	método 2	2.54	4.19	2.45	0.75	5.63
	método 3	4.25	8.12	9.56	13.6	14.8
	método 4	34.8	37.0	57.2	23.0	32.3
	método 5	2.74	5.28	4.31	3.74	2.30
	método 6	3.31	3.78	4.13	3.29	4.79
		F. antipyretica	*P. riparioides*	*B. rivulare*	*F. polyphyllus*	*S. undulata*
Hg (ng g^{-1})	método 1	221	196	197	402	213
	método 2	80.0	81.1	69.5	136	110
	método 3	70.4	74.0	268	469	241
	método 4	388	340	446	902	356
	método 5	28.5	38.2	15.2	29.3	24.1
	método 6	61.4	73.9	61. 6	107	78.1

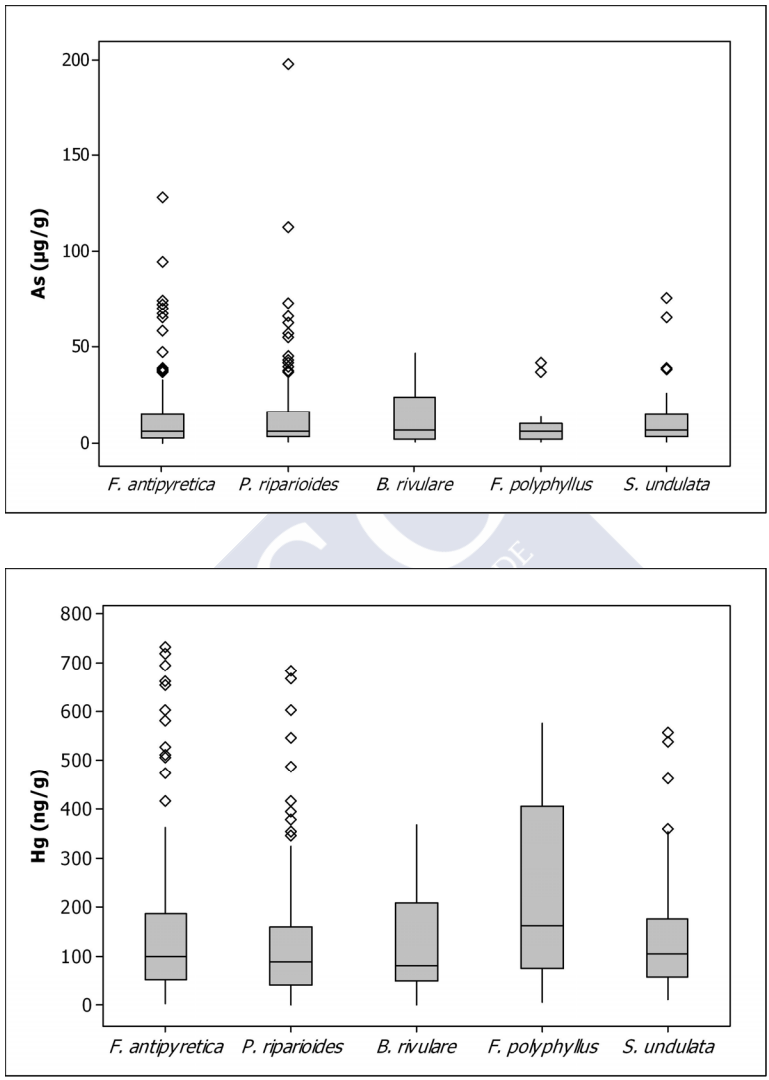


Figura 1. Diagramas de cajas de las concentraciones de As y Hg en diferentes especies de briófitos acuáticos de ríos gallegos.

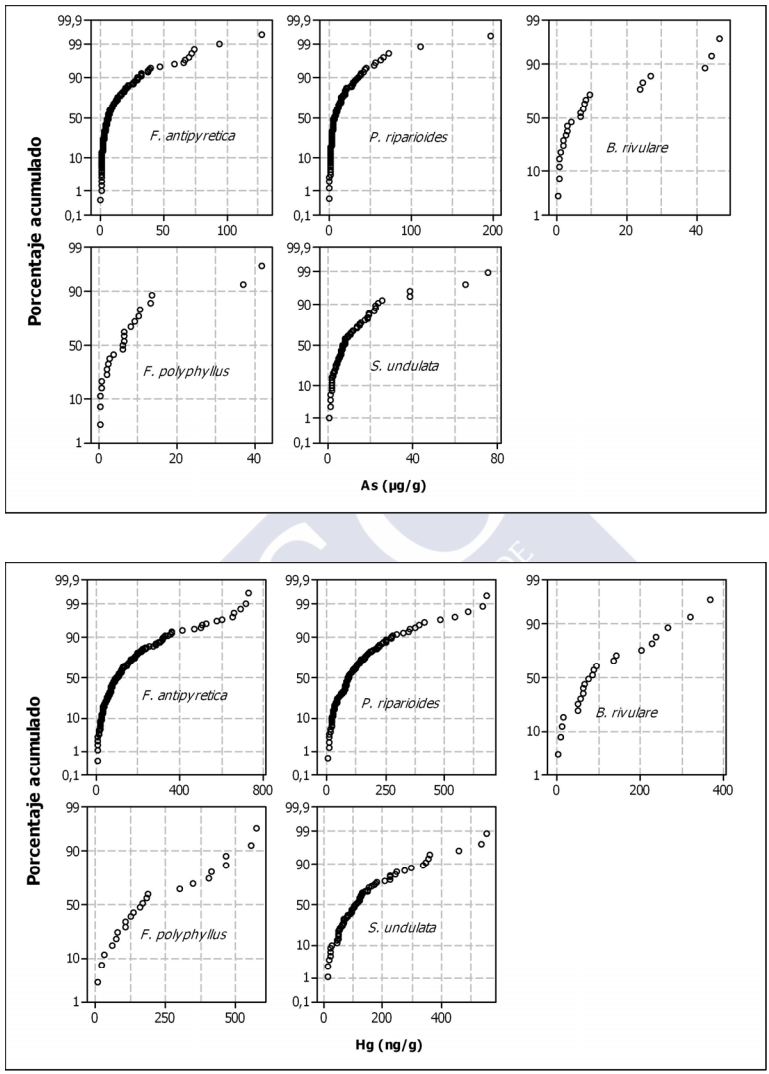
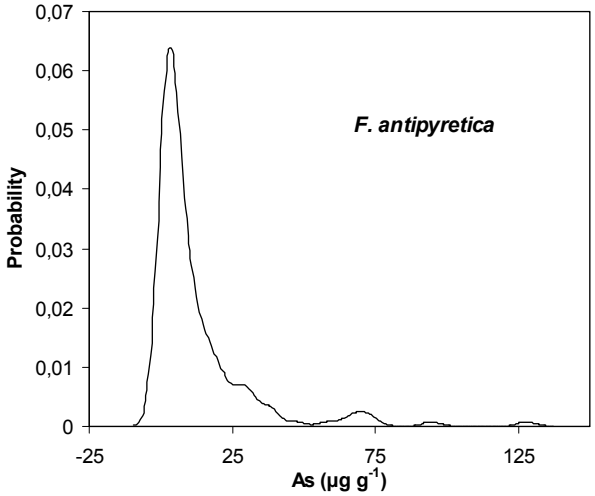
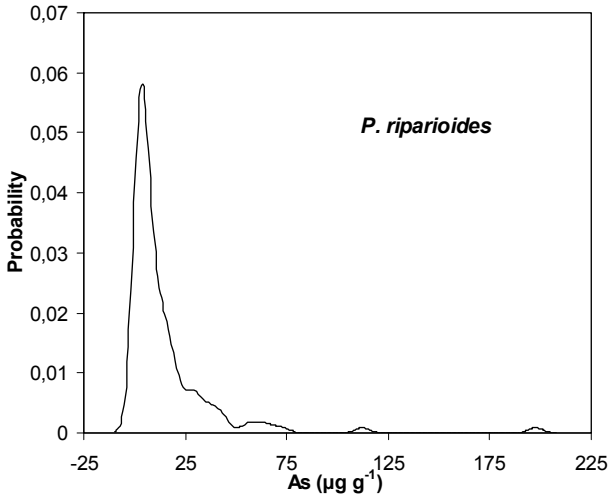
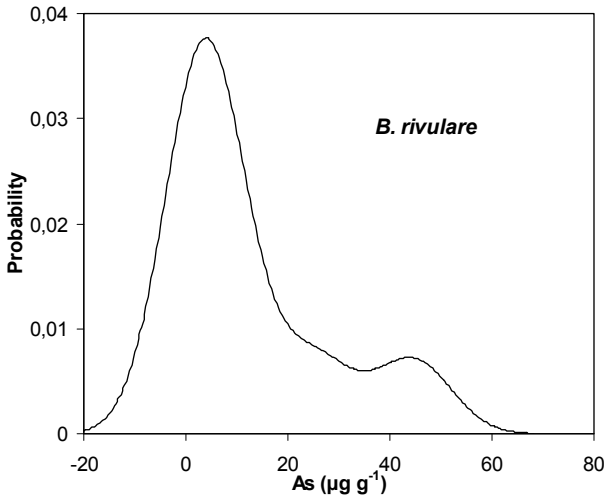
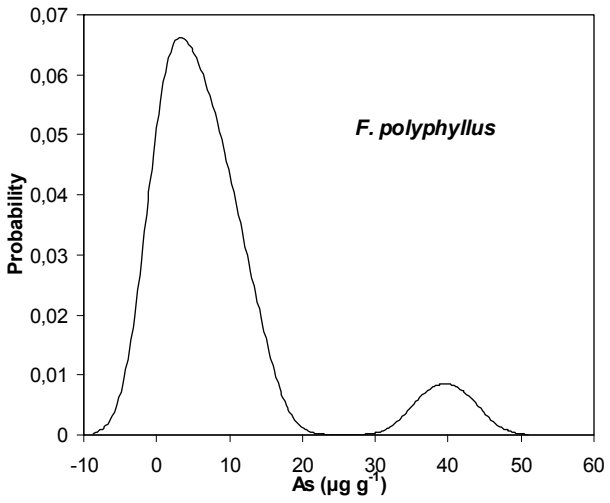


Figura 2. Gráficas de frecuencias acumuladas de las concentraciones de As y Hg en diferentes especies de briófitos acuáticos de ríos gallegos.


0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
Probability
F. antipyretica
-25
25
75
125
As (µg g-1)


0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
Probability
P. riparioides
-25
25
75
125
175
225
As (µg g-1)


0,04
0,03
0,02
0,01
0
Probability
B. rivulare
-20
0
20
40
60
80
As (µg g-1)


0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
Probability
F. polyphyllus
-10
0
10
20
30
40
50
60
As (µg g-1)

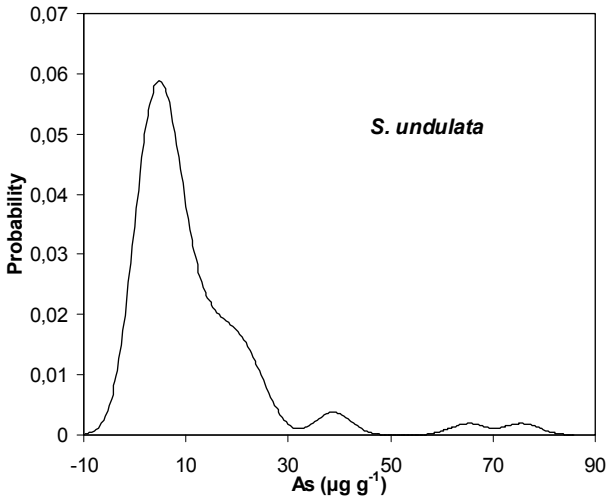
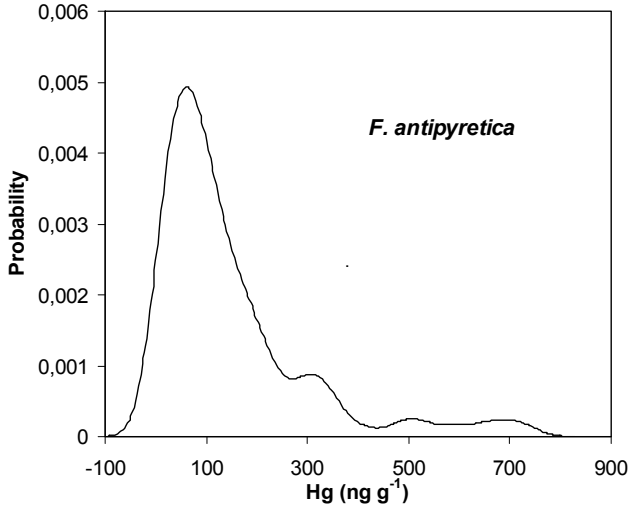
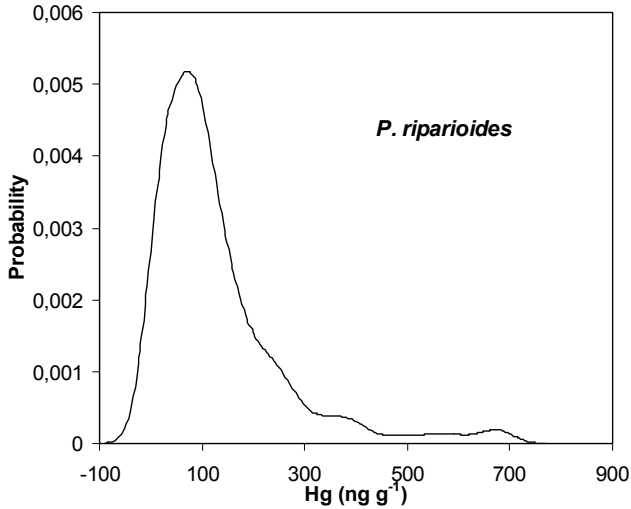
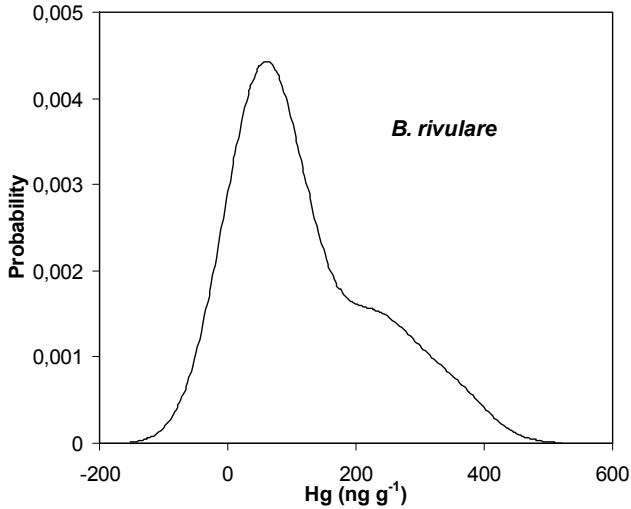
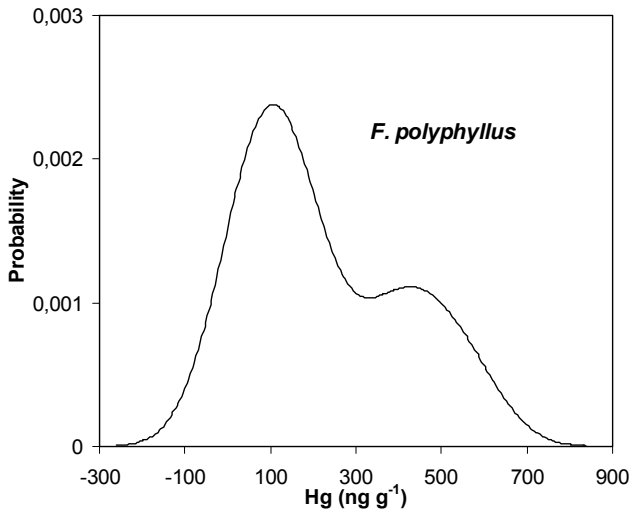


Figura 3a. Distribuciones estimadas por suavizado por núcleo de las concentraciones de As en diferentes especies de briófitos acuáticos de ríos gallegos.


0,006
0,005
0,004
0,003
0,002
0,001
0
Probability
F. antipyretica
-100
100
300
500
700
900
Hg (ng g-1)


0,006
0,005
0,004
0,003
0,002
0,001
0
Probability
P. riparioides
-100
100
300
500
700
900
Hg (ng g-1)


0,005
0,004
0,003
0,002
0,001
0
Probability
B. rivulare
-200
0
200
400
600
Hg (ng g-1)


0,003
0,002
0,001
0
Probability
F. polyphyllus
-300
-100
100
300
500
700
900
Hg (ng g-1)

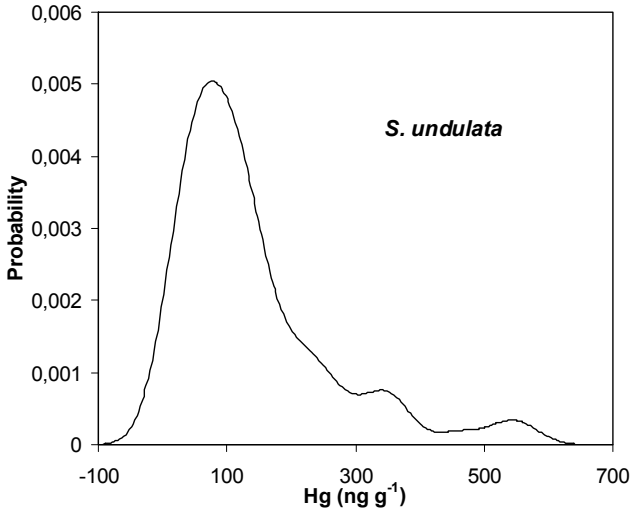


Figura 3b. Distribuciones estimadas por suavizado por núcleo de las concentraciones de Hg en diferentes especies de briófitos acuáticos de ríos gallegos.

Capítulo 6

Biomonitorización activa de arsénico, mercurio y antimonio en el curso bajo del río Eume mediante trasplantes del musgo acuático Fontinalis antipyretica *Hedw.*

Biomonitorización activa de arsénico, mercurio y antimonio en el curso bajo del río Eume mediante trasplantes del musgo acuático *Fontinalis antipyretica* Hedw.

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Abstract

En este trabajo se estudia la acumulación de As, Hg y Sb en trasplantes del musgo acuático *Fontinalis antipyretica* en el río Eume. Las muestras del briófito procedían de un río limpio y se expusieron durante 4, 11, 19 y 28 días. Los resultados más interesantes se encontraron para As y Hg. Para ambos elementos los trasplantes incrementaron su concentración claramente respecto a la inicial y en las estaciones de las que se disponía análisis de musgo autóctono, el briófito trasplantado acumulaba mayor concentración. Los resultados encontrados para el Sb desaconsejan el empleo de *F. antipyretica* para biomonitorizar este metaloide.

Introducción

El empleo de biomonitores para la evaluación de la calidad de las aguas fluviales es frecuente desde hace décadas (ej. Kirchmann y Lambinon, 1973; McLean y Jones, 1975). Dentro de las diferentes especies utilizadas, los briófitos son de las más usadas como bioindicadores acumuladores, dadas las características que presentan (Zechmeister et al., 2003). El empleo de estos organismos tiene una serie de ventajas respecto a la evaluación de la calidad de las aguas de un río mediante los convencionales análisis químicos del agua. Las concentraciones de muchos contaminantes en agua pueden ser muy bajas y por tanto difíciles de analizar, pueden ser también muy variables en el tiempo, y por otra parte un análisis químico del agua no nos informa de la biodisponibilidad de los contaminantes. Estos problemas pueden ser solventados mediante el uso de bioindicadores, puesto que estos solo asimilan la fracción biodisponible, concentran los niveles de contaminantes en agua y dan una medida mucho más estable en el tiempo.

La biomonitorización activa (mediante trasplantes) presenta ciertas ventajas frente a la biomonitorización pasiva (empleo de organismos nativos). Con este tipo de biomonitorización se puede disponer de muestras en lugares tales como en la proximidad de focos de contaminación en los que puede que no existan de forma natural los organismos que se pretenden emplear como bioindicadores. Mediante la biomonitorización activa se puede conocer el estado fisiológico y niveles de contaminantes de las muestras antes de la exposición. Se sabe con exactitud el tiempo de exposición. Se puede disponer las muestras en puntos exactos determinados previamente. En lugares contaminados los organismos trasplantados suelen ser más sensibles que los nativos, dada la falta de adaptación a este tipo de ambientes, por lo que la acumulación de contaminantes suele ser mayor y también serán mayores los efectos fisiológicos (Carballeira et al., 2003).

El presente estudio se llevó a cabo en el río Eume, afectado por la explotación a cielo abierto de lignito de As Pontes de García Rodríguez (15 km^2) y su escombrera asociada (210 ha) en la que abundan los sulfuros metálicos. El río también recibe numerosos vertidos incontrolados de los efluentes urbanos de As Pontes.

Material y métodos

En la cuenca del Eume se seleccionaron cuatro estaciones de muestreo, tres en el cauce principal y una en un afluente conocido como arroyo de Brea (Fig. 1). La primera estación está situada entre la localidad de As Pontes y la cola del

Embalse del Eume, el resto están situadas río abajo de dicha presa. El musgo trasplantado (*Fontinalis antipyretica* Hedw.) procedía de un tramo limpio del río Brandelos (afluente del río Ulla). El musgo se colocaba en bolsas de malla con una luz de 1 cm y ancladas al lecho de río mediante piedras. El experimento se desarrolló en verano de 1991, cuando tanto la mina como la escombrera estaban activas. Los tiempos de exposición fueron 4, 11, 19 y 28 días. El último día se recogió musgo autóctono de la misma especie a la utilizada en los trasplantes en las estaciones 2 y 4.

Los análisis se realizaron con los 2 cm apicales, las muestras fueron digeridas con ácido nítrico en bombas de teflón y horno microondas (a alta presión y temperatura) según López y Carballeira (1993) y Wehr et al. (1983). La lectura de los extractos fue realizada mediante espectroscopía de fluorescencia atómica, As y Sb (PSA Excalibur), Hg (PSA Merlin Plus). Para evaluar la eficiencia del método, se analizó en paralelo material de referencia certificado del Community Bureau of Reference BCR-61 (el musgo acuático *Platihypnidium riparioides*).

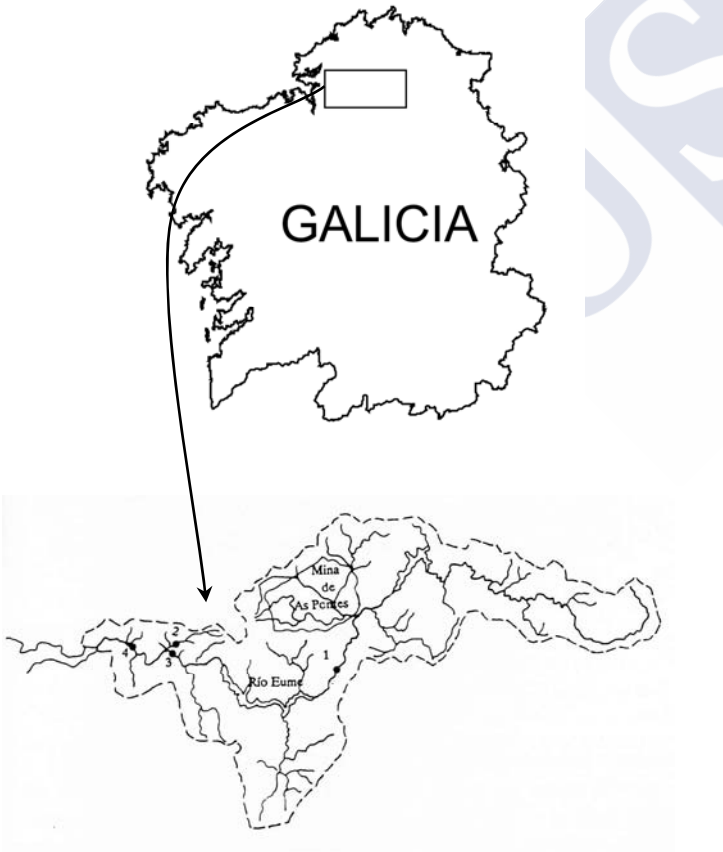

Fig. 1. Situación de los trasplantes de *F. antipyretica* en diferentes puntos de la cuenca del río Eume.

Resultados y discusión

El musgo trasplantado cargó claramente As y Hg con relación a la concentración inicial en todas las estaciones (Fig. 2). Inicialmente se observa una acumulación más rápida en el caso del Hg, multiplicándose su concentración inicial en los 4 primeros días por un factor de entre 50 (en Eume-1) y 95 (en Eume-3). A partir de ese momento las concentraciones permanecen más o menos estables o aumentan aunque no de una manera tan importante. Para As la concentración se incrementa de una manera más gradual y tiende a hacerse más o menos asintótica, sobre todo en Eume-1 y Eume-4. El Sb mostró un comportamiento diferente, en Eume-1 y Eume-4 la tendencia es a una carga más o menos continua, a lo largo de la incubación. En Eume-2 y Eume-4 este metaloide muestra una evolución irregular, con altibajos. Estos resultados concuerdan con los de Díaz et al. (2012), que encuentran una gran afinidad de *F. antipyretica* por el Hg y desaconsejan el empleo de esta especie como biomonitora de contaminación por Sb.

Para As las concentraciones más elevadas se encuentran claramente en Eume-1, lo cual es consistente con su situación más próxima a los focos de contaminación. Los niveles son más bajos en el resto de las estaciones y con diferencias no muy importantes entre ellas. Para Hg también se encuentran las concentraciones más altas en Eume-1, aunque para este metal no se encuentra una diferencia tan importante con el resto de estaciones. Para este metal Eume-2 muestra las siguientes concentraciones más elevadas, lo cual puede parecer en principio sorprendente, dado que este lugar se encuentra en un afluente limpio apartado del curso principal, y por tanto no debería estar afectado por los focos de contaminación situados río arriba. Sin embargo el Hg se dispersa muy bien por vía atmosférica y la central térmica de As Pontes libera grandes cantidades de este metal (Carballeira y Fernández, 2002; López-Alonso et al., 2003; Nóvoa-Muñoz et al., 2008). Podría suceder que gran parte del Hg acumulado por el musgo procediera de deposición atmosférica, Otero-Rey et al. (2003) muestran que casi la totalidad (>99.8 %) del Hg presente en el carbón que es quemado en la central sale con los gases de la chimenea, el As también es volátil, pero menos que el Hg, estos autores estiman que un 24% del As del carbón sale con los gases. Respecto al Sb, una vez más encontramos datos difíciles de explicar, la estación supuestamente más contaminada, Eume-1, es la que presentó los niveles más bajos junto con Eume-2.

Una comparación con las concentraciones encontradas en las muestras de musgo autóctono (Fig. 2) nos permite observar que para As y Hg la acumulación fue mayor en el musgo trasplantado, a pesar de que evidentemente el tiempo de exposición fue mucho menor. El musgo trasplantado, que procedía de un lugar aparentemente limpio, no tenía las adaptaciones que pudiera tener el musgo autóctono al desarrollarse en un medio más contaminado. Esta circunstancia también ha sido observada por Samecka-Cymerman et al. (2005) para *F. antipyretica* y por Fermández y Carballeira (2000) y Fernández et al. (2000) en musgos terrestres. Una vez más con el Sb tenemos unos resultados diferentes, en Eume-2 la concentración inicial en el musgo trasplantado era mayor que el autóctono, y en Eume-4 los niveles en las muestras trasplantadas siempre estuvieron por debajo del autóctono.

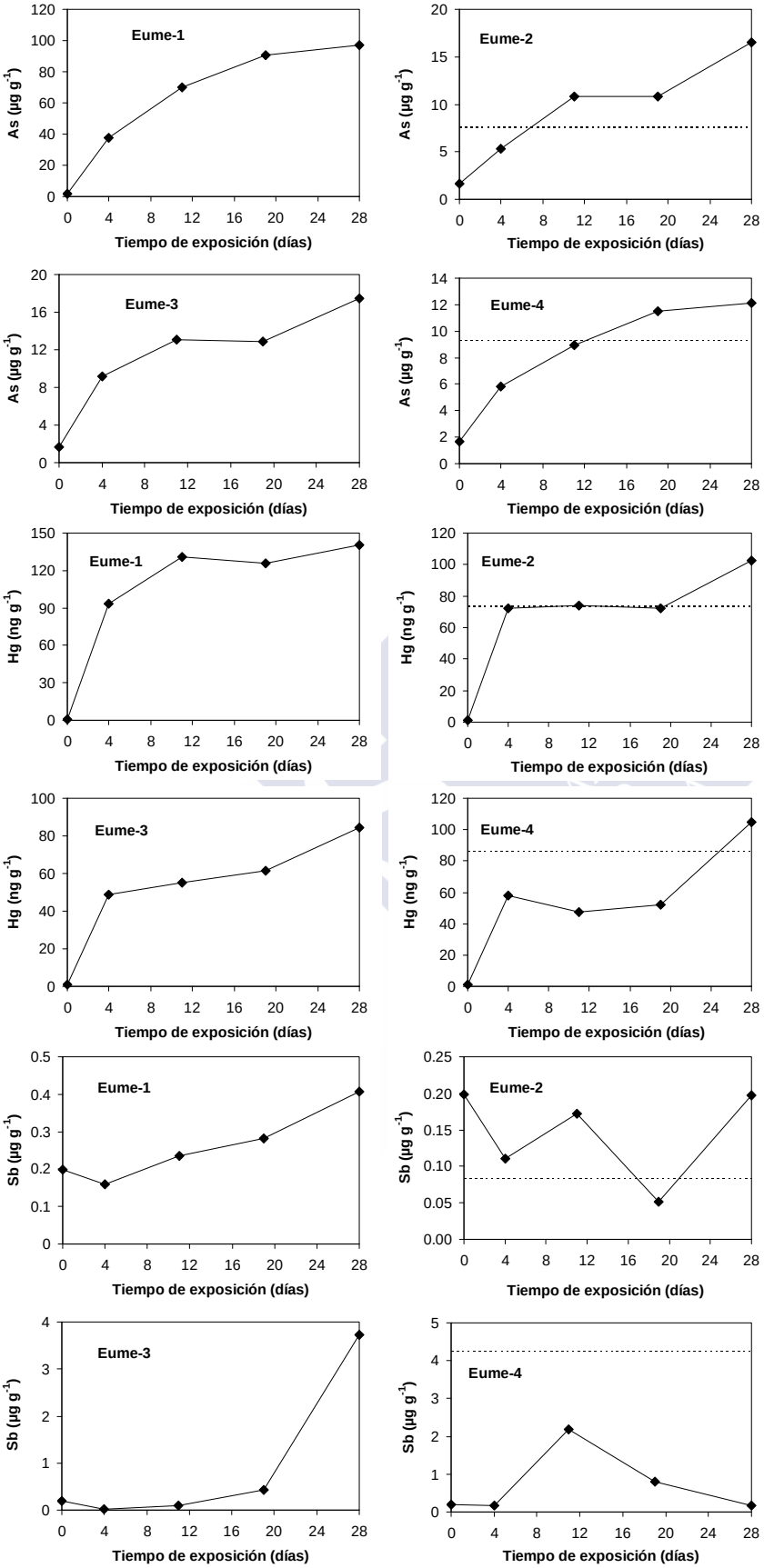

Fig. 2. Evolución temporal de las concentraciones de As, Hg y Sb en musgo procedente de un río limpio y trasplantado en diferentes puntos de la cuenca del río Eume. La línea punteada en las estaciones 2 y 4 corresponde a la concentración presente en musgo autóctono en dichas estaciones el día 28.

La concentración máxima encontrada en este estudio (140.7 ng g^{-1} en Eume-1) para Hg, resultó ser algo más de el doble de la estimada por Díaz et al. (2012) como nivel de fondo para esta especie en ríos gallegos (61.4 ng g^{-1}), por lo que no creemos que la contaminación por Hg en la zona sea severa. En cambio para As, la concentración máxima encontrada (96.9 µg g^{-1} en Eume-1) resultó ser unas 29 veces su nivel de fondo (3.31 µg g^{-1}). Para Sb no existen calculados niveles de fondo para la zona de estudio.

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Capítulo 7

Efectos fisiológicos de la exposición a arsénico, mercurio, antimonio y selenio en el musgo acuático Fontinalis antipyretica *Hedw.*

Physiological effects of exposure to arsenic, mercury, antimony and selenium in the aquatic moss *Fontinalis antipyretica* Hedw.

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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 chlorophyll *a* fluorescence 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 chlorophyll fluorescence 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 fluorescence were generally only observed in the moss exposed to the highest concentrations of these elements in solution. Net photosynthesis and fluorescence were also related to tissue concentrations of these elements in the moss. However, the same concentration of Sb in tissue had very different physiological effects depending on the initial concentration to which the moss was exposed in solution. Temporal trends in 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; fluorescence; bryophytes.

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 ng l^{-1} for Sb and Hg) (Drasch et al., 2004; Koch et al., 1999; Rish, 2004; Schrauzer, 2004; Stoeppler, 2004). However, they can be found at much higher concentrations in certain areas as a result of natural or anthropogenic sources. For example, high concentrations of arsenic (10 – 5000 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. Cenci, 2000; Cesa et al., 2009; Empain, 1973; Wehr and Whitton, 1983). The moss *Fontinalis 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 (Cenci, 2000; Fernández et al., 2006; Vázquez et al., 1999). Thus, the effects of variables such as organic pollution (Martínez-Abaigar et al., 1993; Vázquez et al., in press), 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 Carballo 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; Branquinho et al., 1997b; 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 chlorophyll fluorescence. 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). 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±2ºC). 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 containing solutions with different concentrations of As, Hg, Sb and Se: 0 (control), 0.1, 1, 10, 100, 1000 and 10000 µg l^{-1}, prepared from standard solutions of respectively As_2O_5, $Hg(NO_3)_2$, $SbCl_3$ and SeO_2 (Merck and Panreac). All experiments were carried out in a cool chamber at 16 ± 0.1 ºC, 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. For a more detailed description of the incubations, see Díaz et al. (2012). At the end of each incubation period, the physiological variables and the elements were determined as explained below.

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, 1993; Wehr and Whitton, 1983). The concentrations of As, Sb and Se were determined by atomic fluorescence (PSA Excalibur). The concentration of Hg was determined by atomic fluorescence (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 hours at 16 ± 0.1 ºC 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 º 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 (>5000 µmol photons m^{-2} s^{-1}, 0.8s pulse length, actinic white light) was applied through a fibre-optic cable at an angle of 60º 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 minutes. This length of time is considered to be sufficient to allow all 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 photosystem II (PSII) as the ratio of $Fv/Fm = (Fm - F_0)/Fm$ (Bolhàr-Nordenkampf et al., 1989), where F_0 and Fm 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 Fv/Fm 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 (Csintalan et al., 1999; Lichtenthaler and Miehé, 1997). 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.

3. Results and discussion

3.1 Effects on net photosynthetic rate

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., in press). The photosynthesis and respiration rates and fluorescence data are expressed as percentages, calculated relative to the control for the same incubation period. This procedure corrects for the “transplant effect”,

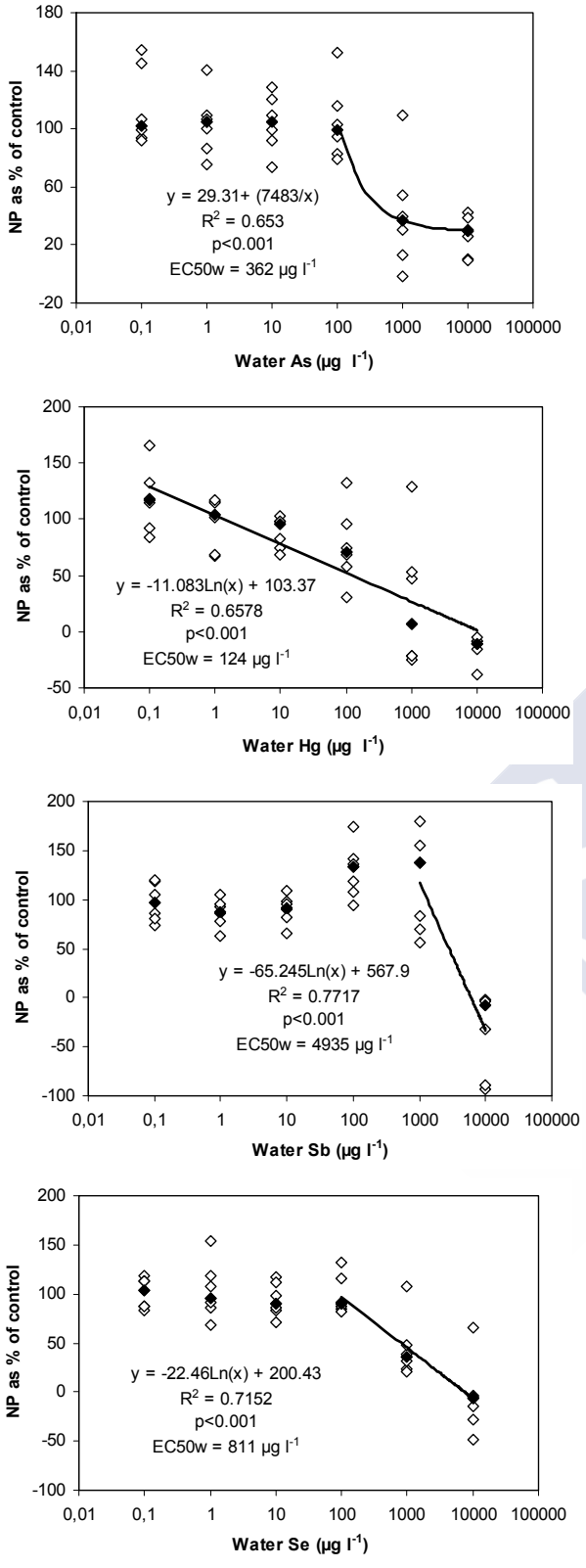

Fig 1. Net photosynthetic rate (NP) of *F. antipyretica* previously exposed to different concentrations (0.1 to 10000 µg l^{-1}) of As, Hg, Sb and Se in solution; the results are expressed as a percentage of the control value. Black diamonds represent the median values for each exposure concentration. The median effective solution concentrations for NP ($EC50_w$) calculated from regression equations are also shown.

i.e. stress caused by relocation of specimens and by the laboratory conditions under which the experiments were performed. In general, net photosynthesis (NP) decreased with increasing concentration of the elements in solution (Fig. 1), although the trend varied depending on the element considered. This trend 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 clearest and most constant decrease in the NP with increasing concentration of element was observed with Hg, from the lowest 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 1000 µg l^{-1}, and a decrease at 10000 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 (1000-10000 µg l^{-1}), which are not usually found in natural environments. However, for Hg the relationship was manifested from lower concentrations.

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 the results shown in the graphs in Fig. 1. This value was deducted from the regression equations that best fit the data in the scatter plot, by means of the curvilinear regression option of the IBM SPSS Statistics 20 software. The entire set of points was only fitted 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, only the final exposure concentrations were fitted. The most satisfactory fits were generally obtained with logarithmic equations, except for As, for which a inverse equation was most appropriate. Although the results thus calculated are of limited interest, since the points used for each exposure concentration correspond to various incubation times, we believe this is an appropriate approach for comparing the relative toxicity of the elements studied.

The $EC50_w$ values (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.

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

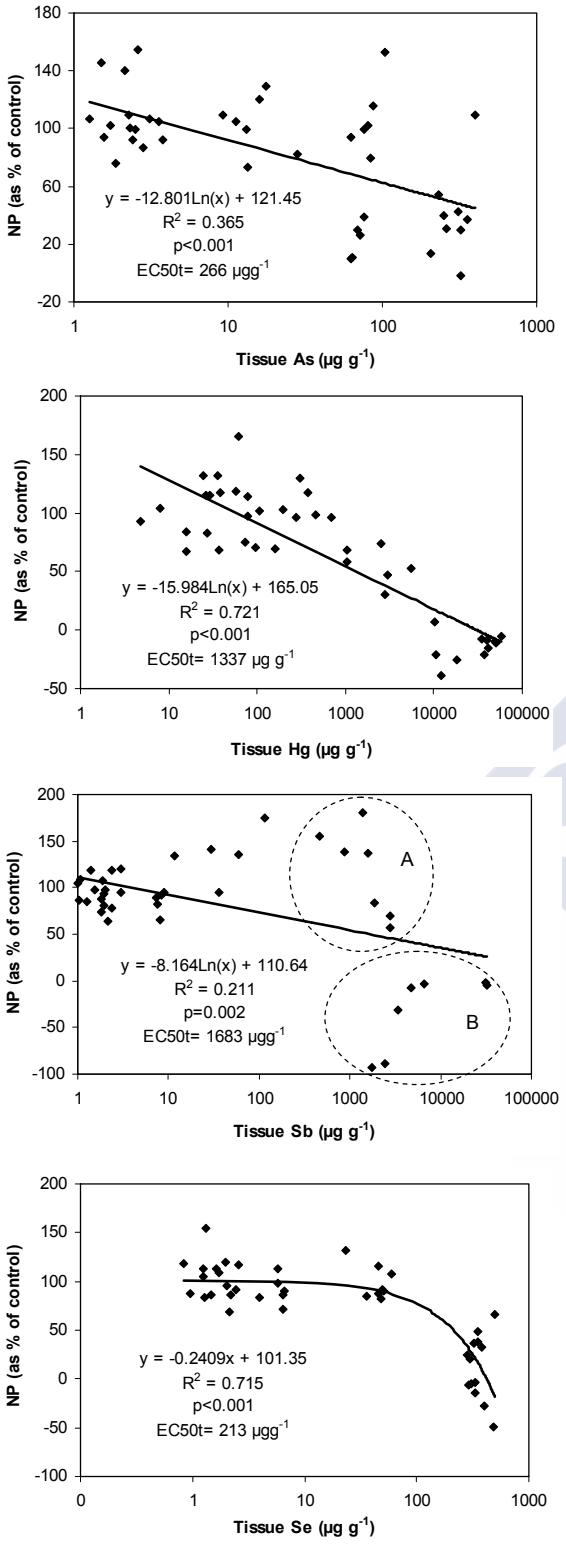

Fig 2. Regressions between the net photosynthetic rate (NP), expressed as a percentage of the control value, and tissue concentrations of As, Hg, Sb and Se in the moss F. antipyretica after the exposure period. Median effective tissue concentrations for NP (EC50t) estimated from regression equations 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.

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 Sb > Hg >> As > Se. Therefore, 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: Díaz et al. (2012). These results clearly differ from those for the concentrations in solution, which indicated (as explained above) that Hg was the most toxic element. For Sb, at the highest concentrations in solution, the moss samples in which similar tissue concentrations were achieved showed different responses (Fig. 2). Those samples incubated in solutions containing 1000 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.

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 1000 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 1000 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 (24h LC100) of Hg for the liverwort *Scapania undulata* was between 500-1000 µg l^{-1}, i.e. within the same range of concentrations 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 1000 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 1000 µg l^{-1},

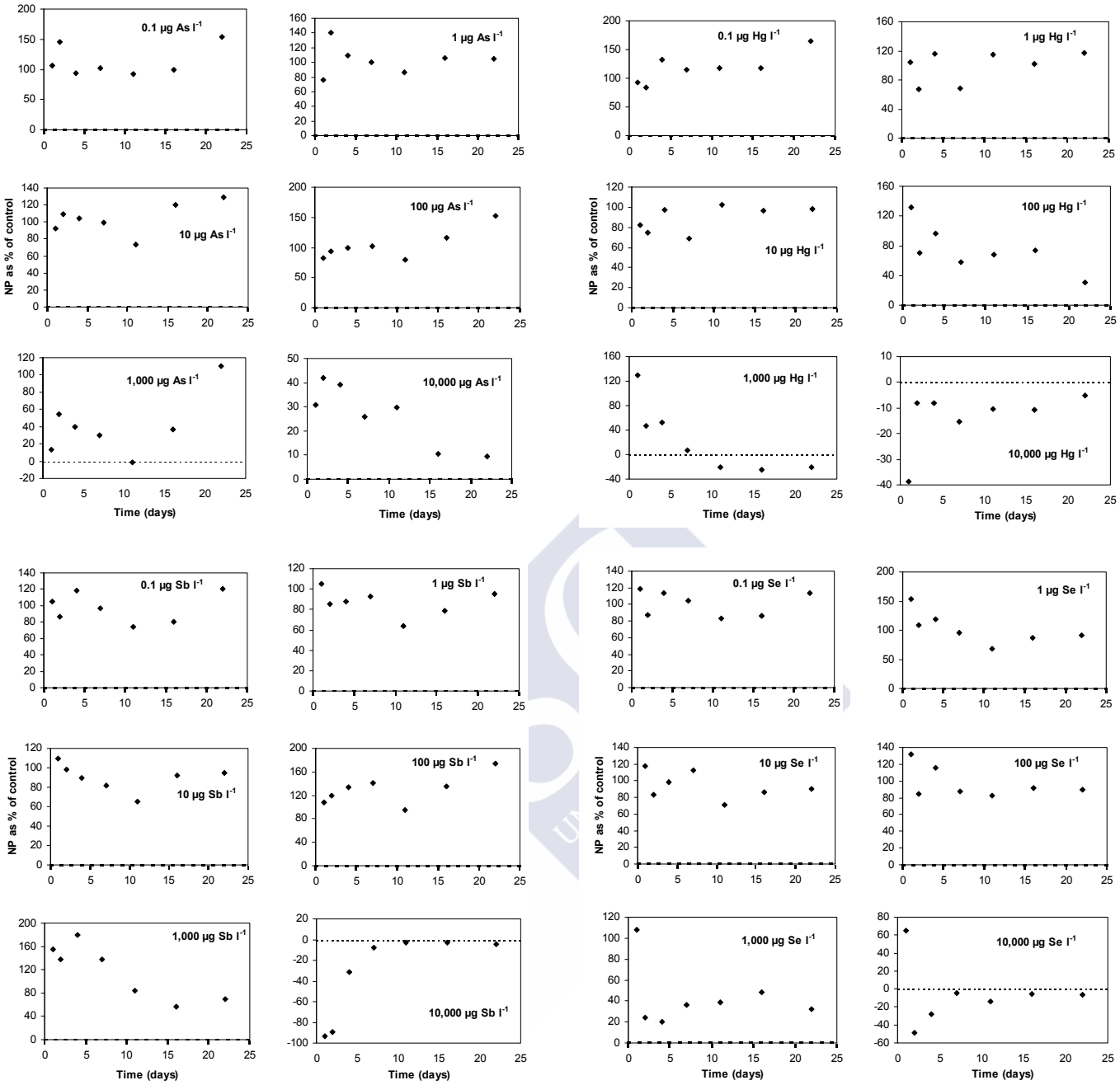

Fig. 3. Temporal changes in the net photosynthetic rate (NP), expressed as a percentage of the control value, in *F. antipyretica* exposed to different concentrations of As, Hg, Sb and Se in water. The dotted line, which represents NP = 0, is shown for visual reference.

there was a sharp decrease in NP between first and second day of incubation, with constant values thereafter. At 10000 µg l^{-1}, the response was similar, but with lower values of NP.

These results again show the higher toxicity of Hg, as this is the only element that caused a decrease in the NP at 100 µg l^{-1} in water. 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 1000 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 1000 and 10000 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 *Rhytidiadelphus squarrosus* to different concentrations of various metals, observed greater inhibition of

photosynthesis in measurements made after 24 hours than in measurements taken after 30 min.

3.3. Effects on dark respiration rate

Unlike net photosynthesis, the dark respiration rates (DR) 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). A similar response has been observed in other studies in which bryophytes were exposed to different elements (Brown and Wells, 1990; Sommer and Winkler, 1982).

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. Brown and Whitehead (1986) exposed *Rhytidiadelphus squarrosus* to different concentrations of Hg and also observed an initial increase in DR followed by a gradual decrease. For Hg, a clear trend was only observed at 10000 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 sampled 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 damage.

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).

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

3.4. Effects on chlorophyll fluorescence

The value of Fv/Fm 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).

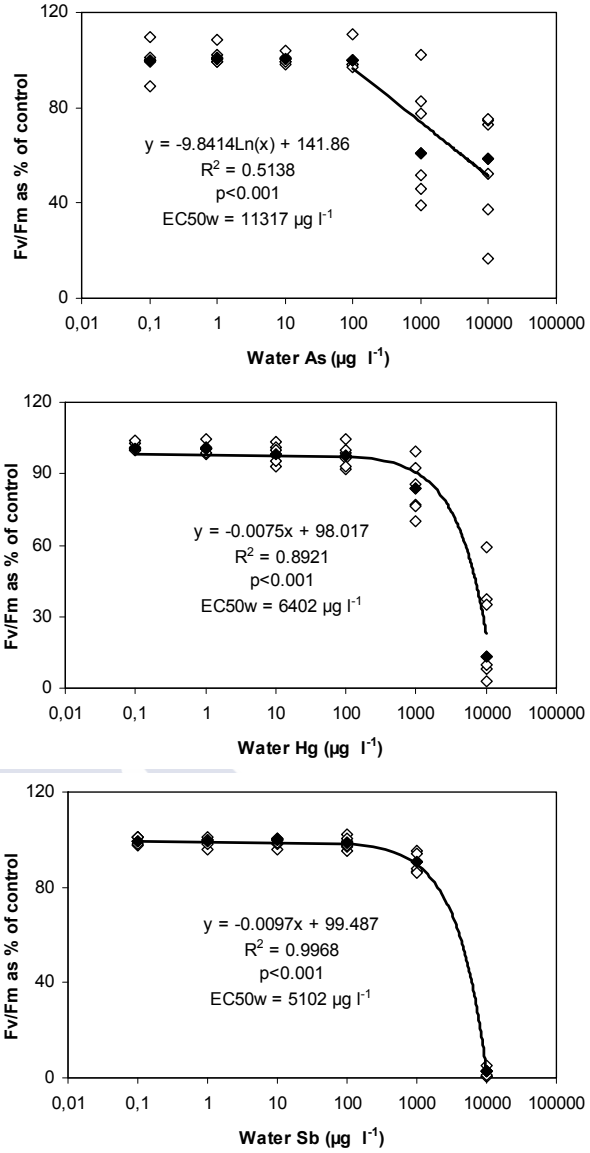

Fig 4. Chlorophyll fluorescence expressed as F_v/F_m of *F. antipyretica* previously exposed to different concentrations (0.1 to 10000 µg l^{-1}) of As, Hg, Sb and Se in solution; the results are expressed as a percentage of the control value. Black diamonds represent the median values for each exposure concentration. The median effective water concentrations for NP ($EC50_w$) calculated from regression equations are also shown.

The Fv/Fm ratio was only affected at the highest exposure concentrations (1,000 and 10,000 µg l^{-1}) of all the elements (Fig. 4). The data, especially for Hg and Sb, were less variable than the net photosynthesis data (Fig. 1). One possible explanation for this is that the fluorescence values are each means of 4 measures, as explained in material and methods. A great advantage of the fluorescence technique is that it is very easy 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 calculated for Fv/Fm were much higher for As and Hg, and very similar for Sb in comparison with net photosynthesis. However, Sb was identified as the most toxic element on the basis of the fluorescence and the least

toxic on the basis of NP. For both physiological parameters, Hg was more toxic than As. Moreno-Jiménez et al. (2009) also reported that Hg was more toxic than As to shoots of two species of shrubs.

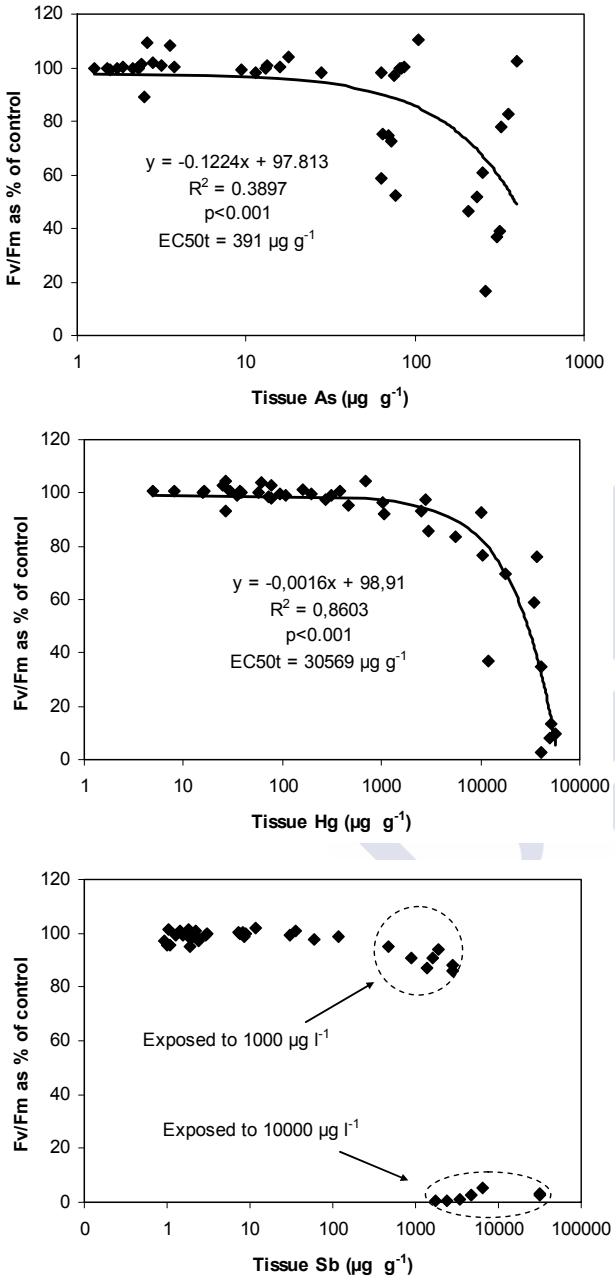

Fig 5. Regressions between chlorophyll fluorescence, expressed as F_v/F_m , and tissue concentrations of As, Hg, Sb and Se in the moss *F. antipyretica* after the exposure period. Median effective tissue concentrations for NP ($EC50_t$) estimated from regression equations are also shown.

In relation to tissue concentrations, the Fv/Fm ratio showed a clear pattern for Hg (Fig. 5), which began to decrease with tissue levels above 1000 µg g^{-1}. For As, the values began to decrease at tissue concentrations above 60 µg g^{-1}, although the data were highly variable. 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. 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. For Sb, two set of data can be distinguished; in the samples exposed to the highest concentration in water the Fv/Fm values were almost 0% of the controls, and the remaining data with values similar to the controls. For the samples in which tissue Sb reached 1500 – 3000 µg g^{-1}, the responses differed greatly depending on whether these concentrations were reached this by exposure for several days to 1000 µg Sb l^{-1} (with Fv/Fm values about 90% of controls) or if the concentrations were reached more quickly after exposure to 10,000 µg Sb l^{-1} (with Fv/Fm values almost 0% of controls). This is similar to the results for net photosynthesis, 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.

The cellular location of a toxic element is important for the physiological effects, and elements accumulated in a intracellular position usually have the most negative effects (Branquinho et al., 1997a; Brown and Wells, 1990; Sidhu and Brown, 1996). It is possible that the high Hg tissue levels observed before a decrease in Fv/Fm began to be evident in the moss were due to the fact that most of this metal was located extracellularly. Satake and Miyasaka (1984) observed that a high proportion of the Hg accumulated in the liverwort *Jungermannia vulcanicola* was trapped as particulate matter within the cell wall. This may explain why the effect of Hg on the moss physiology (both fluorescence and net photosynthesis) 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 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; Esteban et al., 2008; Israr et al., 2006). Moreover, the toxic Sb(III) form, which was used in the experiments, can be converted in plants to a less toxic Sb(V) form (Babula et al., 2008).

The Fv/Fm ratio did not indicate any temporal trends for exposure up to 100 µg l^{-1} (Fig. 6), and values were very close to the control values. At the two highest exposure concentrations of As, the Fv/Fm ratio values were highly variable, although they were almost always lower than the control values. For both Hg and Sb and an exposure

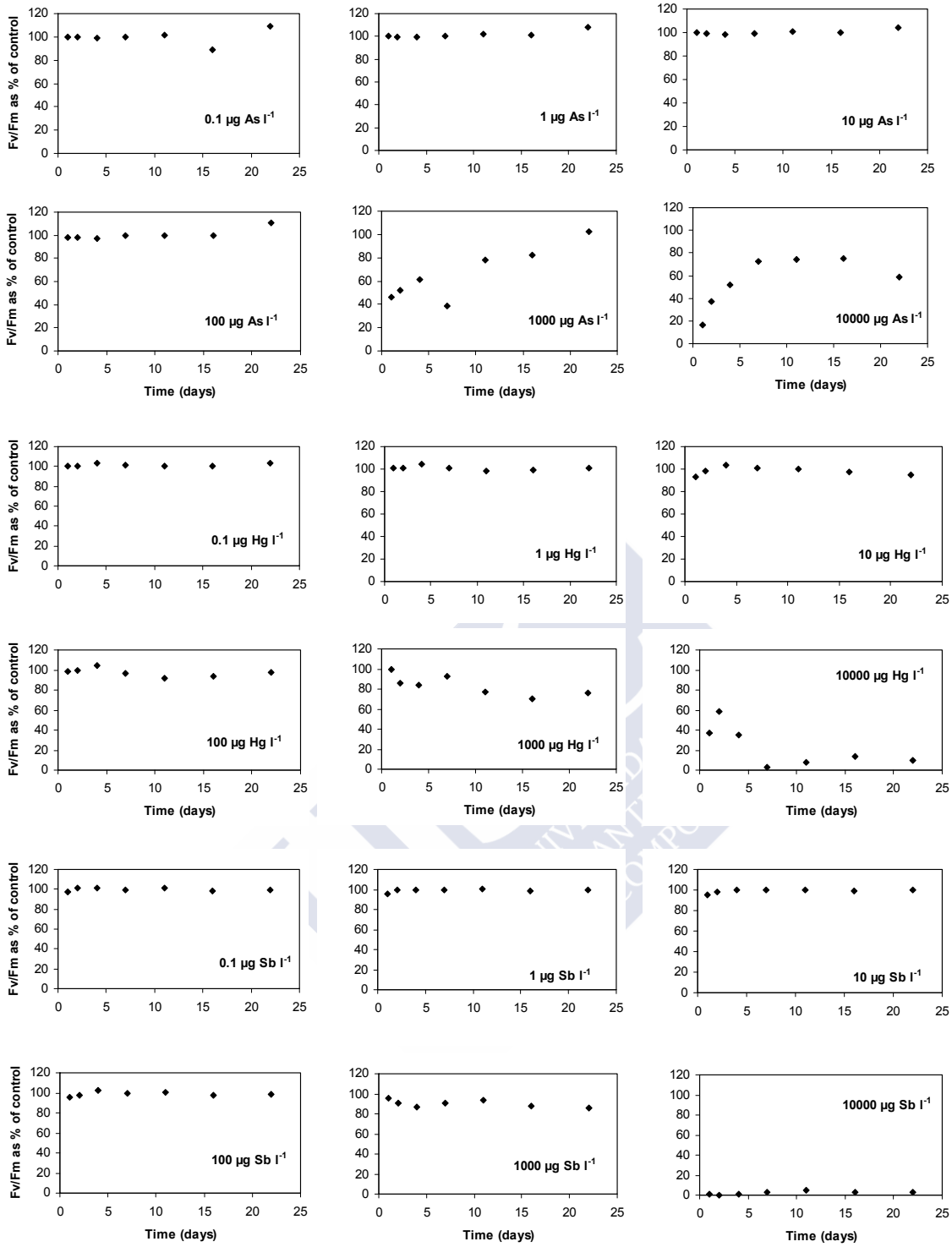

Fig 6. Temporal changes in chlorophyll fluorescence (Fv/Fm), expressed as a percentage of the control value, in *F. antipyretica* exposed to different concentrations of As, Hg, Sb and Se in water.

concentration of 1,000 µg l^{-1}, there was a slow decrease in Fv/Fm, which was most evident for the former. In the moss samples exposed to Hg at a concentration of 10,000 µg l^{-1}, the Fv/Fm values were about 50% of the control values in the first days of the incubation, followed by values close to 0; for Sb at this concentration, the values of the ratio were always close to 0.

4. Conclusions

The physiological responses of mosses differ depending on the element, concentration, exposure time and physiological parameter considered. In general, the moss physiology only appear 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 was the most toxic followed by As. 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 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. On the other hand, although Hg was accumulated in much higher amounts, with clear effects on the physiology of the moss, comparison of tissue concentrations showed that it was proportionally less toxic than the other elements.

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.

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Discusión general

Esta Tesis se ocupa de diferentes cuestiones que habían quedado pendientes dentro de la línea de investigación sobre *el empleo de briófitos en la biomonitorización de ecosistemas fluviales* que sigue el Grupo de investigación en Ecotoxicología de la Universidad de Santiago de Compostela.

Los dos primeros capítulos presentan una cierta independencia del resto. En el primero se abordó un tema poco tratado en el campo de la biomonitorización acuática: la contaminación térmica. Se evaluaron los efectos de temperaturas elevadas sobre el musgo acuático *Fontinalis antipyretica*, así como su resiliencia. Otro aspecto novedoso de este capítulo es que, por primera vez, las respuestas obtenidas fueron caracterizadas mediante técnicas empleadas en el campo de la Ecotoxicología, calculando los ETx y ECx para la temperatura. El interés de esta aproximación es que suministra medidas normalizadas de las respuestas al estrés térmico, lo que facilita las comparaciones entre poblaciones y entre especies, así como entre las alteraciones observadas en diferentes variables fisiológicas. En el segundo capítulo se estudia, en condiciones de laboratorio, el efecto de la acidez en la bioacumulación de aluminio por *Fontinalis antipyretica*. La acidificación de las aguas continentales es un problema relativamente frecuente en países desarrollados. Este tipo de contaminación es problemática por el efecto directo que una alta concentración de protones tiene sobre los organismos acuáticos, pero también por el efecto indirecto provocado por la movilización de metales tóxicos, siendo el caso del aluminio uno de los más evidentes. De hecho se encontró que el pH ejercía un efecto sobre la acumulación tisular de Al más importante que la propia concentración de este metal en agua.

Los cinco capítulos restantes están relacionados entre sí, al encargarse de diferentes aspectos de cuatro elementos -arsénico, mercurio, antimonio y selenio- que a pesar de su relevancia ecotóxica, han recibido en general muy poca consideración en estudios de biomonitorización con briófitos acuáticos. Se caracterizaron sus cinéticas de carga, se obtuvieron los niveles de fondo regionales, se observaron las diferencias interespecíficas, se utilizaron en biomonitorización activa y se compararon sus respuestas tóxicas. La existencia de elementos traza (no por ello irrelevantes biológicamente) en agua es uno de los problemas que se presentan a la hora de abordar el estudio de la contaminación en ecosistemas acuáticos. Esto hace necesario recurrir a técnicas analíticas complejas y disponer de un equipamiento de laboratorio caro, lo que aumenta considerablemente el coste de la vigilancia ambiental, además de incrementar las posibilidades de contaminación de las muestras durante su procesado. El trabajo

presentado en el capítulo 3, sobre cinéticas de carga, puso de relieve la elevada capacidad de *F. antipyretica* de bioconcentrar As, Hg, Sb y Se, destacando en este aspecto el caso del Hg, para el cual se alcanzaron factores de bioconcentración muy elevados. Las altas concentraciones tisulares determinadas en los biomonitores resuelven los problemas analíticos anteriormente expuestos. Estos resultados están en consonancia con los mostrados en el experimento de biomonitorización activa de esta Tesis (capítulo 6), siendo el Hg el metal que más incrementó su concentración corporal respecto a la inicial. También se observó que la capacidad de bioconcentración relativa disminuía según aumentaba la concentración de exposición en agua. Hay que tener en cuenta que el factor de bioconcentración es el cociente entre la concentración tisular y la concentración en el medio y, por tanto, esta caída en los factores de bioconcentración no tiene porque involucrar una caída en las concentraciones tisulares.

En este trabajo también se encontró que el tiempo necesario para alcanzar el equilibrio en las concentraciones tisulares fue muy variable dependiendo del elemento y de la concentración en agua. Sin embargo, en pocos días de exposición los factores de bioconcentración fueron en general son muy altos. Esto aumenta la utilidad de los musgos en estudios de biomonitorización activa, dado que no serían necesarios tiempos de exposición muy altos, minimizando las posibilidades de deterioro o pérdida de los trasplantes, que es uno de los principales problemas que pueden surgir en este tipo de estudios. Los resultados presentados en el capítulo 6 de esta Tesis muestran una rápida acumulación en *F. antipyretica*, sobre todo de Hg, en los 4 primeros días de exposición de los trasplantes.

Por el contrario, los resultados de las cinéticas de carga y del experimento de biomoniotorización activa desaconsejan el empleo de *F. antipyretica* como biomonitor de la contaminación por Sb, al menos cuando las concentraciones en agua son bajas. El Sb mostró en los experimentos de carga un comportamiento indeterminado. Con las concentraciones de exposición más bajas tras un incremento inicial se produjo una caída en los factores de contaminación, que nunca llegaron a ser muy elevados. Este metaloide también mostró un comportamiento diferente al As y Hg en el experimento de biomonitorización activa del capítulo 6, encontrándose en dos puntos de muestreo que el musgo no cargaba. Incluso en uno de ellos, las concentraciones de Sb tisulares permanecieron por debajo de las concentraciones determinadas en musgo autóctono.

Los resultados expuestos hasta ahora avalan la utilidad de *Fontinalis antipyretica* como biomonitor de la contaminación en ríos por ciertos metales y metaloides. Esta

especie es muy común en ríos gallegos, sin embargo en los cursos de agua de la región podemos encontrar otras especies también relativamente frecuentes. Cuando se diseña un estudio de biomonitorización hay que contar con la posibilidad de que la especie previamente seleccionada no esté presente en los lugares de muestreo escogidos. Una manera de resolver el problema es realizar un intercalibrado de las capacidades de acumulación de diferentes especies. En el capítulo 4 se muestra un trabajo de este tipo, realizado con muestras depositadas en el Banco de Especimenes Ambientales de Galicia (BEAG) que custodia nuestro grupo de investigación. En el BEAG se dispone de numerosas localidades de muestreo donde coexistía más de una especie de briofito. Aunque nuestra intención fue analizar As, Hg, Sb y Se, desafortunadamente las concentraciones de Sb dieron en la mayor parte de las muestras por debajo del límite de detección del espectroscopio de fluorescencia utilizado. Una vez más se pone de manifiesto las dificultades de emplear briófitos acuáticos para la biomonitorización de este metaloide, como ya se puso anteriormente de relieve con relación al experimento de las cinéticas de carga y al experimento de biomonitorización activa. Los resultados de este estudio mostraron la ausencia de diferencias significativas en la capacidad de bioconcentración de As y Hg entre las especies estudiadas.

Los datos analíticos del estudio anterior también sirvieron para establecer los niveles de fondo regionales mediante la aplicación de diferentes aproximaciones estadísticas según se explica en el capítulo 5. El método que pareció más adecuado para determinar el nivel de fondo es el que considera como tal: *la concentración correspondiente a la primera moda de los datos tras ser tratados mediante un suavizado por núcleo*. Los niveles de fondo calculados mediante este método no presentaron diferencias relevantes entre las especies estudiadas, tal y como cabría esperar después de comprobar la ausencia de diferencias entre ellas en la capacidad de acumulación de As y Hg explicada en el anterior capítulo.

El capítulo 6 muestra la utilidad de los briófitos en estudios de biomonitorización activa, es decir, mediante la técnica de trasplantes. El musgo trasplantado desde una localidad limpia a varias localidades contaminadas incrementó siempre las concentraciones de As y Hg, superando las encontradas en los respectivos musgos autóctonos. Una ventaja conocida de este tipo de biomonitorización es precisamente la falta de adaptaciones en los organismos trasplantados -que normalmente proceden de una localidad limpia- a concentraciones altas de contaminantes, por ello suelen cargar más que los organismos autóctonos y también los efectos fisiológicos suelen ser

mayores, aunque de este aspecto no tenemos datos en este estudio. En definitiva, los organismos trasplantados son más sensibles y, por tanto, son más eficaces a la hora de detectar contaminación. Comparando las concentraciones máximas determinadas en los trasplantes con los niveles de fondo establecidos en el capítulo 5, encontramos que para Hg se alcanzó una concentración ligeramente superior al doble del nivel de fondo. En cambio para As la concentración máxima fue muy superior a su nivel de fondo, índice de que la contaminación por este metaloide fue más severa.

Los estudios de biomonitorización con relación a la acumulación de As, Hg, Sb y Se presentados hasta ahora únicamente atienden a las concentraciones tisulares alcanzadas. En el último capítulo de esta Tesis se intentó ir un poco más allá y se estudiaron los efectos fisiológicos que diferentes concentraciones de estos elementos pueden provocar en *Fontinalis antipyretica*. Estos experimentos de toxicidad se realizaron conjuntamente con los experimentos de las cinéticas de carga presentadas en el capítulo 3, dado que se disponía de muestras de musgo sometidas a diferentes concentraciones y tiempos de exposición. Por un lado, se analizó las respuestas toxicas en función de las concentraciones del medio y, por otro, en función de las concentraciones tisulares alcanzadas. La toxicidad de cada elemento fue parametrizada por las concentraciones medianas efectivas (EC50). La toxicidad de los elementos es diferente según se atienda a su concentración en agua o a su concentración en el musgo. Por ejemplo, el Hg siempre resultó más tóxico que el As -tanto para la fotosíntesis neta como para la fluorescencia clorofílica- si se tiene en cuenta la concentración en el agua. En cambio para las concentraciones tisulares, el As mostró ser siempre mucho más tóxico que el Hg.

La mayor toxicidad tisular del As contrasta con el nivel de fondo calculado para este metaloide (Capítulo 5) que fue significativamente mayor que para el Hg. *Fontinalis antipyretica* puede por tanto acumular grandes concentraciones de Hg por encima de lo que se podría considerar normal en una situación de no contaminación antes de que empiece a mostrar efectos fisiológicos. Este hecho, aunque puede considerarse un inconveniente desde el punto de vista del empleo de esta especie como bioindicador-sensitivo, es una ventaja desde la perspectiva de usarla como bioindicador-acumulador, puesto que le permite acumular grandes concentraciones de este metal, como ya se había puesto de manifiesto en el capítulo dedicado a las cinéticas de carga, sin alterar su comportamiento.

Conclusiones

Capítulo 1. *Inercia y resilencia en las respuestas del briófito acuático* Fontinalis antipyretica *Hedw. a estrés térmico.*

- En función del índice D665/D665a, la exposición a 33.5 ºC tuvo un efecto claramente estresante desde el primer momento. A 28.4 ºC se empezaron a ver efectos después del día 15. A 24.9 y 19.8 ºC los efectos fueron débiles o nulos.
- En función de la fotosíntesis neta, *Fontinalis antipyretica* fue capaz de aclimatarse a temperaturas moderadamente altas (19.8 a 24.9 ºC) en tres semanas. Esta aclimatación no tuvo lugar a 28.4 o 33.5 ºC.
- Las plantas expuestas a 30ºC durante 10 días no consiguieron recuperarse durante el tiempo de rehabilitación experimental. Las incubadas a esa temperatura entre 2 y 4 días lograron recuperarse (en función del índice D665/D665a) en unos 15 días, aunque las tasas de fotosíntesis y respiración no se normalizaron hasta el final del experimento.
- El empleo por primera vez de técnicas para el cálculo de parámetros ecotoxicológicos (ET50, $EC50_{96h}$, $EC50_{10d}$) permitieron la caracterización simple y precisa de las respuestas de *F. antipyretica* a la temperatura en términos de inercia y resilencia. El empleo de estas técnicas son útiles para suministrar medidas normalizadas de las respuestas al estrés térmico, facilitando así las comparaciones entre poblaciones y entre especies, y entre respuestas fisiológicas.

Capítulo 2. *Efecto del pH en las cinéticas de carga intra- y extracelular de Al en* Fontinalis antipyretica*. Cambios en los contenidos celulares de K, Mg y Ca.*

- La acumulación de Al, tanto extra- como intracelular, estuvo directamente relacionada con el pH del medio, con las mayores acumulaciones a pH de 4.4. La concentración de Al en el agua fue un factor secundario.
- El efecto claro y rápido del pH del medio en las concentraciones intra- y extracelulares de Al en *Fontinalis antipyretica*, sugiere que este musgo podría ser utilizado para monitorizar ambientes ácidos.
- Para este fin, los modelos de bioconcentración construidos pueden ser útiles, aunque se recomienda su mejora para que suministren resultados más fiables.

- En ambientes muy ácidos (pH<5) el musgo liberó Ca y Mg extracelulares, así como Ca intracelular. El efecto del Al en solución, en el rango utilizado en este estudio, fue de mucha menor importancia.
- El K y Mg intracelulares fueron liberados en todo el rango de acidez y concentraciones de Al utilizados; la intensidad del efecto fue inversamente proporcional al pH del medio.
- La acumulación de Al en el interior de la célula no parece ocasionar la liberación de elementos esenciales intracelulares (Ca, K, Mg).

Capítulo 3. *Cinéticas de carga de As, Hg, Sb y Se en el musgo acuático* Fontinalis antipyretica *Hedw.*

- El modelo de la cinética de carga más común fue tipo Michaelis-Menten. As y Se mostraron los patrones de carga más homogéneos, mientras que Sb mostró un comportamiento muy irregular.
- Los Factores de Bioconcentración tendían a disminuir a medida que aumentaba la concentración del medio.
- A pesar de las diferentes capacidades de bioconcentración y de los tiempos en alcanzar el equilibrio, dependientes del elemento y la concentración en agua, en unos pocos días de exposición se alcanzaron altos Factores de Bioconcentración, particularmente con Hg. Esto pone de manifiesto la elevada capacidad de *F. antipyretica* de bioacumular estos elementos, aunque se presenten en bajas concentraciones en el agua, facilitando así su detección.
- Como el tiempo en alcanzar la concentración corporal en equilibrio con la del medio, depende del elemento y de la concentración de exposición, el tiempo mínimo recomendado para la biomonitorización activa sería muy variable, aunque en unos pocos días se pueden esperar altas concentraciones tisulares de estos elementos, excepto para Sb. Debido a esto y a los patrones de bioconcentración irregulares observados con este elemento, no es recomendable el uso de *F. antipyretica* para biomonitorizar bajos niveles de Sb en agua.

Capítulo 4. *Arsénico y mercurio en briófitos acuáticos nativos: diferencias entre especies.*

- Las concentraciones tisulares de As fueron mucho mayores que las encontradas de Hg.
- No se encontraron diferencias en función de la litología de las cuencas.
- El riesgo ambiental que supone la contaminación por As en los ríos gallegos parece ser más grave que la contaminación por Hg.
- No se encontraron diferencias significativas en las capacidades de bioconcentración de As y Hg entre las especies estudiadas, por lo que el uso combinado de especies parece adecuado para monitorizar los elementos estudiados en los ríos de Galicia y en otras regiones con ríos de características similares.

Capítulo 5. *Niveles de fondo de mercurio y arsénico en briófitos de los ríos gallegos.*

- De todos los métodos ensayados, el más adecuado resultó ser el basado en establecer el nivel de fondo como: "la concentración tisular correspondiente a la primera moda de los datos sometidos a un suavizado por núcleo (*kernel smoothing*)".

Capítulo 6. *Biomonitorización activa de arsénico, mercurio y antimonio en el curso bajo del río Eume mediante trasplantes del musgo acuático* Fontinalis antipyretica *Hedw.*

- El musgo bioconcentró As y Hg en todas las estaciones de trasplante. La bioacumulación de Hg fue más rápida.
- Las concentraciones corporales de As y Hg determinadas en los musgos trasplantados fueron mayores que en los musgos autóctonos.
- No se recomienda el uso de *F. antipyretica* como biomonitor de la contaminación por Sb en los ríos gallegos.

Capítulo 7. *Efectos fisiológicos de la exposición a arsénico, mercurio, antimonio y selenio en el musgo acuático* Fontinalis antipyretica *Hedw.*

- En general, *F. antipyretica* no presenta claros síntomas de estrés hasta que se le somete a concentraciones de exposición en agua muy elevadas (en torno a 1000 $\mu g\ l^{-1}$).
- En función de la EC50 para la fotosíntesis el orden de toxicidad fue: Hg > As > Se >Sb, mientras que para la fluorescencia clorofílica fue: Sb > Hg >As.
- En función de la EC50 de las concentraciones tisulares para la fotosíntesis, el orden de toxicidad fue: Se > As > Hg > Sb, mientras que para la fluorescencia clorofílica fue: As > Hg.
- La bioacumulación de As y Se fue baja, aunque con importantes efectos fisiológicos. El Hg se acumuló en mayores concentraciones aunque con efectos proporcionalmente menores.
- La tasa de respiración no resultó ser una medida de estrés útil dada la ausencia de una tendencia clara con la concentración de exposición o con la concentración tisular.
- La fluorescencia clorofílica mostró menor variabilidad que las respuestas respirométricas.
- Los niveles tisulares de Sb ocasionan diferentes efectos fisiológicos dependiendo de la concentración de la exposición. Parece que el musgo es capaz de aclimatarse a concentraciones de exposición de Sb relativamente elevadas.