TESE DE DOUTORAMENTO

ARSENIC IMMOBILIZATION AND TRANSFORMATION BY BIOFILMS DEVELOPED OVER RIVERBED SEDIMENTS

Asdo.....

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Aos meus pais

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SUMMARY

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SUMMARY

Biofilms are complex communities of autotrophic and heterotrophic organisms immersed in a matrix mainly composed by polysaccharides. They are ubiquitous over wet surfaces and provide protection for microorganisms. In river environments there is evidence that biofilms play an important role in the biogeochemical cycles of nutrients and contaminants. The objective of this work is to evaluate the effect of the fluvial epipsammic biofilm on the retention and transformation of As and to understand its interaction with this metalloid in the Anllóns River (Galicia, NW Spain), where As pollution has been repeatedly observed.

In this work As concentrations between 2 and 489 mg kg^{-1} were detected in soils of the Anllóns River basin. Fractionation studies indicated that As is mainly bound to crystalline Fe oxides. The potential risk of As mobility to water systems was low (< 0.25 % of total As), when it was evaluated by means of the standard leaching methods DIN 38414-S4 and Toxicity Characteristic Leaching Procedure (TCLP), but increased up to 124 times using higher liquid:solid ratios and pHs than those of the standard methods. The addition of 10 mM phosphate increased As leachability up to 1,000 times, and it became up to 2.3 times higher when the contact time with phosphate solutions was lengthened from 24 to 240 hours.

The abundance and composition of the superficial biofilm on the Anllóns riverbed sediments was investigated at four points in winter and summer samplings. The main taxa belonged to Chlorophyta, Cyanophyta, Euglenophyta and Heterokontophyta. The highest total algal abundance and genus richness were observed in summer at the river mouth. Positive relationships were found between the biochemical properties (phytopigments and respiratory activity) and total algal abundances determined by taxonomic identification and counting. Site conditions, particularly the trophic state, were relevant in the development of benthic microflora.

The influence of light availability and water nutrient content on biofilm growth was demonstrated in laboratory experiments using experimental channels, having these factors positive effects in chl-a, total carotenoids, total and biologically active organic carbon, soluble carbohydrates, crude proteins and phosphatase activity.

In laboratory experiments using river water as nutrient supplier and As concentrations from 0 to 500 µg L^{-1}, the epipsammic biofilm increased As^{V} sorption (~97 %) in comparison

with sediments devoid of biofilm (~80 %). The presence of equimolar P concentrations decreased the As sorption capacity of the sediment devoid of biofilm in a 25 %, whereas it had no negative effect in the systems with biofilm.

In microcosms experiments, using bioreactors, aiming at evaluating the effct of the biofilms on As retention and speciation from As^{V} polluted water, up to 91% of As^{V} was removed by the systems with epipsammic biofilms, while only ~70% was removed by the sediments without biofilm. The distribution of As in the biofilm showed that ~71% of the retained As was extracellular, most of it (>99%) in the form of As^{V}. The biofilms also inhibited the reduction of As^{V} to As^{III} in the overlying water and methylate inorganic As.

Also, As transfer from contaminated sediments (106 mg kg^{-1} As) to the water column was reduced by 64 % in the presence of biofilm, which also inhibited the formation of As^{III}. Arsenic retained by the biofilm was equally distributed between extracellular and intracellular compartments. Inside the cells significant concentrations of As^{III}, MMA^{V} and DMA^{V} were detected, suggesting that active methylation (detoxification) processes are occurring in the intracellular compartment.

In conclusion, biofilms play a key role in As biogeochemistry in freshwater environments, favouring As immobilization, especially in environments where As and P occur simultaneously, and promoting As detoxification by inhibiting the reduction of As^{V} to As^{III} and methylating inorganic As.

Keywords: retention, uptake, transformation, speciation, methylation, arsenate, arsenite

RESUMEN

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RESUMEN

Un biofilm es una comunidad de microorganismos inmersos en una matriz polimérica de exopolisacáridos (EPS), que se desarrolla sobre una superficie o interfase húmeda, y que permite un intercambio eficiente de agua, nutrientes y gases entre las poblaciones que constituyen el biofilm y el ambiente exterior (Costerton 2007). Los biofilms epipsámicos, que se forman sobre materiales granulares del lecho fluvial, están constituidos por un agregado de algas, bacterias, hongos y protozoos, rodeados por una matriz porosa de EPS, compuesta por polisacáridos, proteínas, ácidos nucleicos y otros biopolímeros de origen microbiano. Se ha demostrado que los biofilms protegen a las comunidades microbianas del estrés ambiental, caracterizándose por una intensa actividad biológica, llevada a cabo por la microflora autotrófica y heterotrófica (Flemming et al, 2001).

A pesar de su aparente ubicuidad, la existencia de biofilms epipsámicos sobre las superficies granulares en sedimentos fluviales ha sido pocas veces reconocida en los trabajos que estudian el papel de los sedimentos fluviales como fuente o sumidero de potenciales contaminantes. Sin embargo, los investigadores que han estudiado la composición, distribución y papel ambiental de esta capa compleja han destacado su efecto en los ciclos biogeoquímicos de diversos elementos, como carbono y nutrientes (Romaní et al. 2004), o contaminantes metálicos (Sabater et al. 2007; Serra et al. 2009), que a su vez afectan al grado de desarrollo y metabolismo del biofilm.

La importancia del estudio del arsénico (As) en relación con el biofilm se basa en la elevada toxicidad de este elemento, cuya presencia en el ambiente en concentraciones elevadas representa un grave riesgo para la salud humana y de la calidad de los ecosistemas. Por otra parte, la elección de este elemento de estudio se justifica en el elevado riesgo de contaminación por As en la cuenca del Río Anllóns (Galicia, NO España) -cuyos sedimentos de fondo serán utilizados en este estudio-, debido a antiguas labores mineras de extracción de oro asociado a arsenopiritas que han producido contaminación de los sedimentos alcanzando concentraciones de hasta 264 mg kg^{-1} en algunos tramos del cauce (Devesa et al. 2008). Recientes intentos de reactivación de la actividad minera a gran escala en la zona han suscitado de nuevo la preocupación de investigadores y habitantes de la comarca afectada, ante las potenciales repercusiones ambientales de dicha actividad. En estas circunstancias es de gran importancia conocer el riesgo de movilización, biodisponibilidad y toxicidad de las

formas de arsénico en agua y sedimentos, analizando un aspecto menos conocido, como es la influencia del biofilm fluvial en el comportamiento biogeoquímico de este elemento.

La toxicidad del As depende de su forma química (inorgánica u orgánica) y de su estado de oxidación. En general, las formas inorgánicas de As han sido consideradas más tóxicas que las orgánicas (Ng, 2005), con la excepción de las formas metiladas de As^{III}. Entre las formas inorgánicas, el As^{III} se considera en general más tóxico que As^{V} (Wang y Mulligan, 2006), lo que se relaciona con su alta afinidad por los grupos sulfhidrilo de las biomoléculas y por la cisteína de muchos enzimas. Por todo ello, es evidente que al estudiar la interacción entre As y biofilm es de gran relevancia analizar no solo las concentraciones totales en agua o sedimento, sino también determinar las especies químicas en ambos compartimentos.

El fósforo puede, a su vez, desempeñar un papel fundamental en la interacción biofilm epipsámico-As porque: 1) Es un nutriente esencial y tiene un efecto estimulante en el crecimiento de los microorganismos que componen el biofilm. 2) Compite con el As por los sitios de adsorción. Esto puede implicar una disminución de la retención de As por los sedimentos y un incremento de la movilidad de As presente en los sedimentos con altas concentraciones de este elemento, según se ha descrito previamente para sedimentos del río Anllóns por Rubinos et al. (2003, 2010). 3) La presencia de fosfato disminuye la toxicidad del As^{V} (Karadjova et al. 2008; Rubinos et al. 2014).

Esta investigación es continuación de la línea de trabajo sobre calidad de sedimentos fluviales desarrollada por el grupo de investigación GI-1243 GEMAP (anteriormente GI-1242), iniciada con el proyecto “Los sedimentos como registros de contaminación en el sistema hidrológico Río Anllóns-Ría de Laxe" (Ministerio de Ciencia y Tecnología REN2003-08673, 2003-2006), en el que se estudiaron los fenómenos de sedimentación del río Anllóns y se determinó la concentración de nutrientes y elementos traza en los sedimentos de fondo, que se relacionaron con las fuentes de contaminación puntual y difusa (Devesa-Rey et al. 2009; Barral et al. 2012). En este estudio se puso por primera vez en evidencia la contaminación por As derivada de actividades mineras en una explotación aurífera, en la que el oro se encuentra asociado a arsenopirita, situada en las inmediaciones de la localidad de Corcoesto (Devesa-Rey et al. 2008; Rubinos et al. 2010; 2011). Aguas abajo de esta zona llegan a alcanzarse en los sedimentos concentraciones de hasta 264 mg As kg^{-1}, muy

superiores a las concentraciones base de As (5-8 mg kg^{-1}) en suelos y sedimentos, y valores de ecotoxicidad de hasta 20 equitox.

Posteriormente, en el proyecto "Efecto de la resuspensión en la biodisponibilidad y toxicidad de contaminantes en sedimentos fluviales: influencia de las características físico-químicas y biológicas del sedimento (CGL2007-62928, MICINN 2007-2010) se puso de manifiesto que la liberación de As desde los sedimentos contaminados depende del pH del medio. Se observó también que la cinética del proceso está controlada principalmente por la disolución de diversos componentes de los sedimentos, especialmente óxidos e hidróxidos de Fe y Al a pH ácido y materia orgánica a pH alcalino (Rubinos et al. 2010). Este estudio también puso de manifiesto que los aportes de P soluble incrementan la removilización de As en sedimentos contaminados, especialmente cuando se producen simultáneamente con cambios de pH. En el marco de este proyecto se inició el estudio del biofilm epipsámico en el río Anllóns, el cual se profundizó con el proyecto "Estudio a nivel de microcosmos de la detoxificación de As y Cr en biofilms desarrollados sobre sedimentos fluviales y sus implicaciones como mecanismo de biocorrección" (CGL2010-22059, MICINN 2010-2013), en el marco del cual se ha realizado el trabajo de investigación que se presenta en esta memoria.

La hipótesis de este trabajo es que los biofilms desarrollados sobre sedimentos fluviales pueden retener activamente As e inducir cambios en su especiación que conduzcan a una disminución de su toxicidad. En este estudio se pretende contribuir al conocimiento del papel desempeñado por el biofilm epipsámico en la biogeoquímica de As, concretamente en la movilización, biodisponibilidad y potencial toxicidad de este elemento en aguas y sedimentos. Por otra parte, este trabajo ayudará a comprender en profundidad la interacción entre este contaminante y el biofilm en la cuenca del río Anllóns, donde se han observado repetidamente elevadas concentraciones de As en suelos y sedimentos. Asimismo, se obtendrá información que permitirá valorar su potencial aplicación en la biocorrección de aguas y sedimentos contaminados, desde dos perspectivas: inmovilización y detoxificación. El primero de los mecanismos implica la formación de especies químicas menos móviles, y por tanto menos biodisponibles, mientras que el segundo de ellos conlleva la formación de especies menos tóxicas. De hecho, ambos mecanismos pueden ocurrir conjuntamente en la interfase agua-sedimento, y dependen de la naturaleza de los componentes orgánicos y

minerales del sedimento, de la abundancia y composición de la microflora béntica y las propiedades físico-químicas del agua de rio.

Para alcanzar estos objetivos, se llevaron a cabo estudios de campo para evaluar el riesgo de transferencia de As desde suelos enriquecidos a las masas de agua, se monitorizaron y caracterizaron el biofilm fluvial desarrollado sobre los sedimentos del río Anllóns, y se llevaron a cabo estudios en canales experimentales para reproducir y caracterizar los biofilms naturales a nivel de laboratorio. Estos experimentos se completaron con estudios a escala de microcosmos, en biorreactores específicamente diseñados, para estudiar la retención y transformación de As en sistemas agua/sedimentos contaminados con As. El aspecto más novedoso de este trabajo es el estudio combinado de procesos físico-químicos (adsorción) y procesos bioquímicos (reducción, metilación…) que tienen lugar en la superficie del sedimento y en presencia de biofilm epipsámico, los cuales pueden afectar a la biogeoquímica y toxicidad de As en el ecosistema fluvial.

Este trabajo se ha dividido en seis capítulos, con sus propios objetivos particulares y plan de trabajo para alcanzarlos. El objetivo del primer capítulo fue detectar el As litogénico en suelos de la cuenca del río Anllóns, donde se han explotado históricamente mineralizaciones Au-As, y evaluar el riesgo potencial de la movilización de As a las aguas subterráneas y al curso fluvial. Con este fin, 50 muestras de suelos de horizonte C, recogidas a lo largo de la cuenca según una rejilla de 1 km x 1 km, fueron sometidas a un análisis exploratorio, para determinar las concentraciones totales a lo largo de la cuenca. Posteriormente, se realizó un análisis de detalle de las 9 muestras de suelos con las concentraciones de As más altas. La concentración de As en los suelos varió entre 2 y 489 mg kg^{-1}, superando hasta en 8 veces el nivel genérico de referencia en suelos de Galicia (Macías Vázquez y Calvo de Anta 2009). La lixiviación de As fue baja (<0.25 % del contenido de As total), cuando se evaluó siguiendo los métodos estándar de lixiviación DIN 38414-S4 y "Toxicity Characteristic Leaching Procedure (TCLP)". Los estudios de fraccionamiento mostraron que el As se encuentra principalmente unido a óxidos de Fe cristalinos, justificando de este modo su baja solubilidad. El efecto del pH, relación líquido:sólido (L:S), tiempo de contacto y fosfato en la movilización de As fueron estudiados con detalle en esas 9 muestras con las concentraciones de As más altas, mostrando un incremento de la solubilidad de As de hasta 124 veces con el aumento de la relación L:S y del pH, en comparación con la obtenida con el método estándar DIN 38414-S4.

El factor más decisivo en la movilización de As fue la adición de fosfato, en concentración 10 mM, que incrementó la solubilidad de As hasta en 1000 veces en comparación con la obtenida con el método estándar DIN 38414-S4. Este efecto aumentó hasta en 2,3 veces con el incremento del tiempo de contacto de 24 a 240 horas. Se concluye de este capítulo que, aunque los métodos de lixiviación estándares proporcionan información útil en términos de límites legales y permiten comparar datos entre laboratorios, pueden subestimar la movilidad de As en escenarios más realistas, entre los que se encuentran los suelos afectados por actividades mineras o por vertidos de contaminantes. Estos aspectos deberían ser tenidos en cuenta para realizar una evaluación más realista de los riesgos ambientales y para la salud provocados por la movilización de As a sistemas acuosos.

Los objetivos del capítulo 2 fueron: (1) investigar la abundancia y composición del biofilm epipsámico en los sedimentos del río Anllóns, (2) evaluar las relaciones entre los parámetros bioquímicos y los métodos biológicos basados en identificación y recuento, y (3) explorar las relaciones entre el crecimiento del biofilm y las propiedades del hábitat sedimentario, principalmente su estado trófico. Con este propósito, se recogieron muestras de sedimento superficial (0-5 cm) en dos estaciones del año (invierno y verano), en 4 puntos de muestreo a lo largo del curso fluvial. Se determinaron las propiedades físico-químicas de los sedimentos y del agua de poro. El estudio de las propiedades biológicas del biofilm incluyó la determinación de la actividad respiratoria, mediante la medida de la actividad deshidrogenasa (siglas en inglés DHA), y la concentración de fitopigmentos (Chl *a*, Chl *b* y carotenoides), así como la identificación taxonómica de los organismos autótrofos. Para llevar a cabo la identificación taxonómica se emplearon dos métodos de muestreo: pipeta y camisas de pequeño tamaño. A partir de estos se determinaron la abundancia algal total y relativa (siglas en inglés TA y RA, respectivamente) y la riqueza de géneros. Los principales taxones encontrados pertenecieron a Chlorophyta, Cyanophyta, Euglenophyta y Heterokontophyta. La clase algal más abundante fue Bacillariophyceae, que representó más del 86 % de la abundancia total de algas en los sedimentos superficiales. Las mayores abundancias algales totales y los valores más altos de riqueza de género fueron observadas en verano en la desembocadura, donde también se encontraron las concentraciones más elevadas de DHA y fitopigmentos. Los resultados de este capítulo pusieron de manifiesto las relaciones positivas entre las propiedades bioquímicas (actividad respiratoria y fitopigmentos) y las abundancias algales totales determinadas por identificación taxonómica y recuento. Todas las propiedades

analizadas evidenciaron una clara influencia sobre las propiedades relacionadas con la abundancia y actividad biológica de los contenidos en nutrientes y materia orgánica de los sedimentos, poniendo de manifiesto la importancia de las condiciones del sitio, particularmente el estado trófico, en el desarrollo de la microflora béntica.

En el capítulo 3 se investigó la influencia de la disponibilidad de luz y la concentración de nutrientes del agua en el crecimiento del biofilm epipsámico. Para ello, se monitorizó durante 21 días la formación de biofilm epipsámico sobre sedimentos fluviales recogidos en el río Anllóns. Los experimentos se llevaron a cabo en 2 canales experimentales, diseñados específicamente para este trabajo: el canal 1 se alimentó con agua de rio y el canal 2 se alimentó con una disolución enriquecida en nutrientes. Cada canal se dividió en 3 secciones, recibiendo cada una de ellas una diferente intensidad de luz. Para llevar a cabo la monitorización del crecimiento del biofilm, se determinaron a lo largo del experimento en muestras de sedimento enriquecido en biofilm el carbono orgánico total y el C activo biológicamente, los fitopigmentos (clorofila a y b y carotenoides totales), los carbohidratos solubles, las proteínas, la actividad fosfatasa y el fósforo biodisponible. En el canal 1, en el que circula agua de río, se observó un efecto positivo de la luz en la concentración de clorofila a, carotenoides totales, carbono orgánico total y biológicamente activo, proteínas y actividad fosfatasa. En el canal 2 la adición de nutrientes aumentó las concentraciones de clorofila a, carbohidratos solubles y proteínas, en comparación con las secciones que recibieron la misma luz del canal 1, propiedades que también experimentaron un incremento con la mayor disponibilidad de luz dentro del canal. Los resultados de este capítulo demuestran que la formación de biofilm epipsámico en condiciones de mesocosmos depende de la disponibilidad de luz y de la composición del agua. Los canales fluviales experimentales diseñados para estos experimentos *in door,* y alimentados únicamente con agua de rio, pueden ser empleados para obtener biofilm epipsámico en aplicaciones medioambientales o biotecnológicos, con la ventaja de que se pueden evitar las interferencias provocadas por la presencia de aniones competidores, como el fosfato, o de fuerzas iónicas altas debidas a la adición de suplementos de nutrientes.

El As movilizado desde los suelos de la cuenca del río Anllóns puede alcanzar el curso fluvial (capítulo 1), especialmente en la presencia de fosfato, e interactuar de este modo con el biofilm epipsámico, completamente caracterizado en el estudio de campo presentado en el

capítulo 2 y reproducido en canales experimentales a escala de laboratorio en el capítulo 3. Por tanto, la interacción biofilm-As es una cuestión de interés que ha sido abordada en los capítulos 4, 5 y 6. En los capítulos 4 y 5, se estudió el efecto del biofilm en la retención y transformación de As presente en aguas contaminadas, mientras que en el capítulo 6 se estudió el papel en la movilidad y transformación de As presente en sedimentos contaminados.

El objetivo del capítulo 4 fue evaluar el papel del biofilm epipsámico en la retención de As^V disuelto y evaluar el efecto de la presencia de concentraciones equimolares de P, comparando los resultados obtenidos en muestra con biofilm con los observados en sedimento desprovisto de biofilm. El crecimiento del biofilm se llevó a cabo durante 14 días, atendiendo a los resultados obtenidos en el capítulo 3, empleando como medio de cultivo agua de río. Se realizaron experimentos de retención con concentraciones iniciales de As de 0, 5, 25, 50, 100, 250 y 500 $\mu g\ L^{-1}$. El porcentaje promedio de As retenido fue de 78.9 ± 3.5 y 96.9 ± 6.6 % para sedimento desprovisto y enriquecido en biofilm, respectivamente. La presencia de fosfato disminuyó en un 25 % la capacidad de retención del sedimento desprovisto de biofilm, mientras que no tuvo un efecto negativo en la retención en los sistemas con biofilm, e incluso llegó a tener un efecto positivo a la concentración de As más alta ensayada. Los modelos de Freundlich, Sips y Toth fueron los que mejor se ajustaron a los datos experimentales de curvas de adsorción en función de la concentración de As. Se concluye que los biofilms epipsámicos juegan un papel importante en la calidad ambiental de los ecosistemas fluviales, aumentando la retención de As, especialmente en ambientes donde As y P están presentes simultáneamente.

En el capítulo 5 se profundizó en el estudio del efecto del biofilm en la retención, absorción, movilidad y transformación de As^V, utilizando biorreactores específicamente diseñados para tal fin. Con este propósito, se incubó un biofilm nativo sobre sedimentos fluviales durante 21 días en biorreactores dispuestos en cámara de incubación bajo condiciones controladas de luz (ciclos noche:día de 12 h de duración con una intensidad de 40 $\mu mol\ fotón\ m^{-2}\ s^{-1}$), temperatura (20 ºC) y aireación (1 $L\ min^{-1}$). Posteriormente una vez desarrollado el biofilm, se añadieron 500 $\mu g\ L^{-1}$ de As^V a dos biorreactores y a uno de ellos se le adicionó también P en concentración equimolar As:P. Estos dos sistemas con biofilm se compararon con sistemas control preparados con sedimento y agua estéril. La evolución de la

concentración de As en el agua sobrenadante se siguió durante las 2 semanas siguientes, así como su especiación. En presencia de biofilm la eliminación de As^{V} en disolución alcanzó el 91 % de su concentración inicial, mientras que fue solo del ~70 % en el sedimento esterilizado control. La presencia de concentraciones equimolares de P tuvo un efecto positivo en la retención de As por el biofilm, alcanzando la eliminación de As en disolución hasta el 97 %, mientras que no tuvo efecto en los sistemas sin biofilm. En los sistemas con biofilm la especie de As predominante fue As^{V} (~97%), mientras que el As^{III} sólo representó un ~1 % de la concentración de As total disuelto. Las especies orgánicas, MMA^{V} y DMA^{V}, representaron un 0,6 y 0,7 % del As total, respectivamente. En contraposición, en los sistemas desprovistos de biofilm, el As^{III} representó hasta un 39 % del As disuelto y no se detectaron especies metiladas. La inhibición, en presencia de biofilm, de la aparición de de concentraciones relevantes de As^{III} tiene un especial interés desde el punto de vista geoquímico y medioambiental, ya que el As^{III} es considerado generalmente más móvil y tóxico que el As^{V}. El estudio de la distribución de As en el biofilm mostró que ~71 % del As se retuvo extracelularmente, la mayoría en forma de As^{V} (>99.5 %). En el interior de las células del biofilm se han detectado especies metiladas que indican que se están produciendo procesos de detoxificación. Únicamente en los sistemas con biofilm se detectaron especies de As volátiles. Se concluye que los biofilms que recubren los sedimentos fluviales incrementan la retención de As con respecto al sedimento desprovisto de biofilm, inhiben la reducción de As^{V} a As^{III} y metilan el As inorgánico, desempeñando así un papel fundamental en la biogeoquímica de As en ambientes fluviales.

En el capítulo 6 se evaluó la influencia del biofilm epipsámico en la liberación de As procedente de sedimentos fluviales con altas concentraciones de este elemento. Para ello, se realizaron experimentos en microcosmos, cultivando biofilms sobre sedimentos del río Anllóns que contienen 106 mg kg^{-1} de As, comparando su comportamiento con el de sistemas control sin biofilm. La transferencia de As a la columna de agua fue baja (< 0.11 % del As total en el sedimento) y se redujo en un 64 % en presencia de biofilm. La especie de As predominante en el agua sobrenadante fue As^{V} en ambos sistemas. La concentración de As^{III} fue más alta (representado hasta un 12 % del As total disuelto) en los sistemas control, mientras que no se detectó en los sistemas con biofilm. Este aspecto tiene relevancia toxicológica debido a que esta especie reducida tiene generalmente mayor movilidad y toxicidad. Los sistemas control sin biofilm mostraron una mayor movilidad de As en agua, en

disoluciones de sulfato y en ácido débil, y mayor biodisponibilidad empleando dispositivos DGT. El As retenido por el biofilm se distribuyó equitativamente entre los compartimentos extracelular e intracelular. Dentro de las células se han detectado concentraciones significativas de As^{III}, MMA^{V} y DMA^{V}, lo cual sugiere que en el compartimento intracelular tuvieron lugar procesos activos de metilación (detoxificación) .

Se concluye del conjunto de este trabajo que los biofilms epipsámicos desempeñan un papel relevante en la calidad ambiental de los sistemas fluviales y concretamente en la biogeoquímica de As, al (1) Aumentar su retención especialmente cuando As y P están presentes simultáneamente en el medio, (2) Afectar a la especiación de As en la columna de agua, al inhibir la aparición de altas concentraciones de As^{III} y, (3) Al metilar el As inorgánico convirtiéndolo en formas menos tóxicas, como se ha comprobado en el agua sobrenadante de los sistemas con biofilm y en el interior de las células que lo componen, así como por la detección de especies de As volátil. El aumento de la retención y reducción potencial de la toxicidad de As en la presencia de biofilm ponen de manifiesto su importancia en sistemas acuáticos naturales y su potencial aplicación en sistemas biotecnológicos de depuración de aguas.

Palabras clave: retención, absorción, transformación, especiación, metilación, arsenato, arsenito

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1.INTRODUCTION

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1. INTRODUCTION

1.1 Arsenic: A global problem

Arsenic (As) is a metalloid recognized as one of the most toxic elements and a non-threshold class 1 carcinogen (Rosen 1971). The problem of toxicity is agraveted because this element is widely distributed in natural environments (Smedley and Kinniburg 2002). In the earth's crust As is the 20th most abundant trace element (NRC 1977) whose average concentration has been fixed at 1.7 mg kg^{-1} (Wedepohl 1995). The baseline As concentration in soils is generally in the order of 5-10 mg kg^{-1} (Smedley and Kinninburg 2002).

Environmental As problems are commonly the result of mobilization under natural conditions, such as weathering of As-bearing minerals and geothermal sources, but human activities have contributed with a major additional impact with mining activities, fossil fuel combustion and the use of As in pesticides, herbicides, crop desiccants and livestock feed (Smedley and Kinninburgh 2002). The presence of As in soils, sediments and water, even at very low concentrations, may cause serious health hazards, due to its toxicity, increasing the incidence of cancer, and dermatological, vascular and cerebrovascular diseases. In particular, there is increasing evidence of cancer risk associated with chronic exposure of low levels of As (Cantor 1996).

Arsenic becomes problematic from a health perspective principally when it partitions into the aqueous rather than the solid phase. For this reason, As mobilization from soils and sediments is of great environmental concern, especially as a result of the contamination of drinking water and food (Fendorf et al. 2010). Thus, problematic As concentrations (>0.01 mg L^{-1}) have been detected in aqueous systems, and more specifically in groundwater systems (Mukherjee et al. 2012), which may be originated from the dissolution of primary or secondary sulfidic minerals (such as pyrite) containing As, or from its desorption from hydrous metal oxides (Nordstrom 2002), that are common mineral constituents of aquifer matrix in many large, unconsolidated sedimentary systems. Besides mineral dissolution and desorption two other processes have been identified as responsible for the mobilization of As from soils and sediments to the aqueous phase: (1) ion displacement, (2) desorption (or limited sorption) at pH values > 8.5, (3) reduction of arsenate to arsenite and (4) mineral dissolution, particularly reductive dissolution of Fe and Mn (hydr)oxides (Fendorf et al. 2008).

Regarding As toxicity for humans, the acute lethal dose of inorganic As has been estimated to be about 600 μg kg^{-1} day^{-1} (ATSDR 2007). For this group, As exposure generally occurs by ingestion, inhalation, through direct dermal contact or transmited from the mother to the foetus via the placental route (Tchounwou et al. 2003). The harmful effects produced by As on human health are reflected especially in the skin, acting as a sentinel organ for the effects of chronic As exposure (Karagas 2015). Arsenic effects on human health causes include acute (from gastrointestinal distress to death) and chronic adverse effects (damage of the liver, kidney, the respiratory, digestive, circulatory, neural, and renal systems, and the skin, and the occurrence of skin, brain, liver, lung, bladder, kidney and stomach cancers).

Arsenic contamination in drinking water is a major global public health issue and is exerting a devastating effect on human health in several parts of the world, particularly in Asia, where chronic As poisoning has become epidemic with over 100 million people exposed to drinking water with high As concentrations (Wang et al. 2007). A wide range of groundwater concentrations of As has been reported, from less than 0.5 to 5000 μg L^{-1} (Ravenscroft et al. 2009). As for ecotoxicological effects, adverse effects of arsenicals on aquatic organisms have been reported at low concentrations in water (19 to 48 μg L^{-1}) (Eisler 1994). Consequently, the World Health Organization recommends a limit of 10 μg As L^{-1} in drinking water (WHO 1993). According to this WHO guideline this implies that more than 100 millions people are at risk, of which 45 millions, mainly in developing countries from Asia, are at risk of being exposed to more than 50 μg L^{-1} of As, which is the maximum concentration limit in drinking water by the law in most countries in Asia (Ravenscroft et al. 2009). The problem of As polluted waters in different parts of the world is summarized in Table 1.1 (Sharma and Sohn 2009).

In Spain, it is considered that surface waters achieve a good ecological status when the arithmetic mean of the concentrations measured at different times during the year does not exceed the Environmental Quality Standard (EQS) (dissolved As concentration of water samples filtered through 0.45 μm), which is fixed at 50 μg L^{-1} annual average for inland surface waters by the Royal Decree 817/2015 (BOE 2015).

Table 1.1: Arsenic polluted waters in different parts of the world (Sharma and Sohn 2009).

Continent	Location	Arsenic Source	Concentration (μg L^{-1})
America	Pampa, Córdoba, Argentina	Groundwater	100-3,810
	Córdoba, Argentina	Drinking water	>100
	Chile	Drinking water	470-770
	Lagunera region, Mexico	Well waters	8-624
	Peru	Drinking water	500
	Northeastern, Ohio	Natural origin	<1-100
	Western USA	Drinking water	1-48,000
Europe	Hungary	Deep groundwater	1-174
	Romania	Drinking water bores	1-176
	South-west Finland	Well water: natural origin	17-980
Asia	Bangladesh	Well waters	<10->1,000
	Calcutta, India	Near pesticide production plant	<50->23,080
	West Bengal, India	As-rich sediments	3-3,700
	Nepal	Drinking water	8-2,660
	Hanoi, Vietnam	As-rich sediments	1-3,050
	Xinjiang, China	Well water	0.05-850
	Inner Mongolia, China	Drinking waters	1-2,400
	Ronpibool, Thailand	Water contaminated by tin mining waste	1-5,000
	Nakton Si Thammarat Province, Thailand	Shallow (alluvial) groundwater, mining	1.25-5,114
	Mekong River foodplain, Cambodia	Groundwater	1-1,340

The impact on human health of As concentrations in Spanish soils has been recently highligthed by Núñez et al. (2016), who studied the relationship between As and Cr topsoil levels and cancer mortality in Spain, revealing that chronic exposure at low levels of these elements could be a potential risk factor for the development of cancer. In this work an association was found between As soil levels and men and women mortality due to cancers of the stomach, pancreas, lung and brain and non-Hodgkin's lymphomas. Moreover, among men, the association was also observed with cancers of prostate, buccal cavity and pharynx, oesophagus, colorectal and kidney. Figure 1.1 shows the As contents of topsoils determined in this study (a) and the oesophageal cancer mortality in men (left) and women (right), respectively (b). The results of study highlights the importance of determining As levels in soils to perform a rigorous risk assessment of the population in a determined area.

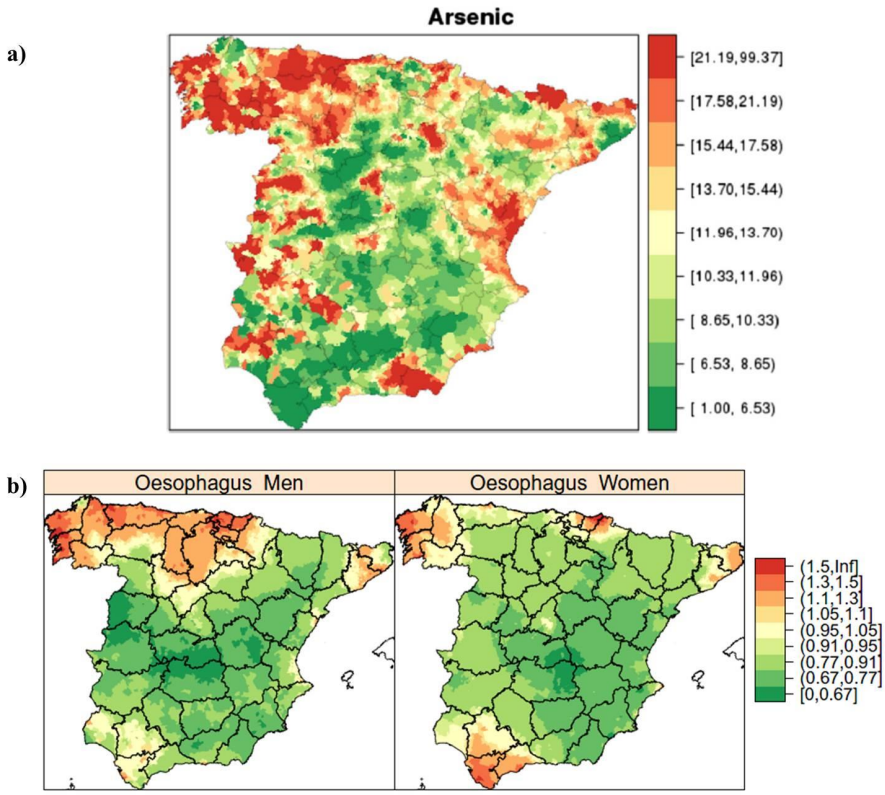

Figure 1.1. a) Arsenic concentrations in topsoils (mg kg^{-1}). b) oesophageal cancer mortality (Inf) in men (left) andwomen (right) over a 10-year period (Núñez et al. 2016).

1. 2. Inorganic and organic As species

Dissolved As can occur in aqueous systems both in inorganic and organic forms. Inorganic As species predominate in soils, sediments and water, whereas organoarsenic compounds prevail in marine organisms (Francesconi et al. 1999). Inorganic As can be present in natural aquatic systems in four oxidation states: + V (arsenate), + III (arsenite), 0 (elemental As), and –III (arsine). The oxidation state is determined by pH and Eh (Fig. 1.2). As^{V} and As^{III} are the common valence states in natural waters. As^{V} is the thermodynamically stable form that generally predominates in oxic surface waters, whereas As^{III} is favoured in environments with low pH and low redox potential (Genç-Fuhrman et al. 2004). In natural

waters and at circumneutral pHs, arsenate and arsenite are present as oxyanions (such as $H_2AsO_4^-$ and $HAsO_4^{2-}$) and as neutral aqueous species (H_3AsO_3), respectively (Anderson and Bruland 1991).

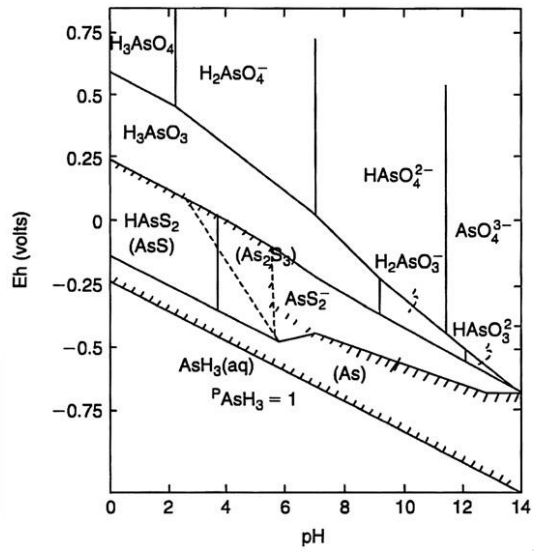

Figure 1.2 The Eh–pH diagram for arsenic at 25 °C and 1 atmosphere with total arsenic 10^{-5} mol L^{-1} and total sulfur 10^{-3} mol L^{-1}. Solid species are enclosed in parentheses in the cross-hatched area, which indicates solubility in parentheses in the cross-hatched area, which indicates a solubility of less than $10^{-5.3}$ mol L^{-1} (from Ferguson and Gavis 1972, and reproduced in Sharma and Sohn 2009).

Arsenic can also occur in organic forms produced by the biological conversion of inorganic As species. Organic species of As include monomethylarsonic acid [MMA^V; $CH_3AsO(OH)_2$], dimethylarsinic acid [DMA^V; $(CH_3)_2AsOOH$], trimethylarsine oxide [TMAO; $(CH_3)_3AsO$], arsenobetaine (AsB), arsenocholine (AsC), arsenosugars (AsS), arsenolipids, etc. (Fig. 1.3) (Duker et al. 2005; Páez-Espino et al. 2009; Tangahu 2011; Wang et al. 2015). However, methylated species are usually not abundant in aqueous solutions compared to inorganic forms of As (Smith et al. 1998; Smedley and Kinniburgh 2002). Thio - and carbonato - complexes of arsenic also exist in anaerobic systems; thiolated forms of arsenic may, in fact, represent an important reactive component within sulfidic environments (Wilkin et al. 2003).

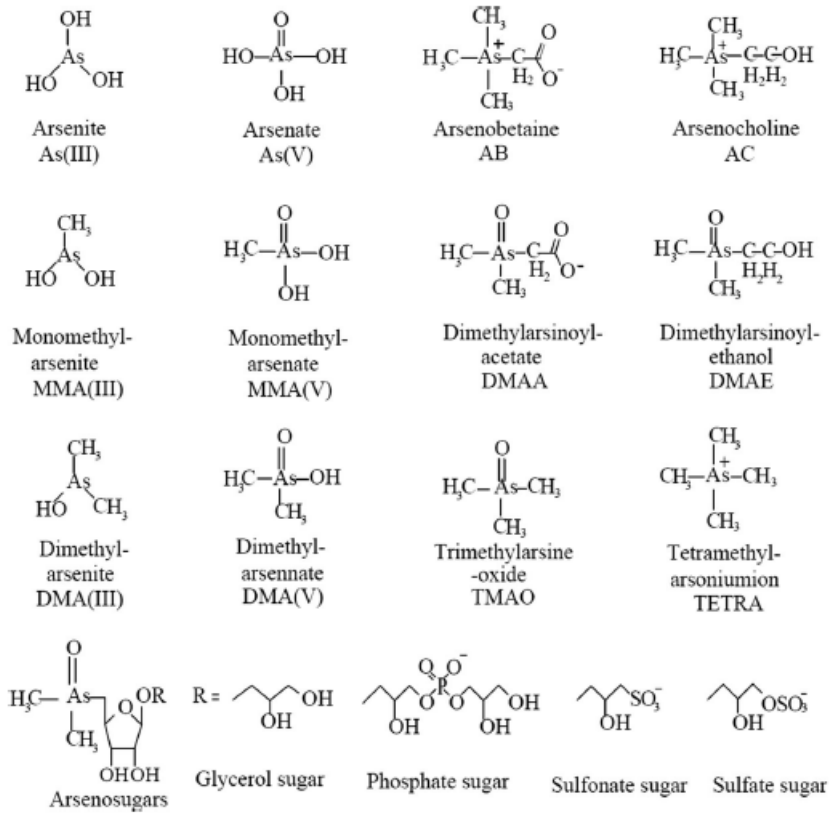

Figure 1.3. Chemical structures of typical arsenic compounds (Wang et al. 2015).

1. 3. Toxicity of inorganic and organic As species

As stated above arsenic is a highly toxic element with well known acute and chronic effects on living organisms. The chemical form and oxidation state influence As acute and chronic toxicity, and the mode of action of such toxicity. Inorganic As is generally recognized as more toxic than the organic forms (Ng 2005), with the exception of methylated As^{III} species (Petrick et al., 2000; Styblo et al., 2000). In general, the toxicity order of As compounds for human cell lines is as follows: dimethylarsinous acid (DMA^{III}) > monomethylarsonous acid (MMA^{III}) > arsenite (As^{III}) > arsenate (As^{V}) > dimethylarsinic acid (DMA^{V}) > monomethylarsonic acid (MMA^{V}) (Hirano et al. 2004; Petrick et al. 2000).

Among inorganics forms, As^{III} is generally considered more toxic than As^{V} (Wang and Mulligan 2006), which is related to its high affinity for sulfhydryl groups of biomolecules and for cysteine of many enzymes. However, many exceptions to this rule have been reported, as several researchers observed equal of higher toxicity of inorganic As^{V} than As^{III} to algae (Cullen et al. 1994a; Karadjova et al. 2008; Knauer et al. 1999; Levy et al. 2005; Pawlik-Skowrońska et al. 2004). As^{V} has also been shown to be more toxic than As^{III} to *Aliivibrio fischerii* (Rubinos et al. 2014), which is commonly used in standard ecotoxicological tests.the In particular, the toxicity of As species for freshwater and marine microalgae and phytoplankton remains controversial (Karadjova et al. 2008). Various papers report that marine microalgae are more sensitive to As^{III}, while freshwater algae are more sensitive to As^{V} (Knauer et al. 1999, Yamaoka et al. 1999; Levy et al. 2005).

This different toxicity of the oxidized and reduced inorganic As species results from their different mode of entry and action in the cells. In both prokaryotes and eukaryotes As^{V} enters the cell through phosphate transporters (Maciaszczyk-Dziubinska et al. 2012; Rosen and Liu, 2009) and replaces phosphate in many biochemical reactions. As^{V} competitively inhibits enzymes that use phosphate or have phosphorylated intermediates (Rosen and Tamás 2010) and it uncouples the formation of ATP (Hughes 2002), ultimately depleting energy cell . In turn, As^{III} is taken up by living cells mainly through aquaglyceroporins (Bhattacharjee et al. 2008; Rosen and Tamás 2010), which are transmembrane proteins that allow the bidirectional transport of water, glycerol and other small uncharged solutes (Hachez and Chaumont 2010). As^{III} toxicity is related to its high affinity for the sulfhydryl groups of biomolecules (Aposhian and Aposhian 2006; Sharma and Sohn 2009), inhibiting important biochemical reactions leading to cytotoxicity (Hughes 2002). As^{III} also leads to the production of reactive oxygen species (ROS) by binding to reduced glutathione (Bhattacharjee et al. 2008).

1.4. Arsenic biogeochemistry in aquatic environments

In aquatic ecosystems (rivers, lakes and groundwater) As may undergo transformations in its chemical form by precipitation or as a consequence of its interaction with mineral surfaces (oxidation/reduction, surface complexation) which can strongly affect the fate, mobility, bioavailability and toxicity of As (Fig. 1.4). In general, As^{V} binds extensively and strongly to

most mineral constituents of sediments, while As^{III} exhibits a limited binding to most soil minerals with the exception of iron (hydr)oxides, for which it has a high adsorption affinity (Dixit and Hering 2003). Aluminum hydroxides and aluminosilicate clay minerals may also retain appreciable As concentrations, exhibiting a strong preference for arsenate relative to arsenite (Manning and Goldberg 1997a; Manning and Goldberg 1997b; Smith et al. 1998). Similarly, reactions of As with Mn compounds have been demostrated; the reaction of As^{III} solutions with Mn oxides results in extensive and rapid uptake, but As is retained as arsenate surface complexes owing to As oxidation by Mn^{III}/Mn^{IV} (Oscarson et al. 1981a; Manning et al. 2002).

Arsenic may be released from solid phases through various mechanisms, which can be broadly grouped into four categories: (1) ion displacement, (2) displacement by hydroxyl in alkaline conditions, (3) reduction of arsenate to arsenite, and (4) mineral dissolution, particularly reductive dissolution of Fe and Mn (hydr)oxides (Fendorf et al. 2008).

Phosphate may be considered a critical factor in the adsorption or release of As from solid phases (Liu, 2001). Phosphate acts inhibiting As^{V} adsorption by Fe and Al oxides (Manning et al. 1996). The strong competition between phospate and arsenate by surface sorption sites is because both act as oxyanions and have similar characteristics, such as quasi-identical pK_a values and charged oxygen atoms (Larsen 1992).

Arsenic speciation in water and sediments may be strongly influenced by microbial activities (Oremland and Stolz 2005), undergoing transformations through biologically mediated reactions. Thus, microorganisms are responsible for As^{V} reduction (arsenate can be used as the ultimate electron acceptor during respiration), As^{III} oxidation (arsenite can serve as electron donor by oxidation to arsenate), bioaccumulation of inorganic arsenicals (iAs), biotransformation of iAs to methylarsenicals and complexation of organoarsenicals inside their cells, and the release to water or the production of volatile As species (Páez-Espino et al. 2009; Rahman et al. 2012). These aspects will be discussed in detail in section 1.5.

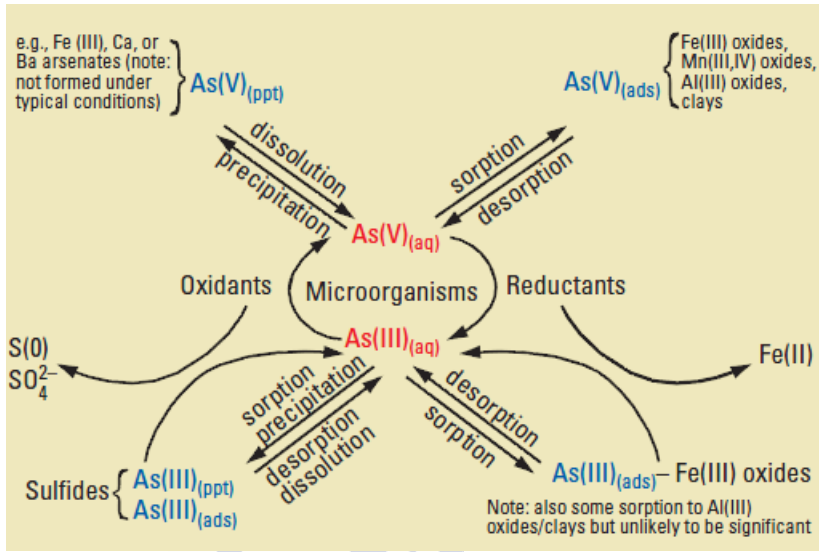

Figure 1.4. Possible routes in the biogeochemical cycling of arsenic (Reisinger et al. 2005).

1.5. Arsenic transformations by microorganisms

Arsenic-microorganisms interactions take place in both extra- and intracellular compartments. On the one hand, oxidation of As^{III} to As^{V} by microorganisms allows the microorganisms to withstand high concentrations of As^{III}, since the affinity of As^{V} by mineral solids is greater than that of As^{III} (Páez-Espino et al. 2009). Also, As sorption to cell surface may occur by means of electrostatic interactions with amide and amino, hydroxyl and sulfydryl groups (Yan et al. 2010; Prasad et al. 2011; Giri et al. 2013) and by complexation, forming inner-sphere complexes on cell surfaces (Giri et al. 2013). On the other hand, As enters cells via phosphate and aquaporin transport systems for As^{V} and As^{III}, respectively (Huang 2014). Once in the cells, effective strategies have been developed by microorganisms to tolerate high As concentrations (Fig. 1.5). Thus As^{V} is reduced to As^{III}, which could be pumped out the cell after reduction (classical *ars* operon detoxification), complexed with thiol compounds and sequestered into vacuoles, or methylated to less toxic organic arsenic species (Páez-Espino et al. 2009; Wang et al. 2015). Besides MMA^{V} and DMA^{V} the products of methylation include trimetilarsine oxide (TMAO) (Duker et al., 2005) and the final product of the methylation pathway, the volatile trimethylarsine ($TMAs^{III}$) (Yin et al., 2011a).

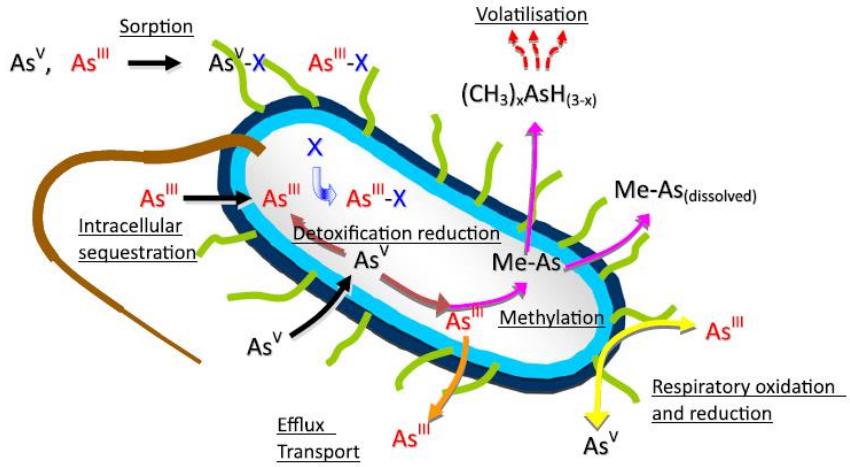

Figure 1.5. Interactions between arsenic and microorganisms (Huang 2014).

Specific models have been proposed for As^V transformations by microalgae in which As^V is taken up by algal cells using a P transport system (Fig. 1.6). Then, inside the cell a detoxification mechanism of As^V takes place which consists in reducing As^V to As^{III}, in methylating it to monomethylarsonic acid (MMA^V), and MMA^V to dimethylarsinic acid (DMA^V) (Cullen et al. 1994a; 1994b; Hellweger et al. 2003; Hellweger and Lall 2004). Finally, As is excreted as As^{III} and/or DMA^V, depending on the algae growth rate and on the phosphate conditions (Helweger and Lall 2004). Methylation of As^{III} is slow and it is more likely to occur in the stationary phase of algae growth (Hellweger et al. 2003).

Although the interactions between As and microorganisms have been extensively studied, as well as As transformations by microorganisms, these studies generally refers to monospecific cultures of algae, bacteria, archaea or fungi at lab scale. For this reason, more realistic studies including multi-specific communities are necessary to reproduce better the As biogeochemistry in aquatic systems, such as riverine environments and microbial communities attached to sediment surfaces.

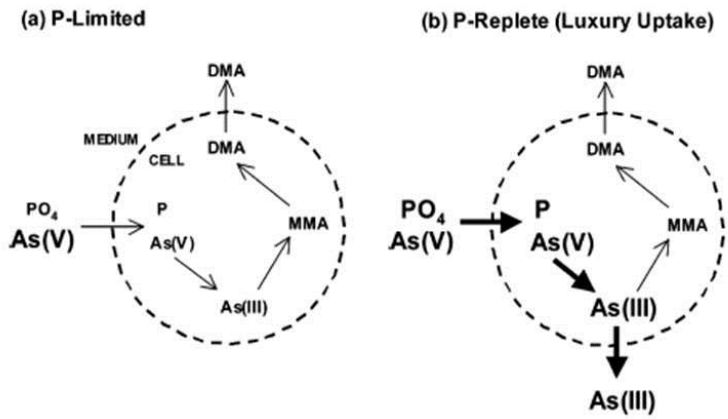

Figure 1.6. As transformations by phytoplankton (Hellweger and Lall 2004).

1.6. Role of biofilms in As biogeochemistry

In natural aquatic environments, microbial cells are often found in complex, surface-attached communities, known as biofilms (Costerton et al. 1995). The primary role of biofilms is the protection of microbial communities in conditions of environmental stress (Decho 2000; Flemming et al. 2001). The term biofilm was initially used in engineering and referred to attached heterotrophic communities (Wetzel 1983), but it has been spread to natural aquatic systems as a general term referring to attached–microbial communities which colonize submerged surfaces in lakes and rivers. In this context, biofilms consist in communities of microorganisms (bacteria, fungi, cyanobacteria, algae and protozoa) embedded in a extracellular polymeric substances (EPS) matrix mainly composed by water and polysaccharides (Costerton, 2007). In addition to water and polysaccharides, extracellular DNA, proteins, lipids, particulate material and detritus are also found in EPS (Costerton et al. 1995; Sutherland 2001a; Lawrence and Neu 2003). The EPS matrix provides mechanical stability to biofilms, mediates their adhesion to surfaces, forms a cohesive polymeric network that interconnects and transiently immobilizes biofilm cells (Wigender and Flemming 2011; Decho 2000; Gebersdorf et al. 2008) and provides protection for predators, toxic substances and physical perturbations.

Biofilms dominate microbial life in streams and rivers, drive crucial ecosystem processes and contribute substantially to global biogeochemical fluxes (Battin et al. 2016). They are crucial in aquatic ecosystems functioning because they are involved in primary production, carbon and nutrient cycling, retention of inorganic and organic nutrients, support of food webs and have excellent ability to degrade and transform pollutants (Mora-Gómez et al. 2016).

In riverine environments, sediment particles offer a large surface area for microbial colonization (Battin et al. 2001). Microorganisms attached to sediment particles are referred to as epipsammic biofilm or epipsammon (Fig. 1.7) (MacIntre et al. 1996).

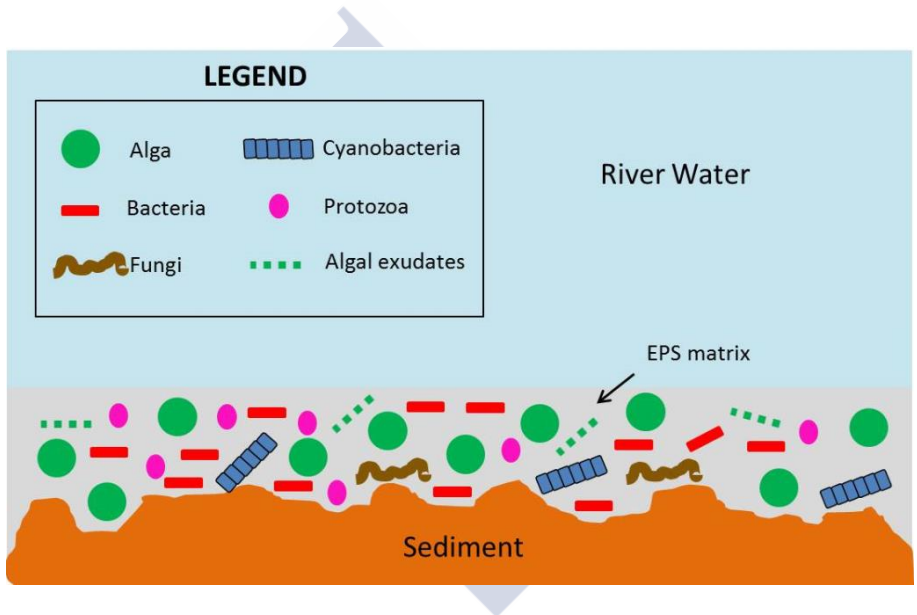

Figure 1.7. Scheme of epipsammic biofilm in riverine systems (Adapted from Mora-Gómez et al. 2016).

Epipsammic biofilms represent the interface between water and granular sediments, affecting the exchange of solutes among these environmental compartments and the physical stability of the sediment (Figs. 1.8 and 1.9). It has been demonstrated that biofilms (1) increase sediment stability and limit their resuspension (Sutherland et al. 1998; Stal 2003; De Brouwer 2005; Flemming 2006; Tolhurst et al. 2006; Ziervogel and Forster 2006; Gerbersdorf and Wieprecht 2015), (2) alters the release of pollutants from bed sediments (Leadbeater and Callow 1992; Gainswin et al. 2006; León-Morales et al. 2006; Gerbersdorf et

al. 2007; Lubarsky et al. 2010) and (3) play a key role in organic matter decomposition (Romaní and Sabater 2001).

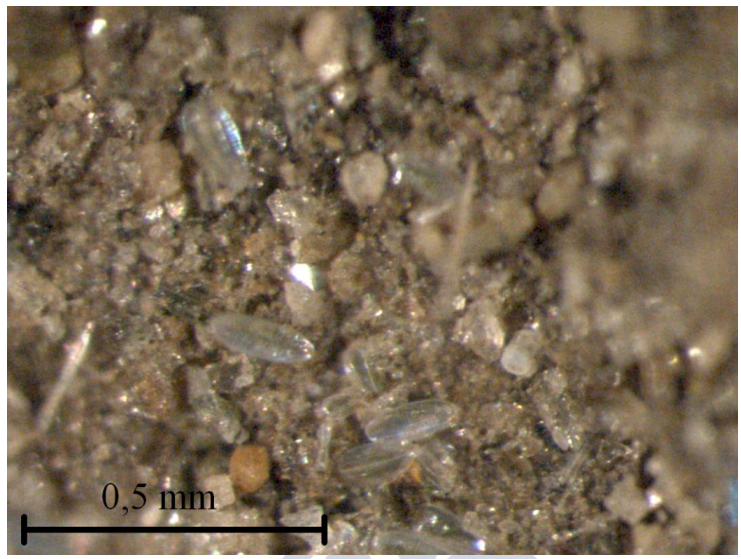

Figure 1.8. Magnifying glass image of epipsammic biofilm growing on sediment particles from Anllóns River.

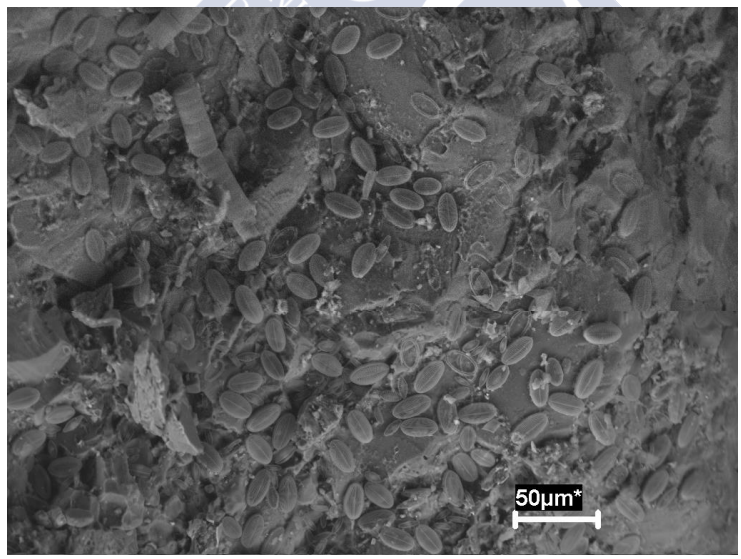

Figure 1.9. SEM image of epipsammic biofilm growing on sediment particles from the Anllóns River.

Biofilms are ubiquitous in freshwater environments, where they can play an important role in the biogeochemical cycles. Nevertheless, despite the importance of epipsammic biofilm in riverine ecosystems, their role is often ignored when the geochemical processes (i.e. retention, mobility, bioavailability and speciation of pollutants) occurring at the sediment-water interfaces are studied. Yet biofilms change chemical exposure in stream ecosystems by influencing the solubility of minerals, the sorption of metals onto particle surfaces, the transformations between oxidized and reduced species, and the metabolism of aquatic biota (Balistrieri et al. 2012). Specifically, it has been demostrated that biofilms play a key role in the retention of metals and metalloids from the overlying waters (Nelson et al. 1996, 1999; Friese et al., 1997; Headley et al. 1998; Decho 2000; Dong et al. 2000; Haack and Warren 2003; Dong et al. 2007; Drahota et al. 2014). Metals and metalloids are removed by biofilms by biosorption, precipitation as sulfides or phosphates and microbial reductive precipitation (van Hullebusch et al. 2003). Biosorption consists of several mechanisms, including ion exchange, chelation, adsorption to cell surface and Eps matrix and diffusion through cell walls and membranes (van Hullebusch et al. 2003). Other authors, among which may be cited Huang (2014), distinguish biosorption (retention of trace elements on the microbial cell surface) and bioaccumulation (intracellular accumulation in cell membranes and cytoplasm). Given its importance in the retention of pollutants, biosorption was postulated as a bioremediation technique for detoxifying various types of polluted waters.

In biofilms, As can be sequestered in extra- and intracellular compartments, as it was explained in section 1.5, with a, important role of EPS matrix. The EPS contains apolar regions from proteins (such as in aromatic amino acids), groups with hydrogen-bonding potential (such as polysaccharides), anionic groups in uronic acids and proteins (e.g.: $-COO^-$, $-HPO_4^-$) and cationic groups in amino sugars and proteins (e.g.: $-NH_3^+$) (Flemming and Sutherland 2001a), and can act as a molecular sieve, sequestering cations, anions, apolar compounds and particles from the water phase (Flemming and Wingender 2010).

The interaction between As and biofilms has received scarce attention, and even less in the case of epipsammic biofilm, as the few studies published refer to biofilm growing on artificial substrates or to rock (epilithic) biofilms. The interaction As-biofilm in aquatic systems can be studied from the point of view of the effect of biofilm on As biogeochemistry (retention, speciation) or from that of As effect on biofilm. In the first case, As behavior in

aquatic systems may be modified by the presence of biofilms. The most immediate effect is As retention; the potential of As enrichment in biofilms has been reported by Drewniak et al. (2008) with concentrations of up to 60 mg kg^{-1} in rock biofilm. Yang et al. (2011) demonstrated that multi-species biofilms, inoculated from a source receiving coal mining effluent, can both sequester and detoxify Se and As. Drahota et al. (2014), studying As adsorption from natural As sources onto natural surface coatings growing on glass slides, confirmed that dissolved As had been sorbed and retained by the biofilm. Tuulaikhuu et al. (2015), who investigated the fate and the toxicity of As on periphytic and epipsammic biofilms using a simplified fluvial system including fish, biofilms and sediment, reported that periphytic biofilms also accumulated As, although it was predominantly retained by sediment. In the second case, the presence of As in aquatic systems has been shown to affects the periphyton communities, byr inhibiting algal growth and photosynthetic capacity, decreasing total biofilm biomass, changing the community composition (selecting tolerant species, reducing species richness and making biofilms more heterotrophic), reducing diatom cell sizes and the ability of the community to use phosphorus (Blanck and Wangberg 1988; Wangberg et al. 1991; Blanck and Wangberg 1991; Rodriguez Castro et al. 2015; Tuulaikhuu et al. 2015; Barral-Fraga et al. 2016).

1.7. Previous studies on As pollution in the Anllóns River

In the Anllóns River catchment (Galicia, NW Spain) (Fig. 1.10), arsenopyrite (AsFeS) mineralizations are associated to gold ores in hydrothermal quartz veins (Nespereira 1978) (Fig. 1.11). In the catchment, gold mineralizations were exploited during the Roman Empire and then from 1895 until 1910 (Fig. 1.12), with intermittent extractions since then. As concentrations in the rocks of the area are usually around 1%, but in mineralized zones with semi-massive arsenopyrite, they can reach up to 10%. In the superficial soil horizons in the mineralized areas, As contents of 4,000 mg kg^{-1} have been detected (Boixet et al. 2007). High As concentrations were also found in the surface and subsurface sediments of the Anllóns River, which were attributed to natural geogenic As enrichment, exacerbated by mining activities (Devesa-Rey et al. 2008a). The highest As contents in the sediments were found downstream from the mining area to the river mouth, with a maximum of 264 mg kg^{-1} (Devesa-Rey et al. 2008a; Rubinos et al. 2010; Rubinos et al. 2011). At some points along the

river course, these high As concentrations have been identified as sources of ecotoxicity (Devesa-Rey et al. 2008b) and no evidence for other potentially toxic elements have been found in the Anllóns sediments (Devesa-Rey et al. 2011).

Figure 1.10. Anllóns River. a) upper strech (A Laracha). b and c) middle strech (Carballo and Verdes). d) river mouth (Ponteceso).

Figure 1.11. Arsenopyrite veins in the gold mine located in the Anllóns River Basin (left) and detail of arsenopyrite-rich quartz vein (right).

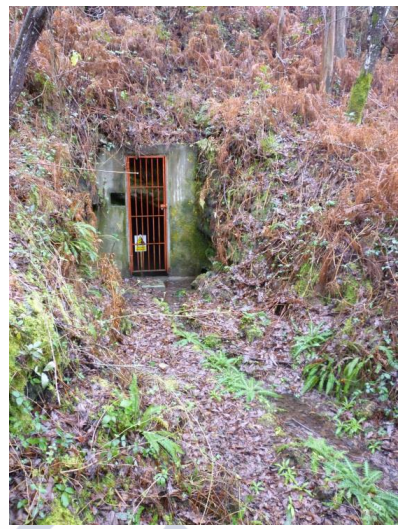

Figure 1.12. Old mining gallery near the Anllóns River course.

Geochemical investigations using sequential extractions showed that most As in the bed sediments of the Anllóns River is associated to low-mobility phases: bound to Fe-oxides and in the residual phase (Devesa-Rey et al. 2008a; Rubinos et al. 2011) (Fig. 1.13).

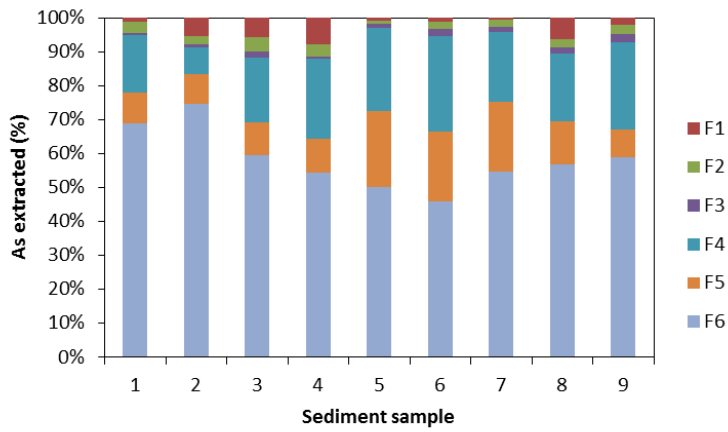

Figure 1.13. As fractionation of Anllóns River bed sediments using the SEP procedure of Lombi et al. (2000). F1: exchangeable, F2: specifically sorbed, F3: associated to Al and OM, F4: bound to amorphous Fe oxides, F5: bound to crystalline Fe oxides, F6: residual phase (Devesa-Rey et al. 2008a).

Corroborating the results of the fractionation, Rubinos et al. (2011) found low As mobility using the DIN 38414-S4 standard procedure. Nevertheless, this behavior was drastically modified by changes in environmental conditions, such as high salinity, alkaline pH, high phosphorus concentrations and high liquid:solid ratio (Rubinos et al. 2010; 2011). Thus, As release from contaminated sediments of the Anllóns River is strongly dependent on the pH. The kinetics of the process are mainly controlled by the dissolution of various components of the sediment, specially oxides and hydroxides of Fe and Al, at acidic pH, and by organic matter at alkaline pH (Rubinos et al. 2010). This study also showed that soluble P increases the remobilization of As in the contaminated sediments, especially when they occur simultaneously with changes in pH. Moreover, it was shown that the presence of phosphate counteracts the acute toxicity of As^{V} to *Aliivibrio fischerii*, so it could be envisaged as a corrective measure for As pollution, facing the risk of health and environmental high concentrations of As^{V} in freshwaters (Rubinos et al. 2014). However, the presence of P did not affect the acute toxicity of As^{III} or that of the organic form DMA^{V} (Rubinos et al. 2014).

Given the high As concentrations found in the sediments of the Anllóns River and the foreseeable presence of biofilms in the interface water-sediments, a detailed study of the interaction As-biofilms deserves special attention and will the goal of the present study.

2. HYPOTHESIS, PURPOSES AND OBJECTIVES

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2. Hypothesis, purposes and objectives

2.1. Hypothesis and purposes

The hypothesis of this PhD research is that biofilms developed on river bed sediments may actively retain As and cause changes in its chemical species, leading to a decrease of its mobility and/or toxicity. Thus, this thesis is expected to contribute to the knowledge of the role of epipsammic biofilms in the As biogeochemistry, and to understand more completely the interaction between this pollutant and the biofilm in the Anllóns River watershed, where As pollution has been previously observed. In this context, it is very important to determine the influence of the riverine biofilm on the mobilization, bioavailability and potential toxicity of As in water and sediments.

The study of biofilm began in the research group GI-1243 GEMAP (formerly GI-1242) with the project entitled "Effect of resuspension in the bioavailability and toxicity of pollutants in river sediments" (CGL2007-62928). In this initial study it became clear that the release of As from contaminated sediments was strongly dependent of pH, and the kinetics of the process were mainly controlled by the dissolution of sediment components, especially oxides and hydroxides of Fe and Al at acidic pH and organic matter at alkaline pH. This study also showed that inputs of soluble phosphorus increase As remobilization from As-rich sediments, especially when they occur simultaneously with changes in pH. In this project, special attention was paid to the effect of biofilms on sediment stability against erosion.

The study of epipsammic biofilm continued with the project entitled "A microcosm scale study of As and Cr detoxification by biofilms developed over river bed sediments and implications for bioremediation" (CGL2010-22059), in which this thesis was framed. The main objective of this project was to study the effect of biofilm developed on river sediments on the retention and transformation of As and Cr, and consequently to assess their potential application in bioremediation of polluted waters and sediments.

Up to date there are no studies about the abundance and composition of epipsammic biofilms in river sediments of north-western Spain and the limited available data on benthic algae in the region's freshwaters refer to epilithic (grown on rock surfaces) or epiphytic (growing on phytobenthos (Margalef 1955, 1956; Ector 1992; Noguerol Seoane 1993; López Rodríguez and Penalta 2004). Nevertheless, the study of biofilm deserves special attention

because, it plays a key role in the biogeochemistry of some elements and because some species of benthic microalgae are indicative of contamination. In particular, benthic diatoms have characteristics that make them well suited for the bio-indication of the quality of river waters, namely their abundance and great taxonomic diversity, their ability to colonize different environments, as well as the preservation of frustules in sediments (López Rodríguez 2005).

The choice of arsenic as an object of study is based on: 1) its high toxicity, so that its presence in the environment in excessive concentrations poses a serious health risk, increasing the incidence of cancer and dermatological, vascular and cerebrovascular diseases 2) high As concentrations have been found in rocks, superficial soils and sediments in the Anllóns River basin, particularly in the As-Au mineralized area. 3) changes in its oxidation state affects its mobility and toxicity.

Phosphorous plays a key role in the interaction between epipsammic biofilm and As and it deserves a particular study because: 1) it is an essential nutrient and it has a stimulative effect on the growth of the microorganisms composing the biofilm. 2) it competes with As for sorption sites, which may involve a reduced retention of As in contaminated waters by the active components of the sediment or increased As mobility from As-rich sediments, as it has been previously reported for Anllóns river sediments by Rubinos et al. (2003; 2010; 2011) 3) P has an alleviative effect of against As^{V} toxicity (Karadjova et al. 2008; Rubinos et al. 2014).

The purpose of this work is to study the effect of the biofilm developed on riverbed sediments on the retention, transformation and toxicity of As, and subsequently to assess its potential application in the bioremediation of polluted river waters and sediments by means of two perspectives: As immobilisation and detoxification. The first mechanism involves the formation of less mobile chemical species, and therefore less bioavailable, chemical species whereas the second mechanism involves the formation of less toxic species. In fact, both processes can act together at the water-sediment interface and are affected by the nature of the mineral and organic components of sediments, the abundance and composition of benthic microflora and the physico-chemical properties of river water. To this end, assays were conducted at field scale to evaluate As-transfer from As-rich soils to the water courses and to monitor the distribution of benthic microflora in the bed sediments from the Anllóns River. Then, experiments were performed in indoor fluvial channels to obtain biofilms and to

explore the light and nutrient effect on biofilm growth. Finally, experiments were done in batch systems and in specifically designed bioreactors to study the retention and transformation of As. The novel aspect of the PhD thesis is the integrated evaluation of physico-chemical (sorption, complexation) and biochemical processes (absorption, reduction, methylation) occurring in the epipsammic biofilm that affect As biogeochemistry and toxicity in the fluvial system.

2.2. Objectives

Our general objective is to improve the knowledge of the effect of the biofilm developed on river sediments on arsenic biogeochemistry, specifically on its retention and transformation. The interaction epipsammic biofilm-As will be studied in freshwater environments, paying attention to the behaviour of the biofilm As polluted waters and As-rich sediments. Particular objectives which will be addressed in the respective six chapters are:

1. *To evaluate the risk of As transfer from As-rich soils to water systems.*
   The aim is to detect high concentrations of lithogenic As in the Anllóns River basin, where Au-As mineralizations have been historically exploited, and to evaluate the potential risk of As mobility to groundwater and to the river course.

2. *To monitor the benthic microflora in bed sediments from Anllóns River.*
   This objective attempts to assess the biological distribution and development in the surface sediment by means of the determination of the dehydrogenase activity, and that of the autotrophic biomass by means of phytopigment analysis and parameters derived from the taxonomic identification, such as algal abundance and genus richness.

3. *To obtain and characterise natural biofilms in experimental fluvial channels under different light intensities and nutrient conditions.*
   The particular aims are to: a) evaluate the influence of light availability and trophic state on the development of biofilms; b) test the suitability of experimental channels to grow biofilms on fluvial sediments, which may be used to perform subsequent

environmental and biotechnological studies; and c) investigate if biofilms can grow in channels fed with river water, without the addition of supplementary nutrients.

4. *To evaluate the effect of a native biofilm developed over riverbed sediments on the retention of As^{V}.*

   The capacity of As^{V} retention of biofilm-enriched sediments will be compared to that of sediments devoid of biofilm, as well as the potential remobilization produced after the retention. as well as the influence of phosphate.

5. *To determine the influence of epipsammic biofilm in the retention and transformation of AsV.*

   In specifically designed bioreactors, it will be studied the mobility and the volatilization of As and the distribution of As within biofilm, as well as As speciation in the various compartments analysed.

6. *To evaluate the risk of As transfer from As-rich sediments to the river water.*

   The role of epipsammic biofilm on As mobilization from As-rich sediments to the aqueous phase was investigated, by analysing: 1) the effect of biofilm on As release from contaminated sediments, determining the changes in the concentration and speciation of aqueous As in the water column and establishing the kinetics of the leaching process. 2) the distribution of the As retained by the biofilm among the cells and the EPS matrix. 3) the volatilization of As and quantify their importance during the incubation experiment. 4) the leaching and bioavailability of As at the end of the incubation experiment in biofilm-rich sediments in comparison with sediment devoid of biofilm.

7. *Finally, in view to the above results, to assess the role of biofilm in the immobilization and transformation of As^{V}.*

3. GENERAL PLANNING

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3. General planning

This dissertation is divided into 6 chapters, which correspond to the six objectives previously defined. A detailed scheme of the work plan is presented in Figure 3.1.

In Chapter 1 a comprehensive study of the As in soils of the Anllóns river basin was conducted. The purpose of this chapter is to evaluate if soils in this basin can reach high As concentrations due to lithogenic enrichment and that As could be mobilized to groundwater and to the river course, posing a risk for environment and human health and is therefore worthy of evaluation. To this end, an exploratory analysis of the total As content and mobility was conducted with 50 soil samples, taken from C horizons of soils along the river course, followed by a detailed study of 9 samples with the highest As concentrations, which were submitted to a complete analysis including As fractionation and leaching under different environmental conditions.

Chapter 2 and Chapter 3 are focused on the study of the epipsammic biofilm from Anllóns River at field and lab scale, respectively. The purpose of chapter 2 was to investigate the abundance and composition of the epipsammic biofilm on the Anllóns River bed sediments, to evaluate the relationships between biochemical parameters and biological indices based on identification and counting, and to explore the relationships between biofilm growth and the properties of the sedimentary habitat, mainly the trophic state. To this end, bed sediment samples (0-5 cm) were collected in two different seasons (winter and summer) at 4 sampling sites along the river course. Physico-chemical properties of pore waters and sediments were determined. Biological properties included the determination of dehydrogenase activity (DHA) and phytopigment (Chl a Chl b and total carotenoids) concentrations, as well as taxonomic identification. For taxonomic identification, two sampling methods were compared: the Pasteur pipette method and a mini-corer method. Total and relative algal abundances and genus richness were calculated. The relationships between the different variables were examined using Pearson correlations and Principal Component Analysis.

Chapter 3 investigates the influence of light availability and water composition on biofilm growth and the suitability of experimental channels for biofilm growth. To this end, the indoor formation of an epipsammic biofilm was monitored during 21 days. The

experiments were carried out in two specifically designed experimental channels: channel 1 fed with river water and channel 2 with nutrient enriched input. Each channel was divided into three sections receiving different light intensities. Bioavailable phosphorous and phosphatase, total and biologically active organic carbon, chlorophyll a and b, total carotenoids, soluble carbohydrates and proteins, as measures of biological activity, were determined in the sediment samples.

Arsenic mobilized from soils may reach the river course and interact with epipsammic biofilm. Consequently, the interaction biofilm-As is studied in Chapters 4, 5 and 6. In chapters 4 and 5, the focus is put on the effect of biofilm in the retention and transformation of As from polluted waters whereas in chapter 6 on the effect of biofilm in the As transfer from As-rich sediments.

In Chapter 4 the role of biofilm onto As^V sorption is investigated, as well as the effect of equimolar P concentrations on As retention. The study of P-As interactions is of interest because P inhibits the retention of As^V, due to the competition for sorption sites in active sediment components, it favours the biofilm growth and it has an alleviative effect against As^V toxicity. To this end, a natural biofilm was grown on sediment samples in batch experiments, using river water as nutrient supplier. Once a mature biofilm was developed, sorption experiments with initial As concentrations 0, 5, 25, 50, 100, 250 and 500 $\mu g\ L^{-1}$ were performed, as well as identical experiments with equimolar P concentrations. In parallel, sorption experiments with sediments devoid of biofilm have been carried out as comparison.

In Chapter 5 the influence of epipsammic biofilms developed on riverbed sediments on the (bio-)sorption and/or (bio-)uptake, mobility, and (bio-)transformation of As^V was studied. With this purpose, a native biofilm was incubated on sediment samples at microcosm level using specifically designed systems. Once the biofilm was developed, 500 $\mu g\ L^{-1}$ As^V exposure concentrations were spiked in two systems, without P and with equimolar As:P concentrations, respectively. Identical control systems incorporating autoclaved sediment were incubated. The evolution and speciation of As concentrations in the overlying water were followed during two additional weeks. The As volatilization during the incubation experiment was also studied, as well as the distribution of As species within biofilms and the remobilization of the previously retained As.

In Chapter 6 the influence of epipsammic biofilms on the concentration and speciation of As released from As-rich sediments was evaluated. With this purpose, a complete study was carried out at a microcosm scale during 42 days, where epipsammic biofilm were grown on natural As-rich sediments, and As leaching and speciation was studied in comparison with sterilized sediments. The As mobility and bioavailability, the distribution of As in extra- and intracellular compartments within the biofilm and As volatilization were evaluated.

An integrative vision of the obtained results will be provided in *General Overview* and finally the final conclusion extracted from this dissertation are presented in *Final Conclusion*.

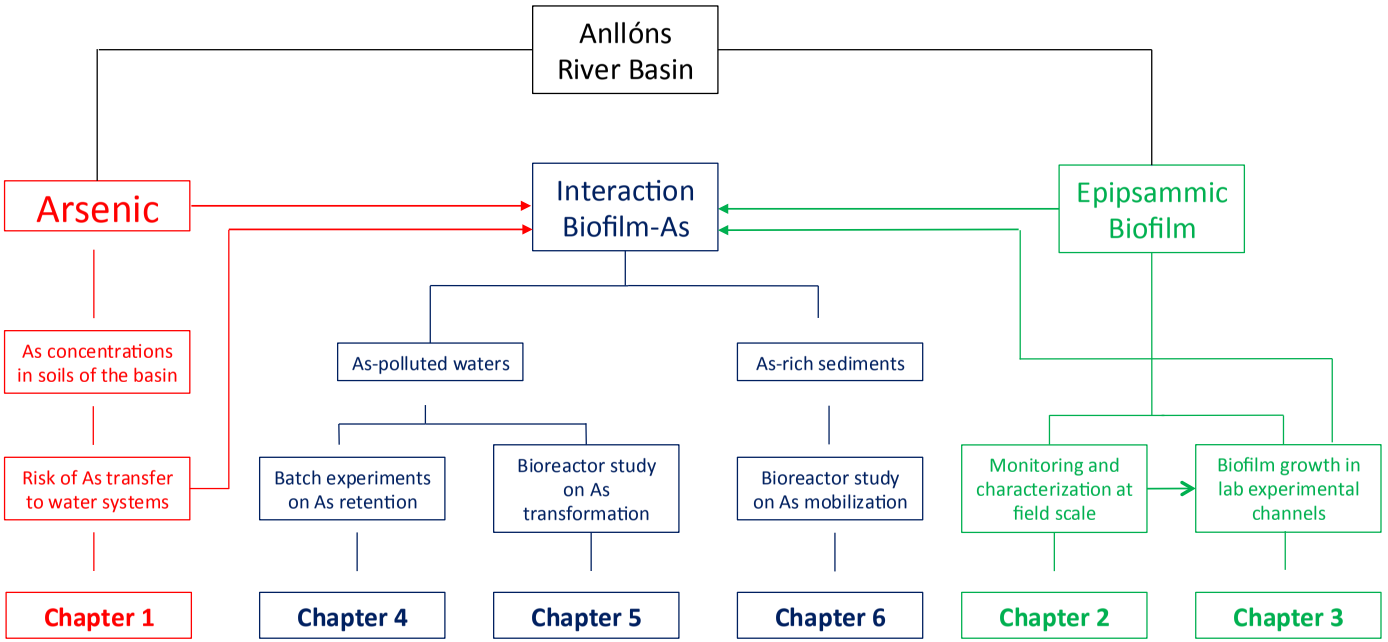

Figure 3.1. General scheme of the work.

4. EXPERIMENTAL

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Chapter 1: Evaluating the risk of arsenic transfer from As-rich soils to water systems

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Chapter 1: Evaluating the risk of arsenic transfer from As-rich soils to water systems

Abstract

The aim of this chapter was to detect lithogenic As in the Anllóns River basin (Galicia, NW Spain), where Au-As mineralizations have been historically exploited, and to evaluate the potential risk of As mobility to groundwater and to the river course. To this end, 50 soil samples of C horizons sampled on a 1 km x 1 km grid in a rectangular area along the river course, including the mineralized zone, were submitted to an exploratory analysis, followed by a more comprehensive study of the 9 soils with the highest As concentration. Total As ranged between 2 and 489 mg kg^{-1}, up to 8 times higher than the generic reference level for Galician soils. Arsenic leachability using the standard leaching methods DIN 38414-S4 and Toxicity Characteristic Leaching Procedure (TCLP) was lower than 0.25 % of total As. Fractionation studies indicated that As is mainly bound to crystalline Fe oxides, which justifies its low solubility. The effect of pH, liquid:solid (L:S) ratio, contact time and phosphate on As mobility was studied in these selected samples, showing that As solubility was favoured up to 124 times by increasing ratios and pH, with respect to that obtained applying the DIN 38414-S4 method. The most decisive factor was the addition of 10 mM phosphate, increasing As leachability up to 1,000 times, and this effect increased up to 2.3 times when the contact time was lengthened from 24 hours to 240 hours. Although standard leaching methods provide useful information in terms of legal limits or comparable data between laboratories, they can underestimate As mobility under more realistic scenarios, such as those occurring in soils affected by mining activities or contaminant spillages; this should be taken into account for a safer evaluation of the environmental and health risk due to As mobilization to aqueous systems.

1. Introduction

Arsenic (As) is a toxic metalloid widely distributed in natural environments (Smedley and Kinniburg 2002). In the earth's crust As is the 20th most abundant trace element (NRC 1977) whose average concentration has been fixed at 1.7 mg kg^{-1} (Wedepohl 1995). The baseline As concentration in soils is generally in the order of 5-10 mg kg^{-1} (Smedley and Kinninburg 2002).

Arsenic mobilization from soils, with its subsequent incorporation into aquatic systems, is of great environmental concern because this element poses a serious threat to human and ecosystem health, especially as a result of the contamination of drinking water and food. Several processes have been identified as responsible for the mobilization of As from soils to the aqueous phase: ion displacement, desorption (or limited sorption) at pH values above 8.5, reduction of arsenate to arsenite, and mineral dissolution, particularly due to the reductive dissolution of Fe and Mn (hydr)oxides and the associated As (Fendorf et al. 2008).

In the Anllóns River catchment (Galicia, NW Spain), arsenopyrite (AsFeS) mineralizations are associated to gold ores in hydrothermal quartz veins (Nespereira 1978). In the catchment, gold mineralizations were exploited during the Roman Empire and then from 1895 until 1910, with intermittent extractions since then. Arsenic concentrations in the rocks of the area are usually around 1%, but in mineralized zones with semi-massive arsenopyrite, they can reach up to 10%. In the superficial soil horizons in the mineralized areas, As contents of 4,000 mg kg^{-1} have been detected (Boixet et al. 2007). High As concentrations were also found in the surface and subsurface sediments of the Anllóns River, which were attributed to natural geogenic As enrichment, exacerbated by mining activities (Devesa-Rey et al. 2008a). The highest As contents in the sediments were found downstream from the mining area to the river mouth, with a maximum of 264 mg kg^{-1} (Devesa-Rey et al. 2008a; Rubinos et al. 2010; Rubinos et al. 2011). At some points along the river course, these high As concentrations have been identified as sources of ecotoxicity (Devesa-Rey et al. 2008b) and no evidence for other potentially toxic elements have been found in the Anllóns sediments (Devesa-Rey et al. 2011). Arsenic fractionation showed that most As in the bed sediments of the Anllóns River is associated to low-mobility phases: bound to Fe-oxides and in the residual phase (Devesa-Rey et al. 2008a; Rubinos et al. 2011). Corroborating the results of the fractionation, Rubinos et al. (2011) found low As mobility in the sediments using the DIN 38414-S4 standard procedure.

Nevertheless, this behavior was drastically modified by changes in environmental conditions, such as high salinity, alkaline pH, high phosphorus concentrations and high liquid:solid ratio (Rubinos et al. 2010, 2011).

The hypothesis of this work is that soils in the Anllóns River catchment can reach high As concentrations due to lithogenic enrichment and that As could be mobilized to groundwater and to the river course, posing a risk for environment and human health and is therefore worthy of evaluation. To this end, total As contents and As mobility were determined in soils from the Anllóns River catchment to evaluate its transfer risk to the aqueous systems. An exploratory analysis of the total As content and mobility was conducted with 50 soil samples, taken from C horizons of soils along the river course, followed by a detailed study of 9 samples with the highest As concentrations, which were submitted to a complete analysis including As fractionation and leaching under different environmental conditions.

Considering that every so often the reopening of the mine is reevaluated, affecting a greater extension than the historical mining area and using an opencast exploitation system, which is more environmentally aggressive than the old gallery system, the results of this study could be of interest for the evaluation of the environmental risk of mining operations which imply soil remobilization and the accumulation in mine spoils, with risk of dust pollution and As lixiviation to water. These results will also be interesting to evaluate the quality of water supplies to the population in the area under study. In fact, concentrations exceeding ten times the legal limit of As in water set at 10 μg L^{-1} were detected in various water sources near the mineralized area. Also, As levels in the waters of the Anllóns River clearly increase downstream from the mineralized area (Costas et al. 2011).The fate of As is of special environmental concern in the catchment, which is partly considered a Site of Community Importance, as defined in the European Commission Habitats Directive (92/43/EEC). Shellfishing at the river mouth is also of great economic and social importance, and should be preserved.

2. Materials and Methods

2.1. Study area

The Anllóns River is located in the region of Galicia (NW Spain); it has a length of about 63 km and flows into the Atlantic Ocean via the Laxe-Ponteceso Ría (Fig. C1.1). The river drains a rural catchment of 516 km^2, mainly dedicated to agriculture, forestry and cattle raising activities. Two main human settlements are located in the catchment: Carballo, with a population of over 31,000 and Ponteceso, with a population of about 6,000.

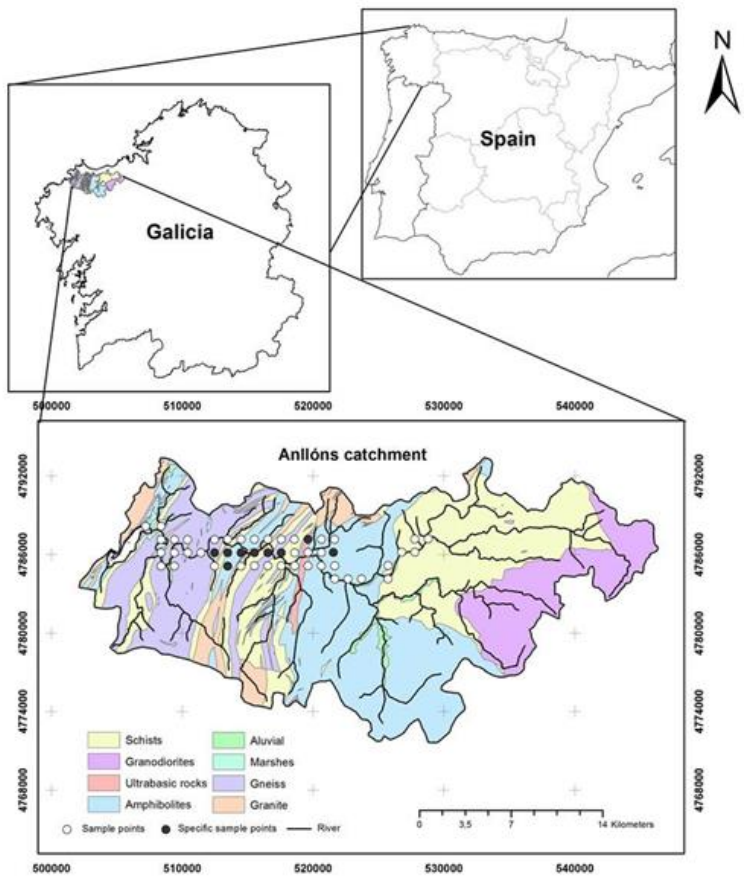

Figure C1.1 Location and geology of the Anllóns River catchment, and location of the sampling points for the exploratory analysis (black and white circles) and for the detailed specific analysis (black circles).

The river runs over schists in the upper stretch, turning into a smooth profile in the middle area of the river, characterized by basic rocks (gabbros and amphibolites). Finally, the lower stretch of the river runs over two mica granite, followed by biotitic gneiss at the mouth, where the largest area of alluvial materials is also found (IGME 1981) (Fig. C1.1). The most abundant soil type in the catchment is Umbrisol, with an umbric A-horizon generally above a cambic B-horizon, thus constituting a Cambic Umbrisol, with andic properties where soils develop over basic rocks. In the areas of scarce organic matter, ochric A-horizons are recognized, resulting in Dystric and Ferralic Cambisols. Leptosols and Regosols are also found in steep areas of the catchment and Fluvisols along the fluvial banks. Gold mining activities were carried out in the area since the Roman Empire, and were intermittently in operation until the 19th Century. In the mineralized areas, Au is associated to As sulphides, which are probably the origin of the high As concentrations detected in the surface sediments downstream from the mineralized area (Rubinos et al. 2003; Devesa-Rey et al. 2008a).

With the purpose of achieving the defined objectives of this study, the experimental procedure outlined in Figure C1.2 has been followed.

2.2. Exploratory analysis

50 soil samples were selected from the "Atlas Geoquímico de Galicia" collection (Guitián Ojea 1992), made up of approximately 30,000 samples -one per square kilometre- covering the entire territory of this region. The purpose of the selection was to cover the area surrounding the Anllóns River catchment, with a more comprehensive study in the As-Au mineralized area. The samples correspond to C horizons, which enable the identification of lithogenic As enrichment in order to evaluate the risk of As transfer, via soil water percolation, to groundwater and to surface waters. The As mobilization as a result of mining activities involving soil excavation and disposal must also be taken into consideration. Soil samples, previously milled and passed through a 200 µm sieve, were again milled in an agate mortar and pestle and passed through a 50 µm sieve, to carry out the corresponding analyses.

2.2.1. Total As concentrations

Total As was determined by X-Ray Fluorescence Spectrometry (custom built, equipped with a Philips high voltage generator and a Mo anode of 2.2 kW as the X-ray source). A certified reference material (BCR-CRM 277b) was employed to check the accuracy of total

As measurements. The differences between the certified and theoretical values were below 5%.

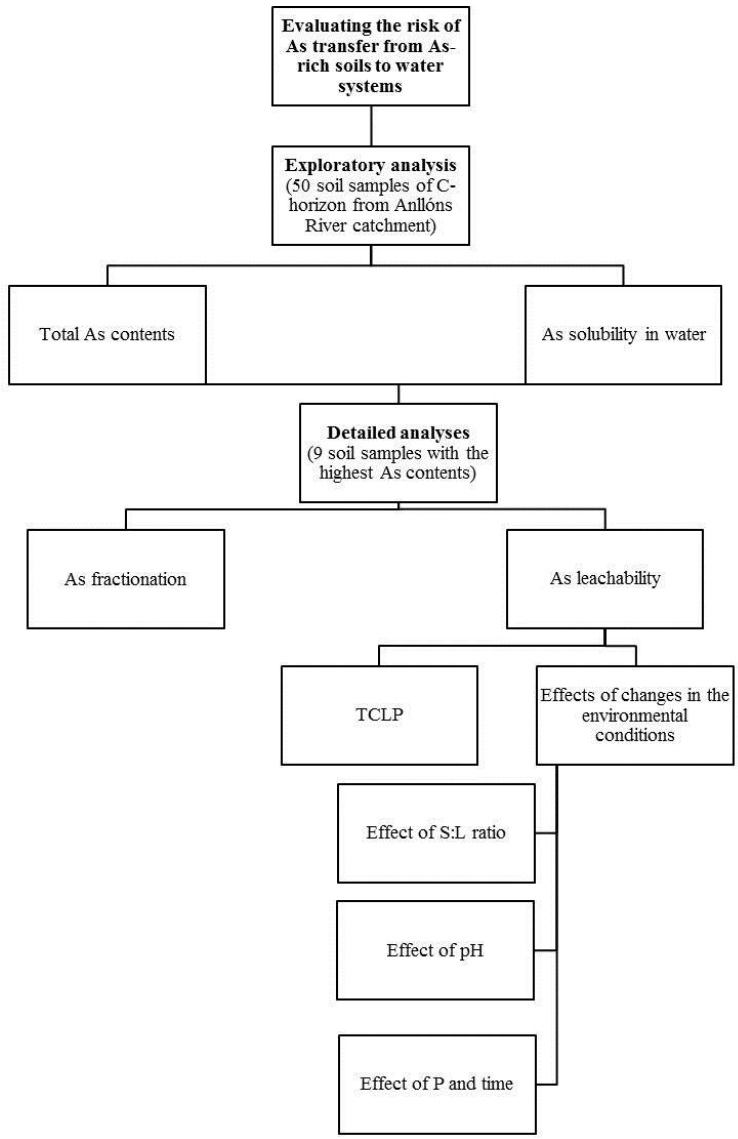

Figure C1.2. Scheme of the experimental procedure.

2.2.2. Mapping of total As contents

Spatial data analysis was carried out using ArcGIS 10.3 software. Inverse distance weighting (IDW) was used in this research to interpolate total As contents. All interpolation methods were developed based on the theory that points closer to each other have more correlations and similarities than those farther away (Yasrebi, 2009). IDW is a linear combination of the observed values, inversely weighted by the distances of the observation locations from the interpolation point. This interpolation method is based on the assumption that the influence of a known data point is inversely related to the distance from the unknown location that is being estimated (Kalivas, 2013).

2.2.3. Arsenic solubility in water

The German standard procedure DIN 38414-S4 (1984) was followed, using Milli-Q water at a 1:10 soil:water ratio and 24 h end-over-end agitation. Subsequently, extracts were filtered using 0.45 µm Whatman Puradisc 25AS^{TM} syringe filters and frozen (-80 ºC) until analysis of total As concentrations by Inductively Coupled Plasma Spectrometry (ICP-MS). A Varian 820-MS ICP-MS, equipped with collision reaction interface (CRI) technology to reduce polyatomic interferences, was employed for this purpose. The Fe concentrations were also determined in the extracts. The detection limits for As and Fe were 3.38 ng L^{-1} and 85.06 ng L^{-1}, respectively.

2.3. Detailed analyses

Nine samples with the highest As contents were selected for a more detailed study of As mobility by determining As solid species using a fractionation sequential procedure and solubility under different experimental conditions. Soil samples were numbered according to their geographical location, from left to right and bottom-up.

2.3.1. As fractionation

The sequential extraction procedure (SEP) described by Lombi et al. (1999) was applied using 1 g of soil and 25 mL of each extractant. This procedure considers the following 'operationally defined' chemical pools: F1: exchangeable (0.05 M $(NH_4)_2SO_4$, 1 h shaking), F2: specifically sorbed (0.05 M $NH_4H_2PO_4$, 1 h shaking), F3: associated to organic matter and Al (0.05 M NH_4F pH 7.0, 1 h shaking), F4: bound to amorphous Fe oxides (0.2 M NH_4-

oxalate pH 3.25, 4 h shaking in the dark), F5: bound to crystalline Fe oxides (0.2 M NH_4-oxalate + 0.1 M ascorbic acid pH 3.25, 30 min shaking in water bath at 96 ºC) and F6: residual phase (calculated as the difference between total As content and the sum of As extracted from steps F1 to F5). The extracts resulting in each phase were centrifuged at 5,000 rpm for 15 min at room temperature, filtered using 0.45 µm Whatman Puradisc 25AS™ syringe filters and frozen (-80 ºC) until analysis of total As concentrations by ICP-MS. All reagents were prepared with deionized Milli-Q water. All experiments were run in triplicate and blanks were run simultaneously.

Total As content of these specific samples was determined by microwave-assisted acid digestion at 150 ºC. To this end, 0.2 g soil was introduced into Teflon™ microwave digestion vessels with 10 mL acid solutions formed by 9 mL $HNO_{3(conc)}$ and 1 mL $HF_{(conc)}$ (Rubinos et al. 2011). After digestion, extracts were brought to a 100 mL final volume with 2.5 % H_3BO_3 solution added to "neutralize" the excess HF and to complex fluoride (forming tetrafluoroboric acid) in solution, as described by Wilson et al. (2006). This dilution with H_3BO_3 solution also makes it possible to maintain the HNO_3 concentrations below 10 %, as required for ICP-MS measurements. Extracts were filtered using 0.45 µm Whatman Puradisc 25AS™ syringe filters, frozen and total As concentrations were measured by ICP-MS.

2.3.2. As leachability

Arsenic mobility was estimated by applying the Toxicity Characteristic Leaching Procedure (TCLP) standard method, which simulates the leaching of waste dumped in a sanitary landfill and represents a method to evaluate the potential toxicity of waste materials. The effect on As mobilization of the modification of environmental conditions, such as pH, solid:liquid ratio, phosphorous and time, was also evaluated. A detailed description of the procedures is given below.

2.3.2.1. TCLP

The EPA Method 1311 (USEPA 1992) was applied, consisting of 24 h extraction in Milli-Q water at pH 4.5, adjusted with acetic acid, using a 1:20 soil:water ratio. After the extraction step, the suspensions were centrifuged at 2,000 rpm for 15 min and the extracts were filtered using 0.45 µm Whatman Puradisc 25AS™ syringe filters and frozen until analysis of As concentration by ICP-MS.

2.3.2.2. Effect of changes in the environmental conditions

The effect of solid:liquid ratio on As mobilization was tested in a 24 h extraction for 1:10 and 1:50 ratios in Milli-Q water. The effect of pH was studied at pH 3, 6 and 9, using 1:10 solid:liquid ratios and 24 h end-over-end shaking. The pH was adjusted with 1M HNO_3 or 1M NaOH; a saline background of 0.01 M $NaNO_3$ in Milli-Q water was used to counteract the differences in ionic strength due to the addition of acid or base and to more closely simulate the conditions of the soil solution. The effect of P (added as NaH_2PO_4) on As mobilization was evaluated by performing an extraction of 1 g soil in 10 mL of 0.01 M P prepared in 0.01 M $NaNO_3$; the suspensions were end-over-end shaken for 24 and 240 h to simultaneously evaluate the effect of phosphate and extraction time.

After the extraction steps, all extracts were filtered using 0.45 µm Whatman Puradisc 25AS^{TM} syringe filters and frozen (-80 ºC) until analysis of total As concentrations by ICP-MS. All experiments were run in triplicate and with the corresponding blanks, at room temperature (20 ± 2 ºC).

2.4. Statistical Analyses

Arsenic mobility for the aforementioned environmental conditions was evaluated using t-Student analysis or by one-way analysis of variance (ANOVA). Single Pearson correlations were calculated to analyse the possible relationships between total As content and As mobilized under the different environmental conditions. Principal component analysis (PCA), a multivariate statistical technique widely used as a tool for reducing the number of dimensions, was applied for a comprehensive study of As mobility in soil samples. Namely, total As and Fe, As fractionation and As mobility under different environmental conditions, as well as Fe mobility in phases F4 and F5 of As fractionation, were introduced as inputs in the PCA analysis. The SPSS 20.0 statistical package was used for all the statistical analyses.

3. Results and Discussion

3. 1. Exploratory analysis

3.1.1. Total As contents

Total As concentration ranged between 2 and 489 mg kg^{-1}, with a mean value of 85 mg kg^{-1}. The statistical distribution of As concentrations can be visually inspected using a box plot, prepared with SigmaPlot software (Figure C1.3a). The median, 25th and 75th percentiles (25, 14 and 94 mg kg^{-1}, respectively) were represented as lines in vertical boxes, with error bars representing the 10th and 90th percentiles (5 and 344 mg kg^{-1}, respectively).

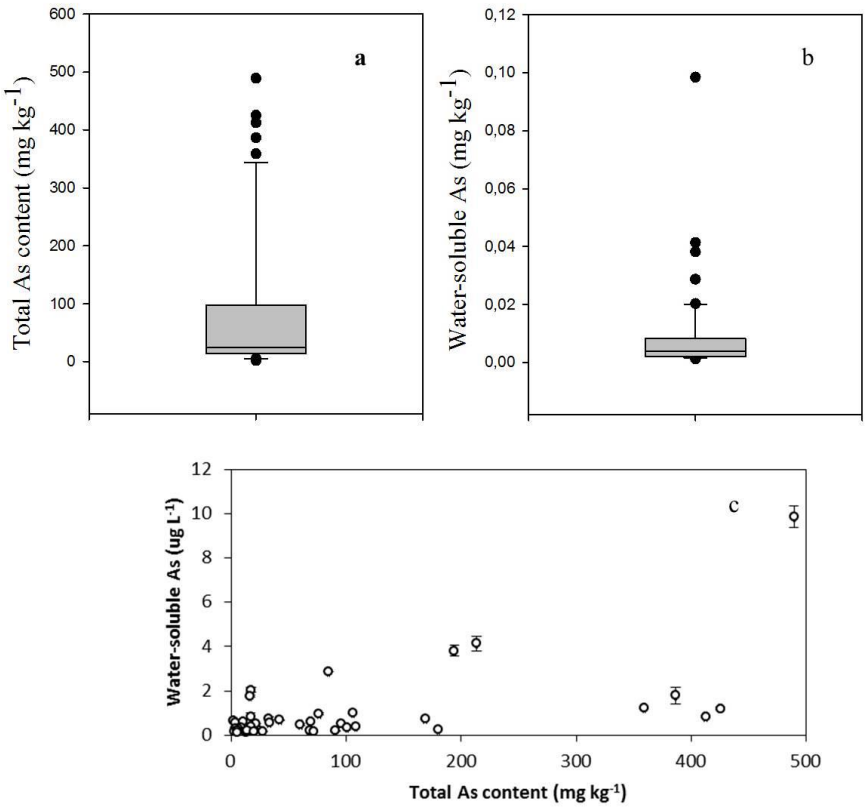

Figure C1.3. a. Box plot for total As content. b. Box plot for water-soluble As. c. Mobilization of water-soluble As in function of total As contents in soil samples.

Five soil samples exceeded this highest percentile (and are considered outliers) and were located in the Au-As mineralized area, according to the geological cartography (IGME 1981). The spatial distribution of As in the soil samples is represented in the map shown in Figure C1.4, prepared using ArcGIS 10.3 software.

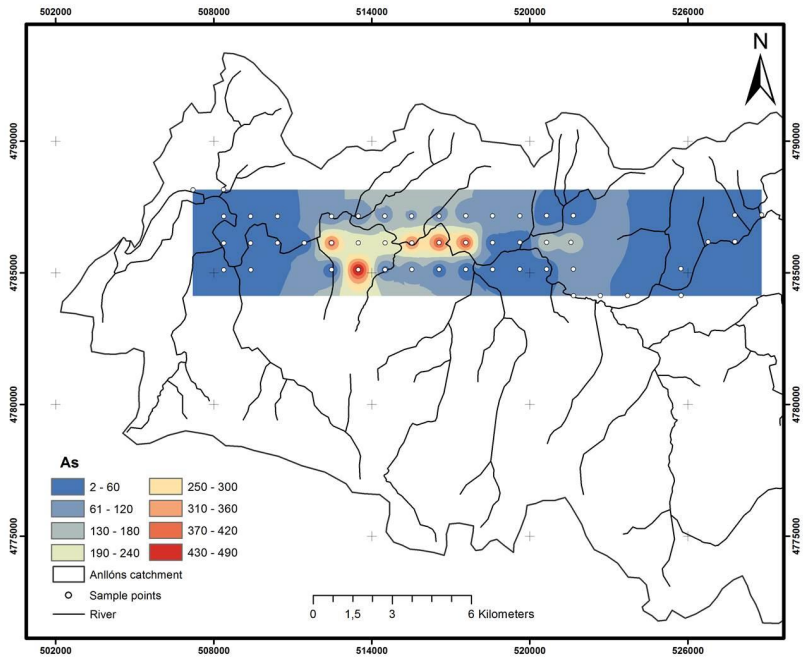

Figure C1.4. Mapping of total As.

Total As concentrations detected in this study were lower than those detected by Boixet et al. (2007) in superficial soils of the mineralized area (up to 4,000 mg kg^{-1}), but are within the range of those detected by Devesa-Rey et al. (2008a) in polluted sediments of this river, reaching a maximum value of 264 mg kg^{-1}.

The general reference level for As is fixed at 50 mg kg^{-1} for soils in Galicia (Macías Vázquez and Calvo de Anta 2009). This As concentration is also considered dangerous in several countries and above this concentration soil remediation procedures are recommended (Adriano 2001). This threshold value was exceeded in 21 soil samples, whereas 23 samples

also exceeded the limit allowed for agricultural soils in Galicia, set at 25-30 mg kg^{-1} (Consellería de Industria y Comercio 1996).

3.1.2. Arsenic solubility in wáter

Water-soluble As concentrations obtained using the DIN 38414–S4 method ranged between 0.13 and 9.85 µg L^{-1}. These concentrations are much lower than the acceptable leaching limit values for waste in inert waste landfills (EC 2003). On a dry weight basis, the As extracted ranged between 1.24 and 98.45 ng kg^{-1}, with a mean value of 8.96 ng kg^{-1}. The median, 25th and 75th percentiles corresponded to 3.88, 1.94 and 8.10 ng kg^{-1}, respectively (Figure C1.3b), while the 10th and 90th percentiles corresponded to 1.65 and 20.05 ng kg^{-1}, respectively. Out of five outliers identified for soluble As, only one was also an outlier for total As. The percentage of As mobilized in these experimental conditions was low and ranged between 0.002 and 0.033 % of total As. No correlation between total As and water-soluble As was observed (Figure C1.3c).

3.2. Detailed analyses

Nine samples with the highest As contents (108-489 mg kg^{-1}) were submitted to a more detailed study including As fractionation and As leachability in various extracts. In these samples, soil pH varied between 5.6 and 7.1 (Table C1.1), total Fe content varied between 1.3 and 6.7 %, whereas Mn concentration ranged between 0.2 and 3.0%. Soil organic matter was not determined because this component is scarce in soil C horizons. No correlation was found between total As and Fe either in the selected samples, or in the exploratory analysis (data not shown). Semiquantitative mineralogical analysis of soil samples using X-ray diffraction did not identify As minerals.

3.2.1. As fractionation

A SEP procedure makes it possible to distinguish trace element fractions with different solubility, which is empirically related to mobility classes (Hlavay et al. 2004). The results of As fractionation following Lombi et al. (1999) are shown in Table C1.1. The following decreasing order of abundance of the fractions was observed: F5>F4~F6>F2~F3>F1.

The predominant fraction was F5 (Fig. C1.5), which represents As bound to crystalline Fe oxides, with percentages varying between 37.4 and 60.7 % with respect to total As. The

following phases, in terms of abundance, were F4 (As bound to amorphous Fe oxides), with percentages varying between 15.8 and 39.8 %, and F6 (As in the residual phase), with values between 2.1 and 30.7 %. The results of the present study are in agreement with those found in Galicia for vineyard soils by Nóvoa-Muñoz et al. (2007), who observed the predominance of the As fraction associated to crystalline Al and Fe oxides. Moreno-Jiménez et al (2010) also observed a predominance of As retained by Al- and Fe hydrous oxides in soils adjacent to an old mine site, showing up to 3,000 mg kg^{-1} total As. However, As distribution in the soils under study was slightly different from that observed in the Anllóns riverbed sediments, where Devesa-Rey et al. (2008a) and Rubinos et al. (2011) found a predominance of the residual phase, with percentages reaching up to 75 % in some samples, although the next most abundant phase was As bound to Fe oxides, representing up to 55%.

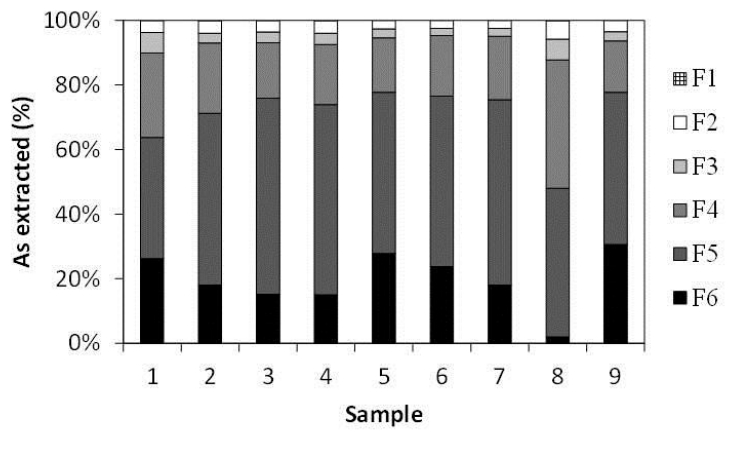

Figure C1.5. As fractionation (% of total As) following the SEP procedure of Lombi et al. (1999). F1: exchangeable, F2: specifically sorbed, F3: associated to Al and OM, F4: bound to amorphous Fe oxides, F5: bound to crystalline Fe oxides, F6: residual phase.

The exchangeable As (F1) extracted with ammonium sulphate was the least abundant fraction, accounting for only 0.01-0.05 % of total As. The specifically sorbed As (F2) extracted with ammonium phosphate was slightly higher and represented between 2.4 and 5.7 % of the total As content. These F1 and F2 fractions are considered the most mobile (Moreno-Jiménez et al 2010) and responsible for As toxicity in soils (Brandstetter et al. 2000). Finally, the As associated to Al and organic matter (F3), extracted with ammonium fluoride, exhibited similar values to F2 and accounted for 2.2-6.4 % of total As.

Table C1.1. Soil pH, total Fe, Mn and As concentrations, and As fractionation in soil samples following the SEP procedure described by Lombi et al. (1999).

Sample	pH	Fe_{XRF} (%)	Mn_{XRF} (mg kg^{-1})	$As_{T,XRF}$ (mg kg^{-1})	F1 (mg kg^{-1})	F2 (mg kg^{-1})	F3 (mg kg^{-1})	F4 (mg kg^{-1})	F5 (mg kg^{-1})	F6 (mg kg^{-1})
1	6.4	2.2	313	489	0.13 (0.04%)	12.19 (3.70%)	20.87 (6.33%)	86.28 (26.18%)	123.37 (37.43%)	86.71 (26.31%)
2	6.6	3.5	401	387	0.16 (0.05%)	13.74 (3.95%)	10.44 (3.00%)	75.50 (21.72%)	184.79 (53.17%)	62.91 (18.10%)
3	7.1	4.6	533	194	0.08 (0.04%)	5.86 (3.49%)	5.70 (3.39%)	28.70 (17.08%)	102.01 (60.71%)	25.69 (15.29%)
4	6.8	4.5	531	213	0.07 (0.05%)	6.21 (3.94%)	5.42 (3.44%)	29.33 (18.60%)	92.83 (58.87%)	23.82 (15.11%)
5	6.3	3.0	372	359	0.09 (0.03%)	8.82 (2.61%)	9.33 (2.76%)	56.89 (16.82%)	168.76 (49.89%)	94.35 (27.89%)
6	6.2	3.2	324	413	0.08 (0.02%)	9.32 (2.44%)	8.43 (2.21%)	71.52 (18.76%)	201.13 (52.75%)	90.83 (23.82%)
7	6.0	2.9	329	425	0.08 (0.02%)	9.53 (2.42%)	9.61 (2.44%)	77.11 (19.60%)	225.82 (57.39%)	71.31 (18.12%)
8	5.6	1.3	200	169	0.06 (0.05%)	7.21 (5.72%)	8.12 (6.43%)	50.21 (39.78%)	57.98 (45.94%)	2.68 (2.13%)
9	6.9	6.7	2981	108	0.01 (0.01%)	3.18 (3.46%)	2.66 (2.89%)	14.56 (15.83%)	43.35 (47.15%)	28.21 (30.68%)

Figures in parentheses in the fractionation analysis indicate percentage of total As solubilised in each step.

Table C1.2. As leachability in TCLP and effect of pH, solid:liquid ratio, phosphorous and time on As mobilization.

		TCLP	Effect of S:L ratio			Effect of pH			Effect of P and time	
Sample	As_T[a] (mg kg^{-1})	As_{TCLP} (mg kg^{-1})	$As_{1:10}$[b] (mg kg^{-1})	$As_{1:50}$ (mg kg^{-1})	$As_{PH=3}$ (mg kg^{-1})	$As_{PH=6}$ (mg kg^{-1})	$As_{PH=9}$ (mg kg^{-1})	$As_{t=24\ h}$ (mg kg^{-1})	$As_{t=240\ h}$ (mg kg^{-1})	
1	329.61	0.84 (0.25%)	0.10 (0.03%)	1.10 (0.33%)	0.12 (0.04%)	0.36 (0.11%)	0.73 (0.22%)	10.50 (3.19%)	17.05 (5.17%)	
2	347.53	0.03 (0.007%)	0.02 (0.005%)	0.52 (0.15%)	0.01 (0.003%)	0.10 (0.03%)	0.67 (0.19%)	10.97 (3.16%)	16.23 (4.67%)	
3	168.04	0.02 (0.01%)	0.04 (0.02%)	0.26 (0.16%)	0.01 (0.006%)	0.12 (0.07%)	0.38 (0.23%)	3.63 (2.16%)	8.48 (5.05%)	
4	157.68	0.03 (0.02%)	0.04 (0.03%)	0.51 (0.32%)	0.02 (0.01%)	0.11 (0.07%)	0.38 (0.24%)	4.54 (2.88%)	7.53 (4.77%)	
5	338.24	0.03 (0.009%)	0.01 (0.004%)	0.20 (0.06%)	0.01 (0.003%)	0.11 (0.03%)	0.62 (0.18%)	8.13 (2.40%)	14.34 (4.24%)	
6	381.30	0.03 (0.009%)	0.01 (0.002%)	0.12 (0.03%)	0.01 (0.003%)	0.09 (0.02%)	0.96 (0.25%)	8.62 (2.26%)	13.38 (3.51%)	
7	393.47	0.02 (0.005%)	0.01 (0.003%)	0.10 (0.02%)	0.01 (0.003%)	0.08 (0.02%)	0.49 (0.13%)	8.21 (2.09%)	14.38 (3.65%)	
8	126.21	0.02 (0.02%)	0.01 (0.006%)	0.07 (0.05%)	0.01 (0.01%)	0.18 (0.14%)	0.89 (0.71%)	3.75 (2.97%)	3.34 (2.64%)	
9	91.96	0.01 (0.006%)	$3.7\ 10^{-3}$(0.004%)	0.05 (0.05%)	$8\ 10^{-4}$ (0.001%)	0.01 (0.01%)	0.05 (0.06%)	1.99 (2.16%)	2.05 (2.23%)	

a. Total As content determined by microwave digestion used for the calculation of the percentages of As extracted.

b. As extracted by DIN 38414-S4 (1984).

In summary, the results of the fractionation procedure revealed that As is mainly associated to amorphous and crystalline Fe oxides, which together represent 63 to 86 % of total As and justify the low water-soluble As concentrations. However, a significant fraction (F1 and F2 representing 2-6 % of total As) may be potentially mobilizable, particularly under specific conditions of pH, solid:liquid ratio, phosphate and time, which have been proved to have drastic effects on As mobility in riverbed sediments containing As of geological origin in this area (Rubinos et al. 2010; 2011).

3.2.2. As leachability

3.2.2.1. TCLP

TCLP solubilized very low amounts of As, with concentrations ranging between 0.27 and 1.63 µg L^{-1} (Table C1.2). The exception was sample 1, for which the As concentration in the TCLP extracts reached 41.86 µg L^{-1}. With the exception of this sample, the values were one order of magnitude lower than those reported by Devesa-Rey et al. (2008a) for this extractant in the Anllóns riverbed sediments. All the values obtained in the present study were also much lower than the maximum allowable concentration for TCLP extracts fixed at 5 mg L^{-1} by USEPA (1996a). The As concentrations are also far from the EC_{20} determined by Rubinos et al. (2014) for As^{V} and As^{III} using the Microtox® acute toxicity bioassay, which correspond to 4.4 and 10.2 mg L^{-1}, respectively.

Table C1.3. Fe leachability in TCLP and effect of pH, solid:liquid ratio, phosphorous and time on Fe mobilization.

		TCLP	Effect of S:L ratio		Effect of pH			Effect of P and time	
Sample	Fe_T (%)	Fe_{TCLP} (mg kg^{-1})	$Fe_{1:10}$ (mg kg^{-1})	$Fe_{1:50}$ (mg kg^{-1})	$Fe_{PH=3}$ (mg kg^{-1})	$Fe_{PH=6}$ (mg kg^{-1})	$Fe_{PH=9}$ (mg kg^{-1})	$Fe_{t=24\ h}$ (mg kg^{-1})	$Fe_{t=240\ h}$ (mg kg^{-1})
1	2.90	1.73 (0.01%)	0.02 (<0.01%)	0.22 (<0.01%)	1.74 (0.01%)	0.07 (<0.01%)	0.56 (<0.01%)	0.10 (<0.01%)	0.20 (<0.01%)
2	4.97	0.40 (<0.01%)	0.01 (<0.01%)	1.67 (<0.01%)	3.96 (0.01%)	<d.l.[b]	<d.l.[b]	<0.01 (<0.01%)	0.02 (<0.01%)
3	6.19	0.77 (<0.01%)	0.85 (<0.01%)	0.43 (<0.01%)	8.34 (0.01%)	0.04 (<0.01%)	0.26 (<0.01%)	0.03 (<0.01%)	0.07 (<0.01%)
4	6.50	5.00 (0.01%)	1.01 (<0.01%)	0.54 (<0.01%)	5.67 (0.01%)	<d.l.[b]	1.62 (<0.01%)	0.03 (<0.01%)	0.10 (<0.01%)
5	4.47	0.59 (<0.01%)	0.06 (<0.01%)	0.34 (<0.01%)	1.19 (<0.01%)	<d.l.[b]	0.66 (<0.01%)	0.10 (<0.01%)	0.10 (<0.01%)
6	4.38	1.01 (<0.01%)	<0.01 (<0.01%)	0.12 (<0.01%)	2.79 (0.01%)	<d.l.[b]	1.59 (<0.01%)	0.15 (<0.01%)	0.08 (<0.01%)
7	4.26	0.74 (<0.01%)	0.01 (<0.01%)	0.09 (<0.01%)	1.62 (<0.01%)	<d.l.[b]	0.16 (<0.01%)	0.08 (<0.01%)	0.10 (<0.01%)
8	1.20	0.50 (<0.01%)	0.05 (<0.01%)	0.17 (<0.01%)	2.22 (0.02%)	<d.l.[b]	1.63 (0.02%)	0.43 (<0.01%)	0.43 (<0.01%)
9	6.59	<d.l.[a]	0.03 (<0.01%)	0.66 (<0.01%)	<d.l.[b]	<d.l.[b]	<d.l.[b]	0.03 (<0.01%)	0.05 (<0.01%)

[a]d.l.: detection limit of Fe in TCLP method equal to $1.7 \cdot 10^{-3}$ mg kg^{-1}.
[b]d.l.: detection limit of Fe in the study of pH effect equal to $8.5 \cdot 10^{-4}$ mg kg^{-1}.

With the exception of sample 1, in which the mobilized As reached 0.84 mg kg^{-1} and represented 0.25 % of total As, TCLP-soluble As expressed in dry weight basis was generally very low, ranging from 0.01 to 0.03 mg kg^{-1}, which represents 0.006 to 0.012 % of total As. These concentrations are in the order of those obtained in the DIN water leaching experiment and also similar to the F1 fraction of the sequential extraction procedure. This low mobility of As in TCLP can be related to As fractionation, which showed a predominant association of As with Fe oxides which were poorly soluble in the acetic solutions (Table C1.3) (the highest concentration of solubilised Fe was 5.00 mg kg^{-1} which represents 0.008 % of total Fe).

3.2.2.2. Effect of changes in the environmental conditions on As mobilization

Effect of solid:liquid ratio

The As concentrations in the extracts were significantly higher ($p<0.05$) for the 1:50 S:L ratio than for the 1:10 S:L ratio (Fig. C1.6a), suggesting that the effect of a higher solution volume prevails over the dilution effect. Nevertheless, the As concentrations were always much lower than the leaching limit values for waste acceptable at landfills, fixed at 0.05 mg L^{-1} for inert waste (EC 2003).

Expressed on a dry weight basis, As mobilization increased between approximately 7 and 29 times with the decrease in S:L ratio (Table C1.2). Therefore, whereas for a 1:10 ratio it varied between <0.01 and 0.10 mg kg^{-1} (representing between <0.01% and 0.03 % of total As), for a 1:50 ratio it ranged between 0.05 and 1.10 mg kg^{-1} (representing between 0.05 and 0.33 % of total As).

Effect of pH

As solubility in 0.01 M $NaNO_3$ at pH 3, 6 and 9 is shown in Table 2. As mobility increased significantly ($p<0.05$) with increasing pH and was 6 to 78 times higher at pH 9 than at pH 3. The maximum As released, observed at pH 9, was 0.89 mg kg^{-1}, accounting for 0.71 % of total As, whereas the lowest percentage, observed at pH 3, only reached 0.001 %. The environmental relevance of the pH effect on As solubility is revealed in Figure C1.6b, where As concentration in the extracts at pH 9 from six soil samples exceeded or were close to the leaching limit value for waste acceptable at landfills for inert waste, fixed at 0.05 mg L^{-1} (EC 2003).

These results agree with other studies reporting higher solubility of As at alkaline pH in soils and sediments (Van Herreweghe et al. 2002; Lager et al. 2005). It is also worth noting that higher As mobility was observed at alkaline pH by Rubinos et al. (2010) in sediments from the Anllóns River, although some samples also showed increased As mobility at acidic pH.

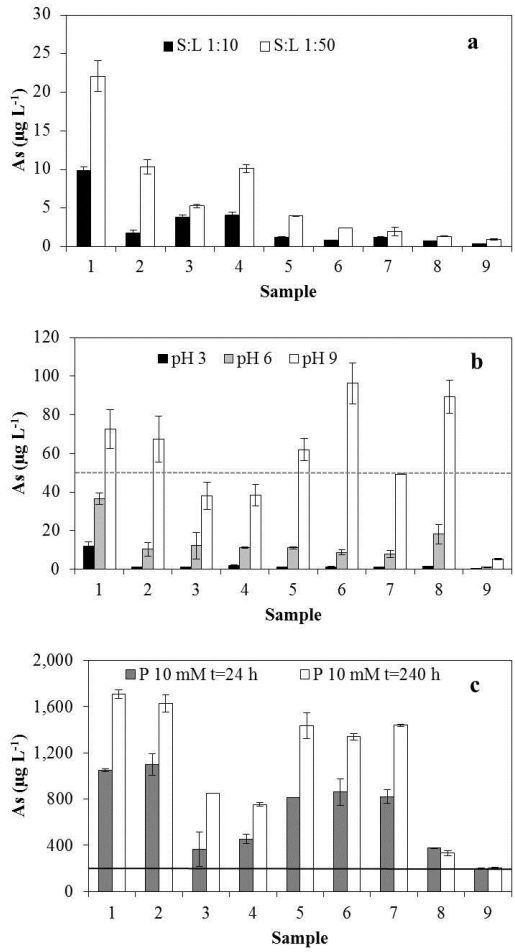

Figure C1.6. a. Effect of solid:liquid ratio (1:10 and 1:50). b. Effect of pH (3,6 and 9). Solid:liquid ratio 1:10, 0.01 M $NaNO_3$, t=24 h. c. Effect of P and time (24 and 240 h). The grey dashed and the solid black lines indicate the leaching limit values for waste acceptable in landfills for inert and non-hazardous waste, respectively (EC 2003).

Alkaline pHs favour As mobility due to the displacement of As by hydroxyl ions competing for sorption sites and to the more negative surface charges of active soil components at this pH, which hinder the subsequent adsorption. Where higher As mobility is observed at acidic pH in comparison with circumneutral pHs, it has been attributed to the partial dissolution of As-bearing Fe and Al (hydr) oxides in soils and sediments (Van Herreweghe et al. 2002; Rubinos et al. 2010). In this study, although As is mainly associated to Fe oxides, less than 0.02 % of total Fe was released at pH 3 (Table C1.3), which justifies that no correlation between Fe and As mobilized at this pH was found. Moreover, at this acidic pH, more positive charges are found on the surface of the active soil components favouring the adsorption of the As predominant monovalent species $H_2AsO_4^-$ and contributing to this low As solubility.

Effect of phosphorous and time

The As mobilized in the presence of 10 mM P concentration ranged between 1.99 and 10.97 mg kg^{-1} (representing between 2.09 and 3.19 % of total As) and was significantly ($p<0.05$) higher, between approximately 100 and 1000 times, than the As mobilized in water following the DIN 38414-S4 method. The effect of phosphate on As mobility, which was also observed for the Anllóns sediments by Rubinos et al. (2011), can be attributed to the competition of phosphate and arsenate for sorption sites; both elements form oxyanions with quasi-identical pKa values and similar effects on the surface charge of the solids (Manning and Goldberg 1996; Hongshao and Stanforth 2001). It is worth noting that this is the only leaching procedure followed in this study in which a linear relationship between the As extracted and total As content in soil was found (R^2=0.88).

When compared to P compartments in the fractionation scheme, the As mobilized by 10 mM P solutions was only slightly lower than the specifically sorbed As extracted in the F2 phase (desorbable with phosphate). It should be noted that this step of the SEP analysis uses a similar P concentration (13.5 mM) and its higher L:S ratio is apparently compensated by a shorter extraction time.

The effect of time on As mobility has been evaluated in the worst case scenario analysed in this study, i.e. using 10 mM P solutions. The As mobilized at 240 h varied between 2.05 and 17.05 mg kg^{-1} (representing between 2.64 and 5.17 % of total As), which implies an

increase of approximately 1.7-fold in As mobility compared to that obtained at 24 h. The exceptions were samples 8 and 9 for which similar results were found at both times. The environmental relevance of the combined effect of P and time is reflected in Figure C1.6c, where all the samples exceed the leaching limit value for inert and non-hazardous waste, fixed at 0.05 mg L^{-1} and 0.2 mg L^{-1}, respectively and some even approach the leaching limit value for hazardous waste (EC 2003).

When compared to P fractions obtained in the SEP procedure, it can be observed that the As extracted in step F2 during 1 h is on average 1.4 times lower than that extracted by solutions containing similar phosphate concentrations during 240 h, which means that As desorbable with phosphate, as estimated in F2, is underestimated by 40 % in these soil samples. These findings are noteworthy, especially for aquatic environments that receive inputs of phosphate, because they clearly show that As solubility may be underestimated in short-time experiments, such as those frequently used in standard leaching tests and sequential extraction procedures.

3.2.3. Statistical Analysis

Significant positive Pearson correlations ($p<0.01$) were found between As extracted by the two standard methods (DIN 38414–S4 and TCLP), but not between them and total As, or with As mobilizable in the most favourable conditions, i.e. alkaline pH and presence of phosphate (Table C1.4). This behaviour raises questions about the predictive ability of these standard methods in terms of environmental risk.

By applying PCA, two principal components (PC) with an eigenvalue >1 were extracted (Fig. C1.7), which explained 74 % of the total variance. PC1 mainly included the variables corresponding to conditions promoting As mobility ($As_{pH=9}$, As-$P_{t=24h}$ and As-$P_{t=240h}$), plus total As and all the As fractions except F3 (As associated with Al and organic matter). In turn, PC2 included the variables related to lower As mobility (As_{DIN}, As_{TCLP}, $As_{1:50}$, $As_{pH=3}$ and $As_{pH=6}$). Total Fe content, as well as Fe solubilized in steps F4 and F5 of the As fractionation (iron oxides) were not correlated and are not grouped with the As related variables. A possible explanation for this is that As mineralizations in this catchment are associated to acid quarzitic veins, which are poor in Fe-rich minerals. Nevertheless, this behaviour is not opposed to the observation that, in the analysed soil samples, As is mainly linked to Fe

oxides, because once As is released by weathering -a process typical of C horizons- from As bearing primary minerals (mainly arsenopyrite), it shows a great affinity for Fe oxides, to which it is bound by adsorption and coprecipitation.

Table C1.4. Significant Pearson correlation coefficients between total As and the As mobilized under the different studied conditions. The level of significance is indicated: **=p<0.01, *=p<0.05.

	As_T	As_{DIN}	As_{TCLP}	As_{pH3}	As_{pH6}	As_{pH9}	$As_{1:50}$	$As\text{-}P_{t=24h}$	$As\text{-}P_{t=240h}$
As_T	1								
As_{DIN}	0.096	1							
As_{TCLP}	0.237	0.895**	1						
As_{pH3}	0.230	0.903**	0.987**	1					
As_{pH6}	0.163	0.828**	0.873**	0.902**	1				
As_{pH9}	0.467	0.074	0.194	0.213	0.412	1			
$As_{1:50}$	0.216	0.918**	0.838**	0.823**	0.739**	0.149	1		
$As\text{-}P_{t=24h}$	0.896**	0.318	0.445	0.437	0.375	0.504*	0.513*	1	
$As\text{-}P_{t=240h}$	0.927**	0.389	0.441	0.434	0.373	0.418	0.519*	0.943**	1

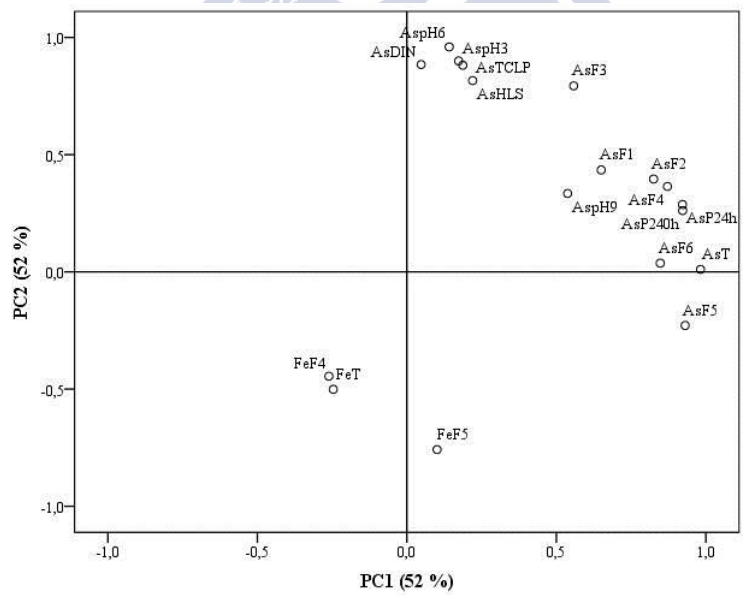

Figure C1.7. Principal Component Analysis for the evaluation of factors affecting As mobility.

3.3. General overview

Arsenic transfer to surface and subsurface waters, and especially to groundwater, may constitute an important risk to aquatic life and even to human health (Fendorf et al. 2008). The results of this study highlight the presence of high but variable concentrations of As in soil samples taken from C horizons in the Anllóns River catchment. The highest As contents were mainly found in or close to the As-Au mineralization area where gold mining operations were carried out at various times.

The mobility of As estimated by the standard procedures DIN 38414–S4 and TCLP was very low (maximum of 0.03 % and 0.25 % of total As, respectively), which is coincident with the results of As fractionation, because the sum of F1 and F2, considered the more mobile fractions, represents 6 % of the total As at most.

Nevertheless, the results of this study indicate that changes in environmental conditions, such as modifications of S:L ratios, pH conditions, the presence of competitive anions (phosphate) and contact time, drastically affect As mobility, increasing As solubility up to 1,600 times, which has to be taken into account in evaluations for environmental and health purposes.

In nature, such changes in S:L ratios may, first of all, be due to changes in the volume of water that percolates through the soil profile, which is related to rainfall. Secondly, the soil may be removed from its location as a preparatory step for mining operations and accumulated in spoil heaps subjected to leaching, or deposited in dumps where S:L ratios are also affected by the density of the tailings. Thirdly, if soil particles reach the river course, S:L ratios may change depending on whether the particles are deposited on the riverbed or in suspension and in this case, S:L ratios may change due to the river flow rate.

Alkaline conditions can occur as a consequence of contaminant discharges over the soil *in situ* or, if they are dumped as mine tailings, due to mining operations using alkaline solutions for metal beneficiation, as occurs in gold processing.

Phosphate concentrations may be increased due to the fertilization of soils *in situ*. Also if As-enriched soil particles reach the river course they can interact with soluble phosphate coming from urban and industrial sewage treatment plants and from fertilizers leached or

eroded from agricultural soils in the river catchment. In fact, in the Anllóns River, diffuse and point sources of P pollution have been identified along the river course, which result in soluble P concentrations of up to 1.4 mg L^{-1} (Rial 2007). In the riverbed sediments, high As concentrations have been detected in many places coinciding with P concentrations of up to 2324 mg kg^{-1} (Devesa-Rey et al. 2009), thus aggravating the risk of As mobility.

Contact times that exceed 24 h, which is the time established in the leaching standards methods, could occur in real conditions with the leaching of soils *in situ* or if they are dumped as mine spoils, or during transportation as suspended particles along the river course or when they are deposited as bed sediments.

4. Conclusions

Total As concentrations up to 8 times higher than the generic reference level of Galician soils are found in the C horizon of soils from the Anllóns River catchment. Based on the low solubility of As in the standard leaching tests DIN 38414-S4 and TCLP (up to a maximum of 0.25 % of total As), and to the predominance of the low mobility As phases in the soils, mainly associated to crystalline Fe oxides, the environmental risk of these high As concentrations may be considered low. Nevertheless, changes in environmental conditions, such as S:L ratios, pH conditions, the presence of phosphate and contact time, bring about an increased As mobility which can reach, under the most favourable conditions, up to 5 % of total As. The effect of phosphate is of particular interest as it can enter the soil and fluvial ecosystems from various sources, increasing the risk of As transfer to the aqueous phases, an effect which is aggravated by long contact times.

These results indicate that the information provided by the standard methods may be considered conservative for a reliable evaluation of the risk of As transfer from soils to water bodies. Although the standard methods provide useful information to compare with leaching limit values and to establish comparisons between researchers from different labs, it is important to perform other tests that represent more realistic scenarios for the assessment of the transfer of pollutants into aquatic systems.

Chapter 2: Monitoring benthic microflora in river bed sediments: a case study in the Anllóns River (Spain)

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Chapter 2: Monitoring benthic microflora in river bed sediments: a case study in the Anllóns River (Spain)

Abstract

The purpose of this chapter was to investigate the abundance and composition of the superficial biofilm on the Anllóns River (NW Spain) bed sediments, to evaluate the relationships between biochemical parameters and biological methods based on identification and counting, and to explore the relationships between biofilm growth and the properties of the sedimentary habitat, mainly the trophic state. Bed sediment samples (0-5 cm) were collected in two different seasons (winter and summer) at 4 sampling sites along the river course. Physico-chemical properties of pore waters and sediments were determined. Biological properties included the determination of dehydrogenase activity (DHA) and phytopigment (Chl *a* Chl *b* and total carotenoids) concentrations, as well as taxonomic identification. For taxonomic identification, two sampling methods were compared: the Pasteur pipette method and a mini-corer method. Total and relative algal abundances (TA and RA respectively) and genus richness were calculated. The relationships between the different variables were examined using Pearson correlations and Principal Component Analysis. The main taxa belonged to Chlorophyta, Cyanophyta, Euglenophyta and Heterokontophyta. The most abundant class was Bacillariophyceae, which represents more than 86% of the total abundances in the superficial sediments. The highest total algal abundance and genus richness were observed in summer at the river mouth, where DHA and phytopigment concentrations were also the highest. The statistical analysis revealed positive correlations between TA and the biochemical parameters (DHA and phytopigments) as well as positive relationships of these three parameters with the physico-chemical properties of the sediments, such as electrical conductivity, fine particles, C, N, S and total P. The results of this study reveal the positive relationships between the biochemical properties (phytopigments and respiratory activity) and total algal abundances determined by taxonomic identification and counting. All of these properties presented evidence of a clear influence of the nutrients and organic matter contents of the sediments, pointing to the importance of the site conditions, particularly the trophic state, in the development of benthic microflora.

1. Introduction

Biofilms covering the surfaces of rocks, mineral grains and plant debris are common in aquatic environments. Biofilms are composed of heterotrophic and autotrophic microorganisms, immersed in a complex matrix of extracellular polymeric substances (EPS). The primary role of biofilms is the protection of microbial communities in conditions of environmental stress (Decho 2000; Flemming et al. 2001).

Epipsammic biofilms represent the interface between water and granular sediments, affecting the exchange of solutes among these environmental compartments and the physical stability of the sediment. It has been demonstrated than biofilms affect the role of sediments as potential sources or sinks for contaminants (Devesa-Rey et al. 2009; Gerbersdorf et al. 2011) and that the presence of biofilms alters the adsorption and release of pollutants by bed sediments (Leadbeater and Callow 1992; Gainswin et al. 2006, Prieto et al. 2013). EPS form adhesive coatings on sediment particles, increasing their erosion threshold, and consequently increasing sediment stability and limiting their resuspension (Sutherland et al. 1998; Stal 2003; De Brouwer 2005; Flemming 2006; Tolhurst et al. 2006; Ziervogel and Forster 2006; Gerbersdorf and Wieprecht 2015). It has been observed that the stabilization of the sediment matrix by biofilms reduces the potential release of pollutants due to resuspension (León-Morales et al. 2006; Gerbersdorf et al. 2007; Lubarsky et al. 2010). Notwithstanding, despite this evidence regarding the influence of biofilms on the physical and chemical properties of the granular bed sediments, their role is often ignored when the geochemical processes (i.e. retention, mobility, bioavailability and speciation of pollutants) occurring at the sediment-water interfaces are studied.

Previous studies of diatom benthic populations indicated that the Anllóns River (NW Spain) was moderately to heavily contaminated (Ector 1992). Research into the chemical composition of the bed sediments of the Anllóns River (NW Spain) confirmed that the basin is affected by diffuse pollution due to agricultural activities and urban and industrial discharges into the water course (Devesa-Rey et al. 2008b; Devesa-Rey et al. 2012). Ecotoxicity was observed at some points in the river course, which in several cases was attributed to high arsenic (As) concentrations (Devesa-Rey et al. 2008b), related to past mining activities in gold-rich areas containing arsenopyrites (Devesa-Rey et al. 2008a, 2010c). Arsenic is of particular concern in this basin as Rubinos et al. (2010, 2011) have

shown that As can be mobilized in the Anllóns riverbed sediments under conditions of high salinity, alkaline pH or high phosphorus concentrations, as well as during high-flow resuspension events. Once mobilized, As may interact with the biofilm. Arsenate -the most common form of As in natural waters- could affect periphyton communities, inhibiting algal growth, photosynthetic capacity, changing community composition, diatom sizes and reducing the ability of the community to retain phosphorus (Blanck and Wangberg 1988; Blanck and Wangberg 1991; Wangberg et al.1991; Rodriguez Castro et al. 2015).

So far, there have been no studies into the abundance and composition of epipsammic biofilms in river sediments of north-western Spain and the limited available data on algae in the region's freshwaters refer to epilithic (growing on rock surfaces) or epiphytic (growing on living plants) phytobenthos (Margalef 1955, 1956; Ector 1992; Noguerol Seoane 1993; López Rodríguez and Penalta 2004). Nevertheless, the study of biofilms deserves special attention because some species of benthic microalgae are indicative of contamination. In particular, benthic diatoms have characteristics that make them well suited for the bio-indication of the quality of inland waters, namely their abundance and great taxonomic diversity, their ability to colonize different environments, as well as the preservation of frustules in sediments (López Rodríguez 2005).

In this work we aim to assess the relationships between biological methods for the evaluation of epipsammic biofilm growth, based on taxonomic identification and counting, and biochemical methods. Respiration, as an index of the overall biological activity in the superficial sediment, and phytopigments, as a measure of the abundance of photosynthetic organisms in biofilms, were selected as the biochemical properties to be tested.

The activity of the enzyme dehydrogenase (DHA) can be used to determine respiratory activity, as it provides a measure of the activity of oxidation–reduction enzymes responsible for dissociating H^+ from H_2O, thus diverting $2e^-$ into the electron transport process. DHA is present in all active microorganisms and can be used in both aerobic and anaerobic environments. DHA has been used to measure the activity of stream and river microbial communities and their responses to disturbances (Trevors et al. 1982; Blenkinsopp and Lock 1990; Ponsati et al. 2014).

The determination of phytopigment concentrations by spectrophotometry is commonly used to estimate biofilm growth over various surfaces (Ortega-Calvo et al. 1995; Tomaselli et al. 2002; Guasch et al. 2004; Serra et al. 2009a; Vázquez-Nion et al. 2013). It has been extensively applied for the measurement of microphytobenthos in sediments. Phytopigments determined by this method were highly correlated with instrumental colour measurements performed with the Anllóns sediments (Sanmartín et al. 2011).

The present study attempts to assess the overall biological activity in the surface sediment by means of the determination of the dehydrogenase activity, and that of the autotrophic biomass by means of phytopigment analysis. The statistical relationships between these biochemical measurements and the parameters derived from the taxonomic identification, such as algal abundance and genus richness, are tested, and the effect of sediment properties (particle size, nutrients and the presence of toxicants) on biofilm growth is also examined. This information would be useful for the evaluation of biofilm abundance and composition on riverbed sediments and for the assessment of its effects on the biogeochemical cycles of the elements, particularly those which, like arsenic, have significant effects on the environment and public health.

2 Materials and methods

2.1 Sampling

Superficial bed sediment samples were taken at four sites in the Anllóns River, distributed along the watercourse between the locality of A Ponte and the river mouth at Ponteceso (Fig. C2.1). The sites were identified with a number and two letters. The number indicates the position from the locality of A Ponte along the watercourse and the letters are abbreviations of the location. The sites were selected taking into account the contamination detected in previous studies (Table C2.1). Thus, site 1AP is influenced by diffuse pollution whereas 2CA and 4PO are located downstream from the biggest towns in the area (Carballo and Ponteceso) and are influenced by waste water treatment plants. Site 3XA is located in the area of Au-As mineralization. Sampling sites 1AP, 2CA and 3XA were mostly shaded by riparian vegetation with *Alnus glutinosa* and *Fraxinus excelsior*, whereas site 4PO was situated in the estuary, without riparian vegetation, and receiving more sunlight.

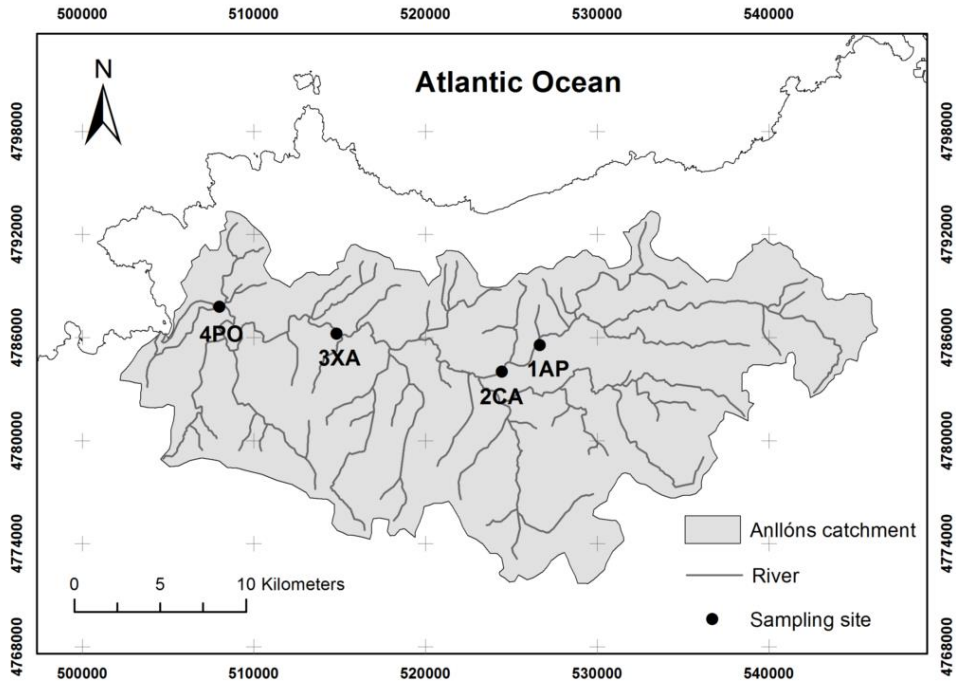

Figure C2.1. Location of four sampling sites along the Anllóns River.

Table C2.1. Name, location and other characteristics of the sediment sampling points.

Sites	Location	Distance* (km)	Elevation	Special feature
1AP	A Ponte (43º13´17.75´´N 8º40´17.77´´O)	0	121m	Diffuse pollution
2CA	Carballo (43º12´27.49´´N 8º41´56.70´´O)	3.13	100m	Wastewater plants
3XA	Xavarido (43º13´40.13´´N 8º49´03.11´´O)	16.86	54m	Mining activity
4PO	Ponteceso (43º14´31.11´´N 8º54´04.99´´O)	32.05	4m	Wastewater plants

* From A Ponte site

Sampling was carried out in February and July 2012 between 10 am and 4 pm of the same day. Sediments were collected with a small plastic shovel from the top 4 cm at various points of the same site, mixed and homogenized to form a complex sample. Sediment samples were stored in thermally insulated airtight plastic boxes and transported to the laboratory, where pore water was removed by centrifugation at 2000 rpm. Subsequently, a portion of the

solid samples was stored at 4ºC until biochemical analyses, and the remainder was immediately freeze-dried and later sieved by 2 mm for the determination of the general properties of the sediment.

2.2 Analyses

Eh and pH in the surface of the sediment were measured *in situ* with a portable device (HANNA HI 9025 microcomputer, Padova, Italy) at the sampling time. Eh values obtained with the Pt-Ag/AgCl electrodes were corrected to refer them to the H_2 by adding 245 mV.

Analyses of the pore water included total phosphorus ($P_{T\text{-}PW}$), which was determined after acid digestion (APHA 2005) with 1 ml H_2SO_4 31% and 0.1 g $(NH_4)_2S_2O_8$ at 120 ºC for 30 min by colorimetric determination using the phosphomolibdenum blue method (Murphy and Riley 1962). Soluble P ($P_{SOL\text{-}PW}$) was measured by colorimetric determination in pore water samples filtered by 0.45 µm. pH and electrical conductivity (EC) were measured with a HANNA HI 9025 microcomputer and HI 9033 device (Padova, Italy), respectively.

With regard to sediment analyses, total phosphorus (P_T) was determined by means of an acid digestion (HF, H_2SO_4, HCl, 10:1:10) followed by colorimetric determination, as mentioned above. Bioavailable P (P_{BIO}) was estimated by extracting the sediment with NaOH 0.1 M using a 1:100 sediment:solution ratio (Wolf et al. 1985). Total C, N and S were determined by elemental analysis using a macrosample Leco TruSpec CHNS instrument (St. Joseph, Michigan, USA) based on the total combustion of the sample and subsequent determination of the combustion gases using a thermal conductivity detector. Grain size distribution was determined by wet sieving and the pipette method as per Guitián and Carballas (1976). Total As content of the sediment samples was determined by microwave-assisted acid digestion (150 ºC), employing Teflon^{TM} microwave digestion vessels containing 0.2 g sediment and 9 ml $HNO_{3(conc)}$ + 1 ml $HF_{(conc)}$, followed by As analysis using hydride generation atomic absorption spectrometry (HGAAS, Perkin–Elmer M2100 coupled with an MHS-10 hydride generation unit, Waltham, Massachusetts, USA), by reaction of acidified samples with 3% $NaBH_4$/1% NaOH as reductant to generate arsine gas (AsH_3).

Measurement of respiratory activity by means of dehydrogenase activity (DHA) is based on intercepting e^- flow through mitochondrial and microsomal electron transport systems using a surrogate electron acceptor, 2,3,5-triphenyltetrazolium chloride (TTC), which has a

slightly higher redox potential than that of the coenzyme UQ – cytochrome b complex, one of several cytochrome compounds used by eukaryotic organisms as terminal electron acceptors, and similar cytochrome electron acceptors are used by prokaryotic organisms (Packard 1971; Broberg 1985). DHA activity was measured in the fresh samples by the reduction of TTC to triphenylformazan (TPF), following the method described by Tabatabai (1982). DHA was measured at 485 mm and 520 mm to avoid possible interferences with photosynthetic pigments (Patil et al. 2000).

Phytopigments (chlorophyll-*a*, chlorophyll-*b* and carotenoids) were solubilized by extraction with dimethyl sulphoxide for 46 min, using an extractant:sediment ratio of 3.6 mL g^{-1} at 57 ºC, following the method optimized by Devesa et al. (2007) for sediment analysis. In the extracts, chlorophyll-*a*, chlorophyll-*b* and total carotenoids were determined spectrophotometrically (Cary 100 Conc. Varian, Santa Clara, California, USA). **Eqs. (1)-(3)** were employed to determine the concentrations of chlorophyll-*a* (Chl *a*) and *b* (Chl *b*), as well as total carotenoids (C_{x+c}), in $\mu g \cdot mL^{-1}$, following Wellburn (1994).

$$\text{Chl } a = 12.47A_{665.1} - 3.62A_{649.1} \quad \text{(Eq. 1)}$$

$$\text{Chl } b = 25.06A_{649.1} - 6.5A_{665.1} \quad \text{(Eq. 2)}$$

$$C_{x+c} = (1000A_{480} - 1.29\text{Chl } a - 53.78\text{Chl } b)/220 \quad \text{(Eq. 3)}$$

where Chl *a* and Chl *b* represent the concentration of chlorophyll-*a* and chlorophyll-*b* respectively, and C_{x+c} represents the concentration of total carotenoids, comprising the oxidized forms (xantophylls) and the reduced forms (carotenes). $A_{665.1}$, $A_{649.1}$ and A_{480} represent the absorbance of the extracts at 665.1, 649.1 and 480 nm, respectively. To estimate the phaeopigments, 0.5% of HCl 1 M was added to the extracts and, after 10 min, the absorbance at 665.1 nm and 649 was measured again. The difference of absorbance between the non-acidified and acidified samples corresponds to the absorbance of the chlorophyll. Each sample was submitted to several extractions (between 2 and 3) until no phytopigments were quantified in the extracts, and the concentrations obtained were added to obtain the total concentration.

The taxonomic identification required specific samplings, which were carried out simultaneously to the sampling of the sediments subjected to characterization. There is no

standard method for biofilm sampling in unconsolidated granular materials such as riverbed sediments. Therefore, in this study, two sampling techniques on sediment surfaces were tested and compared: 1) The Pasteur pipette method: samples were collected from a known area of 1 cm^2 at five points in the area, transferred to glass tubes and made up to a known volume; 2) A novel corer method: sampling was done by introducing in the sediment surface a plastic corer of 2.4 cm diameter x 3.0 cm height, sealed at the top with Prolene® film to preserve biofilm integrity. After collection, the samples were cooled and transported to the laboratory where they were preserved with a solution of 4% formaldehyde. Algae were identified at the lowest taxonomic level (individuals/cm^2) under an optical microscope Olympus BX61 with a Nomarski interferential contrast (Tokyo, Japan) and were quantified using an inverted microscope. Algal abundance was calculated, including 5 replicates of each sample. For the pipette method, an alliquot of 0.1 mL was taken from the glass tubes. For the corer method, 2 mm diameter areas were sampled from each core. In both cases, samples were placed on slides and observed at 20x magnification. Additionally, selected unaltered sediment samples taken with the corer were observed by Scanning Electron Microscopy (SEM) (ZEISS EVO LS 15, Jena, Germany) to study the association of microalgae populations with mineral phases.

2.3 Statistical analysis

The Student's t-test was carried out to analyze the differences between sampling methods for taxonomic identification. As a first step, data were checked for normal distribution. Single Pearson correlations and principal component analysis (PCA) were calculated with the statistic software package SPSS v20.0 to analyze the possible relationships between the biological and chemical parameters analyzed.

3 Results and discussion

3.1 Physicochemical properties of the bed sediments

In Table C2.2 it can be seen that the sediments had neutral to slightly acidic pH (6.1 – 7.0). The oxidation/reduction potential, measured as Eh, ranged between -21.5 and 59.0 mV and was indicative of a moderately anoxic state of the surface layer of the sediment. The pore

waters extracted from the sediments showed pH values slightly higher than those measured *in situ*. EC values fell within the range 0.17-0.47 mS cm^{-1} from samples 3XA to 2CA, whereas sample 4PO showed a higher value (9.46 and 14.25 mS cm^{-1} in winter and summer, respectively) that evidenced the marine influence in the estuary. Total phosphorus ($P_{T\text{-}PW}$) in the pore water varied from 0.21 to 0.48 mg L^{-1}, and exceeded 0.1 mg L^{-1}, that is the maximum acceptable concentration to avoid accelerated eutrophication or to promote algal blooms (USEPA 1986). Soluble phosphorus ($P_{SOL\text{-}PW}$) fell within the range 0.003-0.13 mg L^{-1}. These values would classify the pore water quality with a Good or High ecological status according to Recommendations on Phoshorus Standards for Rivers derived from the Water Framework Directive 2000/60/EC (WFD UK TAG 2008). No remarkable differences in P concentrations were observed among sites or sampling season. Although P concentrations could suffer seasonal variations due to remineralization of organic matter in the water column and sediment, to uptake for phytoplankton growth and to excretion by zooplankton (Laurent et al. 2012), in the Anllóns catchment these effects can be counteracted by P inputs from wastewaters, which are more remarkable in low flow periods, and from agricultural activities which are more intense in spring and summer in this area.

The sediments showed a predominance of the sandy fraction, which always exceed 60%. Site 4PO exhibited the finest texture in both samplings. This fact may be attributed to the decrease in the river slope, and the mixing of freshwaters and saline waters, favouring the deposition of fine materials in the proximity of the estuary.

Carbon (C) and nitrogen (N) also showed the highest values at site 4PO (around 6% and 0.5%, respectively) and the lowest at site 3XA (around 0.6% and 0.04%, respectively) at both sampling times. This may be related to more favourable deposition conditions, and to the highest clay and silt contents -and consequently to the highest surface area- at site 4PO, favouring organic matter (OM) and nitrogen retention. This behaviour has been previously described by Bergamaschi et al. (1997), who reported that C and N were strongly related to sediment surface area, and by Xiaoxia et al. (2005), who reported that total N presented positive correlations with the fine particle content in the surface sediments of the southern Yellow Sea. C/N ratios could be used as an indication of OM origin in riverbed sediments. Thus, C/N ratios <12 are typical for OM associated to algal biomass, and therefore from autochthonous origin (Müller 1977), whereas C/N ratios >12 are indicative of OM rich in

lignin and cellulose as well as poor in N, attributable to terrestrial origin (Lamb et al. 2006). Sediment samples analysed in this study presented C/N ratios from 12 to 16, indicative of the predominance of OM from terrestrial origin. These values are in the range of those reported by Devesa-Rey et al. (2009) for riverbed sediments from 14 sampling sites in the Anllóns River, with values varying from 5 to 36 and with a mean value of 13.4, as well as of those reported by Barral et al. (2012), who found C/N values from 13 to 35, with a mean value of 18.1, for 10 sediments from the same river. Moreover, C/N ratios for soils and suspended sediments in the Anllóns River catchment, analysed by Iglesias et al. (2011), were also in the same range, with values of 14.6 and 10.7, respectively.

Sulphur (S) in the sediments may be present both in inorganic and organic form. In the samples analysed, S contents ranged between 0.02 and 0.78 %, with the highest value at site 4PO, where the estuary conditions favour S reduction and precipitation. S contents were lower in summer, pointing to a biological oxidation of the organic S and its transformation into soluble sulphates.

In the sediments, P_T varied from 205 mg kg^{-1} (1AP) to 1130 mg kg^{-1} (4PO). On average, 71% of the P (51-90%) in the sediments is in bioavailable form, the highest P_{BIO} corresponding to 4PO. This high bioavailability may have a direct effect on the primary productivity and, thus, on biofilm formation (Sterling et al. 2000). P concentrations are slightly lower than those determined by Devesa-Rey et al. (2008b) but similar to those previously obtained by Barral et al. (2012) for the sediments of the Anllóns River. Total P concentrations exceed at two sites the Lowest Effect Level (LEL) established by the Ontario Sediment Quality Guidelines (Persaud et al. 1993), which is set at 600 mg kg^{-1}, although they did not exceed the Severe Effect Level (SEL), set at 2000 mg kg^{-1}. Below SEL, the sediment is considered clean to marginally polluted and it is expected that this level of contamination will have no effect on the majority of sediment-dwelling organisms.

Table C2.2. Physico-chemical properties of the sediments analysed.

		WINTER				SUMMER			
	Sampling sites	**1AP**	**2CA**	**3XA**	**4PO**	**1AP**	**2CA**	**3XA**	**4PO**
In situ	**pH**	6.6	6.5	6.1	7.0	6.4	6.0	6.7	7.1
	Eh (mV)	10.6	22.0	39.2	-21.5	26.5	59.0	9.0	-13.5
Pore waters	**pH**	6.8	6.3	6.9	7.4	6.4	6.7	6.7	6.7
	EC (mS cm^{-1})	0.31	0.47	0.19	9.46	0.18	0.23	0.17	14.25
	$P_{T\text{-}PW}$ (mg L^{-1})	0.22	0.48	0.28	0.29	0.27	0.23	0.30	0.21
	$P_{SOL\text{-}PW}$ (mg L^{-1})	0.13	0.09	0.03	0.04	0.05	0.03	0.07	0.003
Bed sediments	**Particle size (%)**								
	Clay	12.2	7.0	5.0	18.2	1.1	0.8	0.7	9.4
	Silt	13.7	6.0	4.0	18.5	4.7	6.6	6.0	29.5
	Sand	74.1	87.0	91.0	63.3	94.2	92.6	93.3	61.0
	C (%)	5.04	1.28	0.60	6.09	0.80	0.99	0.66	6.73
	N (%)	0.34	0.11	0.04	0.51	0.05	0.07	0.04	0.51
	S (%)	0.05	0.29	0.14	0.78	0.02	0.04	0.02	0.43
	C/N	15	12	14	12	15	14	16	13
	Total P (mg kg^{-1})	618	421	345	628	205	365	290	1130
	Bioavailable P (mg kg^{-1})	405	266	302	547	132	327	165	575
	As (mg kg^{-1})	11.8	12.1	42.4	21.6	7.3	13.4	43.3	40.6

Grain size fractions were classified as coarse sand (2–0.2 mm), fine sand (0.2–0.05 mm), coarse silt (0.05–0.02 mm), fine silt (0.02–0.002 mm) and clay (<0.002 mm).

Arsenic (As) in the sediments ranged between 7.3-43.3 mg kg^{-1}, and was higher in samples 3XA and 4PO, downstream from the Au-As mineralization area. These values were lower than the maximum values detected in the sampling campaign performed by Devesa-Rey

et al. (2008b), which reached 264 mg kg^{-1}. The As concentrations found in the bed sediments were also lower than reference As levels for soils in Galicia, fixed at 50 mg kg^{-1} (140 mg kg^{-1} in soils over slates with arsenopyrite) (Macías and Calvo 2009) and lower or close to the thresholds of the European Water Framework Directive for suspended matter and sediment (40 mg kg^{-1}) (EU-WFD 2000). The values were also lower than the "Effects Range Median" (ERM) (the level at which half of the studies reported harmful effects) set at 70 mg kg^{-1} As by Long et al. (1995). Nevertheless, with the exception of site 1AP, these values exceeded in all cases the "Effects Range Low" (ERL) (the lowest concentration of a metal that produced adverse effects in 10% of the data reviewed) set at 8.2 mg kg^{-1} by the same authors. Arsenic mobility is a key factor in the toxicity of this element. In the sediments of the Anllóns River, As exhibited low mobility because it was found to be mainly associated to the least mobile fractions: bound to Fe-Al oxides and in the residual phase (Devesa-Rey et al. 2008a; Rubinos et al. 2011), although it can increase in conditions of increased pH, higher salinity, higher phosphorous concentrations or higher liquid:solid ratios (Rubinos et al. 2010, 2011). Devesa-Rey et al. (2008a), applying the Toxicity Characteristic Leaching Procedure (TCLP) to sediments of the Anllóns River, determined As concentrations in extracts lower than Criteria Continuous Concentration (CCC) for As set at 150 µg L^{-1} by USEPA (1996b).

3.2 Biological characterization of the bed sediments

Dehydrogenase activity (DHA) showed the maximum value at site 4PO in both seasons (1457 and 3075 mg kg^{-1} d^{-1} in winter and summer, respectively), followed by site 2CA (Table C2.3). Both sites are characterized by their nutrient richness, which could justify their greater biological activity. DHA values were in the range of activity values reported by Filimon et al. (2013) for sediments in Serbian water streams with varying levels of metal pollution and slightly higher than those reported for a wide range of soils in the world (Dick et al. 1996). Filimon et al. (2013) pointed out the difficulty to classify polluted sites from the assessment of enzymatic activities, which depend on season and location, and also the difficulty to extrapolate to field surveys the linear relationship found in lab experiments between microbial enzymatic activities and levels of trace elements in soils and sediments. Thus, although it is possible to make comparisons between sites with similar characteristics over time or after the impact of a potential contaminant, a particular DHA value does not classify a site as contaminated.

Table C2.3. Biological properties of the sediments analysed.

	WINTER				SUMMER			
Sampling sites	**1AP**	**2CA**	**3XA**	**4PO**	**1AP**	**2CA**	**3XA**	**4PO**
DHA (mg TPF kg^{-1} d^{-1})	441	745	n.d.[1]	1457	51	305	81	3075
Chl *a* (ug g^{-1})	2.16	n.d.[2]	n.d.[2]	1.14	2.18	9.12	0.71	34.6
Chl *b* (ug g^{-1})	1.50	0.44	0.10	1.94	1.32	4.85	0.16	18.7
Total carotenoids (ug g^{-1})	1.40	0.34	0.29	0.72	1.62	5.03	0.23	21.4

n.d.: not detectable

Detection limits: [1]DHA: 0.5 mg TPF kg^{-1} d^{-1}

[2]Chl a: 0.05 ug g^{-1}

All the evaluated phytopigments (Chl *a*, Chl *b* and total carotenoids) presented a similar behaviour (Table C2.3). Phytopigment concentrations were similar to those previously found by Devesa-Rey et al. (2009) and Sanmartín et al. (2011) for the same river, which ranged between 3.4 and 83.8 mg kg^{-1}, and 2.2 and 60.1 mg kg^{-1}, respectively. The concentrations were slightly lower than those obtained by Gerbersdorf et al. (2007) in the sediments of the Neckar River (Germany), where Chl *a* varied from 35 to 197 mg kg^{-1}.

The highest phytopigment values corresponded to site 1AP in winter and particularly to 4PO in summer, when the phytopigment concentrations at this site reached values up to 5-times higher than at the other sites. Sanmartín et al. (2011) also reported the highest phytopigment contents at 4PO. It seems that the brackish water in the estuary favours the development of the autotrophic population at this site. In fact, most estuaries worldwide are turbid and highly productive due to constant allochthonous nutrient inputs (Pinckney et al. 2001). The abundance of nutrients at 1AP and 4PO and the greater light availability due to the absence of riparian vegetation at 4PO may explain the higher growth of autotrophic populations at these sites. Positive relationships between N and P and algal biomass were already demonstrated in different studies (Biggs and Close 1989; Dodds et al. 1997, 2002; Chételat et al. 1999, 2006; Biggs 2000), and also positive correlations between nutrients and phytopigments were previously reported for the Anllóns riverbed sediments (Devesa-Rey et al. 2009; Devesa-Rey et al. 2010c). Phytopigments were also positively correlated with oxalate-extractable Cu and Zn, which are essential elements for the growth and metabolism of

the benthic microflora (Devesa-Rey et al. 2009). Nevertheless, at high concentrations these trace metals could also become toxic (Soldo and Behra 2000) and could induce a shift in the community composition with the dominance of green algae in Cu or Zn-exposed phototrophic biofilms (Genter et al. 1987; Serra et al. 2009a; Tlili et al. 2010 and Tlili et al. 2011).

The taxonomic identification of algae found in the surface sediments employing the Pasteur pipette and the corer method is summarised in Table C2.4. The main taxa identified belonged to the divisions Cyanophyta, Heterokontophyta (Bacillariophyceae), Euglenophyta and Clorophyta. Total algal abundances (TA) and genus richness (GR) were calculated for both sampling methods (Fig. C2.2). TA and GR could only be determined for two sites in winter (1AP and 4PO) due to the low amount of microalgae found in 2CA and 3XA, whereas in summer these parameters could be determined for the four sites. TA was similar using both sampling methods, being only higher ($p<0.05$) with the Pasteur pipette method for 1AP in winter and summer and for 3XA in summer, when algal abundances were lower. GR was similar using both sampling techniques except for site 1AP in winter, which showed higher GR ($p<0.05$) with the pipette method. In comparison, the corer method generally exhibited higher precision for TA (lower values of relative standard deviation) and enabled the direct observation of unaltered sediment surfaces by SEM, for the study of the distribution of microalgae populations and their close association with mineral phases (Fig. C2.3). Site 4PO showed the highest TA and GR values in both seasons, although algal growth was remarkably higher in summer, which is in accordance with the highest phytopigment and nutrient concentrations and light availability at this site. These results were also in accordance with the data reported by Aguilera et al. (2007) for the benthic eukaryotic community of the Río Tinto (Huelva), who stated that total cell abundances were generally highest in September, decreasing dramatically in January, whereas diversity remained fairly constant during the year at most sampling stations.

Table C2.4. Taxonomic identification of the autotrophic population of the Anllóns River bed sediments

		WINTER				SUMMER			
Division	**Genus**	**S1AP**	**S2CA**	**S3XA**	**S4PO**	**S1AP**	**S2CA**	**S3XA**	**S4PO**
Cyanophyta	*Geitlerinema*		+				+		
	Lyngbya			+					
	Phormidium	+		+	+		+	+	+
	Pseudanabaena	+					+		+
Chlorophyta	*Closterium*						+		
	Oedogonium		+				+	+	+
	Oocystis	+			+				
	Scenedesmus				+		+		+
Euglenophyta	*Euglena*	+			+		+	+	+
	Phacus		+						
	Trachelomonas	+			+	+	+		+
Heterokontophyta	*Achnantes*				+	+	+	+	+
	Amphora				+	+	+	+	+
	Bacillaria								+
	Cocconeis	+			+	+	+	+	+
	Cymbella						+	+	+
	Denticula								+
	Diploneis				+				+
	Entomoneis				+				+
	Eunotia	+				+	+	+	
	Fragilaria					+	+	+	+
	Frustulia							+	+
	Gomphonema					+	+	+	
	Gyrosigma				+			+	+
	Melosira	+	+	+	+	+	+	+	+
	Navicula	+	+	+	+	+	+	+	+
	Nitzschia	+		+	+		+	+	+
	Pinnularia	+	+	+	+	+	+	+	+
	Pleurosigma				+				+
	Rhoicosphenia				+		+	+	+
	Stauroneis						+	+	
	Surirella	+			+		+	+	+
	Triblionella				+				+
	Ulnaria	+	+	+	+		+	+	+

(+) the genus was identified by the two sampling methods.

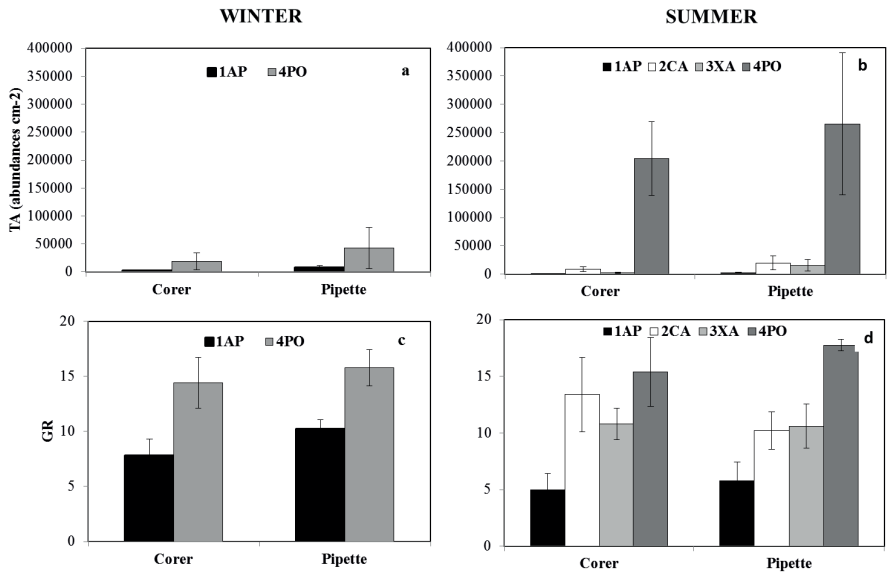

Figure C2.2 Total abundances (abundances/cm^2) (a and b) and Genus Richness (c and d) in winter and summer, respectively.

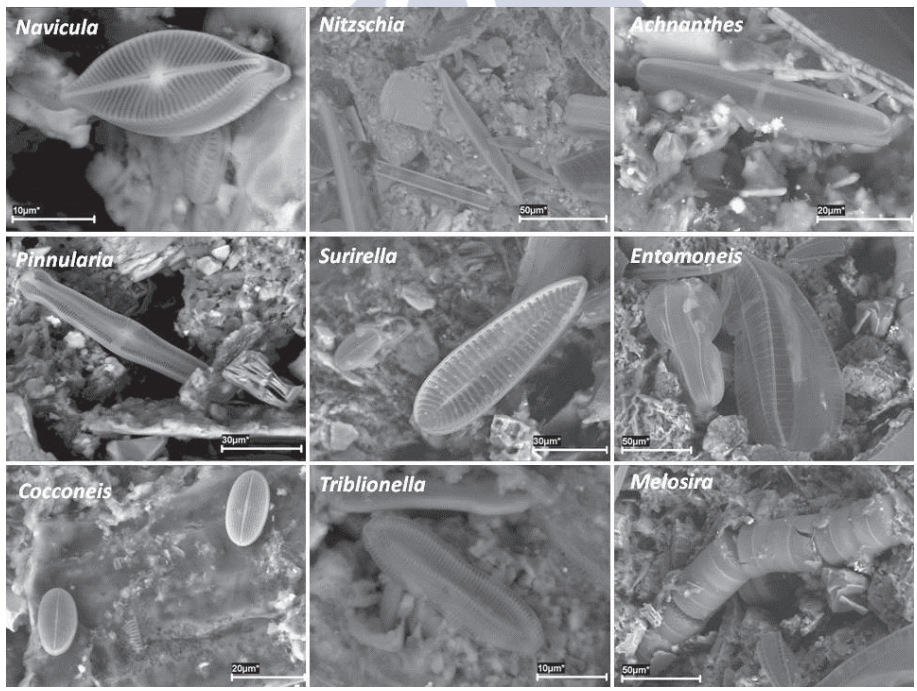

Figure C2.3 SEM images of the main taxa of algae found in the bed sediments.

The relative algal abundances (RA) were determined for all the sites in summer but only for 1AP and 4PO in winter, due to the low amount of microalgae found in 2CA and 3XA in this season (Fig. C2.4**)**. The relative algal abundance varied with the sampling site and time, as well as with the sampling technique. The most abundant division was Heterokontophyta and, specifically, the most abundant class was Bacillariophyceae, that in general showed RA>98%. Exceptions were site 1AP in winter (employing the pipette method) and site 2CA in summer, where between 8-14% belonged to other divisions (Cyanophyta, Chlorophyta and Euglenophyta). As revealed in this study, benthic microalgae are often dominated by diatoms on sandy sediments (Hickman and Round 1970; Colijn and De Jonge 1984; Aberle and Wiltshire 2006) whereas green algae and cyanobacteria occur rarely or only at some seasonal stages (Hillebrand and Kahlert 2001; Aberle and Wiltshire 2006).

Among Bacillariophyceae, *Navicula* was usually the predominant genus using the corer method. The only exception was at site 1AP in the winter sampling, for which benthopelagic *Melosira* was the most abundant genus. More variable results were found using the pipette method. In this case, *Navicula* only predominated in 3XA and 4PO (Fig. C2.4), whereas *Melosira, Achnantes* and *Ulnaria* (*U. ulna*) prevailed in the other cases. The predominance of *Navicula* in our study, particularly at site 4PO, where it showed the highest RA (66-97%), can be explained by the tolerance of the *Navicula* species to organic pollution and to eutrophic conditions in water (Lange-Bertalot 2001; Segura-García et al. 2010), as well as to brackish and electrolyte rich waters (Ehrlich 1995; Cox 1996; Lange-Bertalot 2001). Studying diatom populations, Ector (1992) already indicated that the Anllóns River is moderately contaminated in spring, to heavily contaminated in summer, due to urban and industrial effluents. Furthermore, De la Peña (2003) observed contamination downstream from the town of Carballo, and attributed the predominance of tolerant species such as *Navicula minima* and *Gomphonema parvulum* to the influence of eutrophication and organic pollution.

The influence of seasonality could be observed in the general increase of *Navicula* in the summer sampling, as well as of *Achnantes* and *Cocconeis* at 1AP. The increase of *Navicula* could be explained by its tolerance to contamination, which increases in summer in the Anllóns River (Ector 1992). The dominance of species susceptible to contamination, such as Achnantes *minutissima*, was reported by De la Peña (2003) for unpolluted places in the Anllóns River, such as 1AP. Typical brackish species were observed at site 4PO (at the river

mouth), such as *Achnanthes brevipes*, *Entomoneis* sp., *Diploneis* sp, *Melosira nummuloides*, *Pleurosigma* sp, *Surirella striatula*, *Triblionella* sp., which suggests the adaptation to osmotic stress and the predominance of the saline-resistant species.

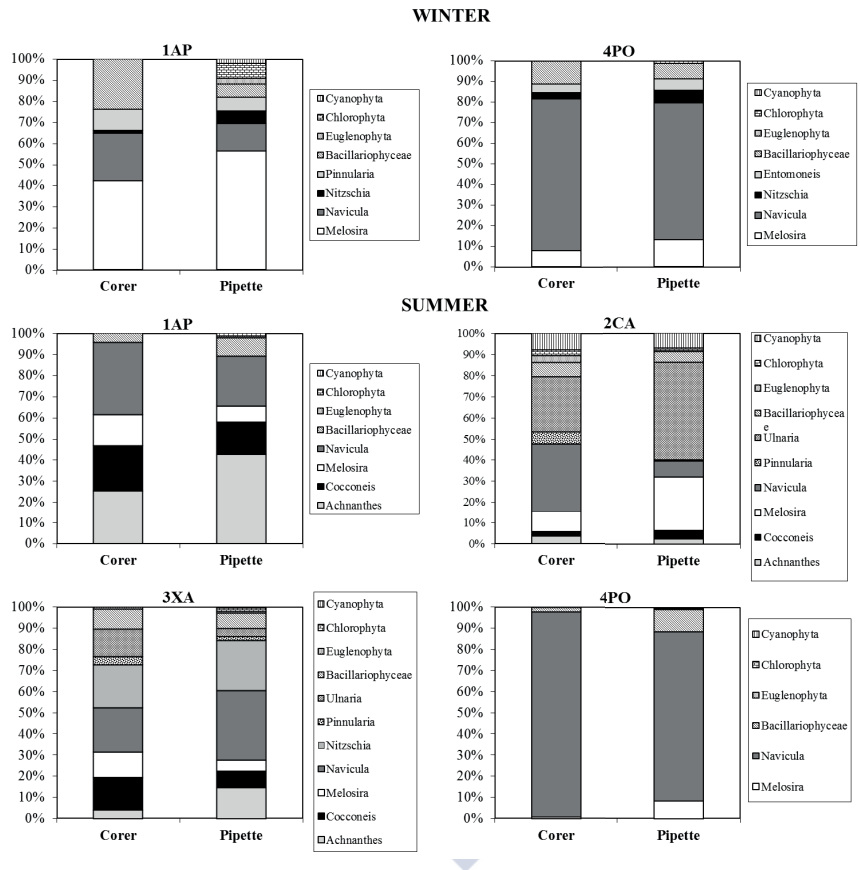

Figure C2.4. Relative algal abundances. Cyanophyta, Chlorophyta and Euglenophyta include all the genera belonging to each of these phyla. Bacillariophyceae include all the genera belonging to this class, whose abundance is <3%, whereas the genera whose relative abundance is >3% are represented individually.

Among the freshwater microalgae groups, Chl *a* is present in all photosynthetic algae whereas Chl *b* is present in the Chlorophyta (green algae) and Euglenophyta divisions (Leavitt and Hodgson 2001). Bacillariophyceae (diatoms), the predominant class in this study, presents, as major photosynthetic pigments, chlorophyll a and c, along with fucoxanthin (carotenoid) as an accessory pigment (Gómez et al. 2009). Despite the predominance of diatoms in the biofilms of the Anllóns River, significant concentrations of Chl *b* were found

in this study, which cannot be attributed to these microalgae class. Moreover, the ratios Chl *b*:*a* ranged between 0.22 and 1.71, which are similar to those published by Devesa-Rey et al. (2009) and Sanmartín et al. (2011) (0-1.79 and 0.62-1.23, respectively) for bed sediments from Anllóns River. Nevertheless, these ratios were much higher than those reported by Schlüter et al. (2006), who quantified phytoplankton groups in lakes, analysing 20 different algal cultures, obtaining Chl *b*:*a* ratios of 0.23-0.41 and 0.20-0.23 for Chlorophytes and Euglenophytes, respectively. Hence, the higher ratios found in this study could suggest the contribution of aquatic and terrestrial plants to Chl *b* concentrations. This is in agreement with the C/N ratios obtained in this study, which were indicative of the allochthonous origin of the organic matter.

3.3 Relationships between variables

Pearson correlations were calculated for the data obtained for the summer sampling, which was the most complete (Table C2.5). Total and soluble P, and pH of the pore water, as well as total As in the sediments are not shown in the table as they did not present significant correlations ($p<0.05$) with the other parameters. Eh and pH only showed a negative correlation between them; EC showed positive correlations with the content of fine particles, and with nutrient content (C, N, P and S) which were also positively correlated with the content of fine particles, which may promote OM and nutrient deposition. Among the biological parameters, the concentrations of phytopigments, DHA and TA values showed positive correlations between them as well as with EC, fine particle content and the nutrient content.

Principal Component Analysis (PCA) was initially carried out for all the variables analyzed in the summer sampling. Two principal components (PC) with an eigenvalue >1 were extracted (Fig. C2.5), which explained 95.6% of the total variance. PCA corroborated the importance of site conditions on the benthic microflora, given that DHA, phytopigments, TA and GR were placed in the same quadrant as nutrients, OM and fine particle classes of the sediments. This association can be explained because site conditions favourable for fine particle deposition are also prone to the accumulation of OM and nutrients, due to low energy and sorption phenomena, and these conditions seem to be favourable for the development of biofilms. Sand content and Eh are in the opposite quadrant, indicating that those sites with a

Table C2.5. Significant Pearson correlation coefficients between physico-chemical parameters of sediments and biological parameters, in the summer sampling. The level of significance is indicated: **=p<0.01, *=p<0.05

SUMMER	pH	Eh	EC	Clay	Silt	Sand	N	C	S	P_T	P_B	Chl a	Chl b	TotalCarot	DHA	TA_{core}	$TA_{pipette}$
pH	1																
Eh	-0.986*	1															
EC			1														
Clay			1.000**	1													
Silt			0.998**	0.996**	1												
Sand			-0.999**	-0.998**	-1.000**	1											
N			0.999**	0.999**	0.998**	-0.999**	1										
C			0.999**	0.999**	0.998**	-0.999**	1.000**	1									
S			0.999**	0.998**	0.999**	-1.000**	1.000**	1.000**	1								
P_T			0.989*	0.985*	0.996**	-0.994**	0.991**	0.991**	0.993**	1							
P_B										0.956*	1						
Chl a			0.973*	0.972*	0.980*	-0.978*	0.982*	0.981*	0.982*	0.988*	0.975*	1					
Chl b			0.973*	0.972*	0.979*	-0.978*	0.982*	0.982*	0.981*	0.986*	0.973*	1.000**	1				
TotalCarot			0.979*	0.979*	0.983*	-0.983*	0.987*	0.987*	0.986*	0.988*	0.966*	0.999**	1.000**	1			
DHA			0.997**	0.996**	0.999**	-0.999**	0.999**	0.999**	1.000**	0.996**		0.987*	0.986*	0.990**	1		
TA_{corer}			0.996**	0.995**	0.999**	-0.998**	0.999**	0.998**	0.999**	0.996**		0.989*	0.988*	0.992**	1.000**	1	
$TA_{pipette}$			0.998**	0.996**	1.000**	-1.000**	0.998**	0.998**	0.999**	0.995**		0.979*	0.978*	0.982*	0.999**	0.998**	1

coarser texture, which also have less favourable OM accumulation and, therefore, lower O_2 consumption due to OM decomposition, and higher Eh, are less suitable for biofilm growth.

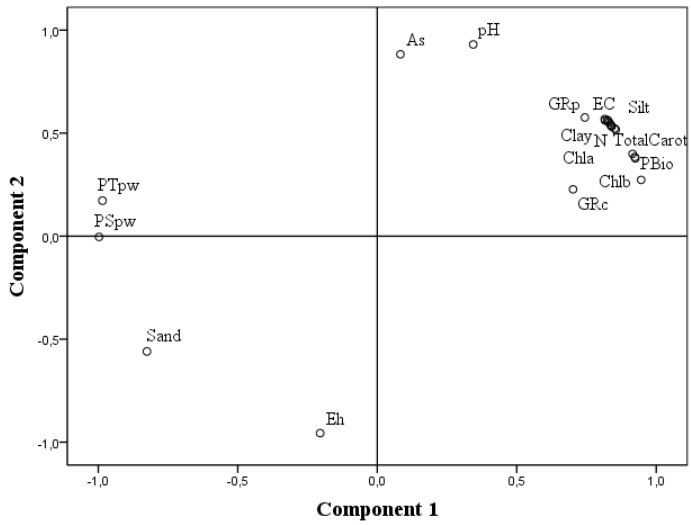

Figure C2.5 Principal Component Analysis for physicochemical and biological parameters

In conclusion, the results corroborate the control of biological activity by site conditions, mainly by the trophic state (N, P and OM) and suggest that a comprehensive study of nutrients, phytopigments and benthic microflora could contribute to a better understanding of the ecological status in riverbed sediments.

4. Conclusions

The results of this study reveal the positive relationships between the biological properties (phytopigments and respiration) and total algal abundances determined by taxonomic identification. Similar results are obtained when sampling with the Pasteur pipette method and the corer method, but the latter enables the observation of microalgae distribution over mineral surfaces by SEM. Bacillariophiceae, namely the genus *Navicula*, are the most abundant algae in the analysed sediments. A clear influence of the nutrient (N, P) and OM contents of the sediments is observed in the development of benthic microflora, pointing to the importance of the site conditions, particularly of the trophic state.

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Chapter 3: Biofilm formation on river sediments under different light intensities and nutrient inputs: a flume mesocosm study

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Chapter 3: Biofilm formation on river sediments under different light intensities and nutrient inputs: a flume mesocosm study

Abstract

This chapter investigates the influence of light availability and water composition on biofilm growth. To this end, the formation of an epipsammic biofilm on riverbed sediments was monitored during 21 days. The experiments were carried out in two specifically designed experimental channels: channel 1 fed with river water and channel 2 with nutrient enriched input. Each channel was divided into three sections receiving different light intensities. Total and biologically active organic carbon, chlorophyll a and b, total carotenoids, soluble carbohydrates, proteins, phosphatase and bioavailable phosphorous were determined in the sediment samples. In channel 1, fed with river water, a positive effect of light availability on chlorophyll a, total carotenoids, total and biologically active organic carbon, crude proteins and phosphatase activity was observed throughout the experiment. In channel 2 the addition of the nutrients increased the concentrations of chlorophyll a, soluble carbohydrates and proteins, in comparison with the sections receiving the same light in channel 1. These properties also increased with light availability. This study demonstrates that epipsammic biofilm formation in mesocosm conditions depends on light availability and the overlying water composition. The designed experimental fluvial channels fed only with river water can be employed to obtain epipsammic biofilm for use in environmental and biotechnological experiments, thus avoiding the affectation of competitor aninons or high ionic strength due to the presence of supplementary nutrients.

1. Introduction

There is growing interest in the study of biofilms on riverbed sediments, because of their important role in river ecosystems. From an ecological perspective, biofilms protect microbial communities from environmental stress (Flemming and Wigender 2001). Microenvironments with specific physical and chemical characteristics are created in biofilms, where an intense biological activity is generated by autotrophic and heterotrophic microflora, with important implications in the cycles of the elements (Romaní et al. 2004). From a physical point of view, biofilms increase sediment stability against water erosion (Stal 2003; Underwood and Paterson 2003; Droppo 2009; Gerbersdorf et al. 2009a, 2009b). This stabilization is attributed to the exopolysaccharide matrix which surrounds cells. In circumstances favouring resuspension, which usually coincide with episodes of increased water flow caused by heavy rains, sediment erosion may be mitigated by the presence of biofilms. Sediment stabilization limits the interaction of the resuspended particles with the surrounding water and controls the release or sorption of contaminants (Eggleton and Thomas 2007). Finally, from a biotechnological point of view, biofilms are a novel area of interest with regard to the bioremediation of wastewaters originating from industrial, mining and urban activities; this is due to their efficiency and low cost of implementation when compared to common physical and/or chemical treatments (Pal and Paul 2008).

Biofilm biomass and composition may vary in response to environmental factors. Irradiance, temperature, water flow and nutritional status constitute environmental key factors for the growth of phototrophic biofilms, and this is demonstrated by their effects on photosynthetic and respiratory activity, and on the production of extracellular polymeric substances (EPS) (Zippel et al. 2007). In riverine systems, Villeneuve et al. (2011) found that bacterial and algal densities were highly dependent on seasonal factors (temperature and light availability) and chemical water quality. As for light availability, it is known that photosynthetically active radiation is essential for photosynthesis in order to store energy and reductive capacity for primary production (Zippel et al. 2007). Consequently, a positive effect of light availability on biofilm algal growth has been described by Guasch and Sabater (1994) and by Hill (1996), among others. As for nutritional status, it has been observed that an increase in inorganic nutrients (mainly nitrate and phosphate) favours algal growth as proved by increased algal biomass and chlorophyll concentrations (Perrin et al. 1987; Borchardt

1996; Dodds et al. 1997; Dodds et al. 2002; Tank and Dodds 2003). Previous work by Devesa-Rey et al. (2009) showed that phytopigment concentration in the surface sediments along the Anllóns River watercourse was directly related to the concentrations of organic matter, total N, total P and available P in sediments.

Mesocosm experiments conducted in experimental channels make it possible to carry out ecosystem-level research under controlled conditions (Roussel et al. 2007). Mesocosm studies have greater ecological relevance than small scale laboratory experiments, although less experimental control and replication (Clements and Neuman 2002). In recent years, experimental fluvial channels have been used to study the growth of freshwater and marine phototrophic biofilms, using microscope slides as a substratum and characterized by gravimetry, microscopy, taxonomy, molecular biology and chemical analysis (Zippel et al. 2007), to investigate the effect of biofilms on the chemical processes in sediments (Woodruff et al. 1999; Gainswin et al. 2006; Serra et al. 2009a), to determine the influence of environmental conditions and toxic substances (metals, herbicides and bactericides) on the biofilm (Guasch et al. 2004; Guasch et al. 2007; Navarro et al. 2008; Ricart et al. 2009; Serra et al. 2009b; Ricart et al. 2010; Serra et al. 2010; Bowes et al. 2012; Bonnineau et al. 2013; Proia et al. 2013), to study the interaction between toxicants and grazers (Muñoz et al. 2001; Real et al. 2003; López-Doval et al. 2010), and also to evaluate the effect of biofilms on sediment stability (Stone et al. 2011) and on the toxicological effects of re-suspended pollutants (Brinkman et al. 2010). However, the use of experimental indoor fluvial channels to study the growth of biofilms developed on sediments is scarce and mainly focused on the study of the effects of biofilms on chemical processes in sediments, as in Woodruff et al. (1999) and Gainswin et al. (2006).

Chemical and biochemical methods can be used to follow the growth of the epipsammic biofilm. Given that algae cells, together with the EPS matrix, comprise the majority of freshwater biofilm biomass in good lighting conditions -bacterial biomass is usually lower- (Romaní 2010), phytopigments and EPS concentrations may be suitable parameters to evaluate the development of biofilms. Chlorophylls, together with carotenoids, are the main photosynthetic pigments, but whereas chlorophyll-a can be found in all photosynthetic algae, chlorophyll-b can only be found in green algae and Euglenophytes (Leavitt and Hodgson 2001). In turn, the EPS matrix is a crucial structural parameter for biofilm integrity, stability

and architecture (Hamilton 1987; Romaní et al. 2004). Polysaccharides are the main components of the EPS matrix (Sutherland 2001b) and can make up between 50–90% of their total organic matter (Denkhaus et al. 2006). Proteins, nucleic acids, (phospho)lipids and humic substances have also been identified in biofilms (Flemming and Wingender 2003). Sutherland et al. (1998), studying the effect of biofilms on the erodibility of sublittoral sediments, stated that soluble carbohydrates (also called colloidal carbohydrates) are considered a measure of EPS or mucopolysaccharides secreted by diatoms. However, the analysis of total carbohydrates by the classical phenol-sulphuric acid assay (Dubois et al. 1956) includes intracellular, extracellular and particle-bound carbohydrate material (Underwood et al. 1995) and does not provide a good estimation of EPS.

The present study was conducted in order to: a) evaluate the influence of light availability and trophic state on the development of biofilms; b) test the suitability of experimental channels to grow biofilms on fluvial sediments, which may be used to perform subsequent environmental and biotechnological studies; and c) investigate if biofilms can grow in channels fed with river water, without the addition of supplementary nutrients. The latter is a relevant point because subsequent studies, such as contaminant adsorption, may be affected by the presence of competitor anions or high ionic strength.

Biofilms growing directly on sediments reproduces the conditions of river ecosystems and seems appropriate to be used in mesocosm studies with environmental or biotechnological purposes. The information from this study will also increase the knowledge regarding biofilm development in natural streams, its variation under different light regimes and the influence of the overlying water composition, which may be affected by hydrological conditions and soil use in the basin.

2. Material and methods

2.1. General description of the riverbed sediments

The river bed sediments employed for this study were collected in the Anllóns River. Various studies have been conducted on the composition of the Anllóns River sediments (Devesa-Rey et al. 2008a; 2010a; 2010b; Iglesias et al. 2011), as well as on the development

of biofilms along the river course (Devesa-Rey et al. 2009; Sanmartín et al. 2011; chapter 2). *Bacillariophyceae* (diatoms) comprise up to 98% of the autotrophic population at the sampling stations, whereas the heterotrophic population evaluated by the most probable number (MPN) reaches values of up to 1.2 10^6 MPN g^{-1} (Devesa-Rey et al. 2010c).

Bed sediment was collected at Ponte de Eguas (43° 13' 24.46'' N 8° 45' 44.61'' W), located 8 km downstream from the town of Carballo (with a population of over 25,000). Local geology is composed by metamorphic rocks (peridotite, serpentinite and piroxenites). A complex sample was taken with a small plastic shovel from the top 4 cm, picking sediment at various points at this site. The 4 cm deep layer is considered the active layer under the water-sediment interface, suffering physical and biochemical exchanges between sediments and water. This is also the depth where biofilms generally develop (Moreno and Niell 2004). The sediment sample was transported under a water layer in thermally insulated airtight plastic boxes to avoid oxidation. Once in the laboratory, the sample was stored wet at 4 ºC until deposited in the experimental channels, forming a layer of about 3-4 cm. The main characteristics of the sediment are: 86.2% sand fraction, 7.1% silt fraction and 6.7% clay fraction, 13.9 g kg^{-1} organic matter (OM), 629.6 mg kg^{-1} total nitrogen (N), 471.9 mg kg^{-1} total phosphorous (P), pH 5.5 (sediment:water ratio 1:2.5) and 51.9 g kg^{-1} total Fe.

2.2. Experimental design for the mesocosm study

Biofilm formation was monitored in two experimental indoor fluvial channels, which were 130 cm long, 13 cm wide and 10 cm high (Fig. C3.1a). The lighting system consisted of 5 lamps (Mazda Fluor Lumière du Jour C9 TF 65 85 W, Philips, Amsterdan, Holland). Each channel was divided into three sections (named 1, 2 and 3), with different light intensities (40, 30 and 20 µmol photon m^{-2} s^{-1}, respectively), the last two obtained by shadowing with 1 and 2 sheets of semi-translucent paper laid over the sections 2 and 3, respectively (Fig. C3.1b). Light intensity was measured by a portable lux meter (photo-radiometer) Model HD 2302.0 (Delta Ohm, Padova, Italy). Experiments were run with a 16:8 h light:dark cycle.

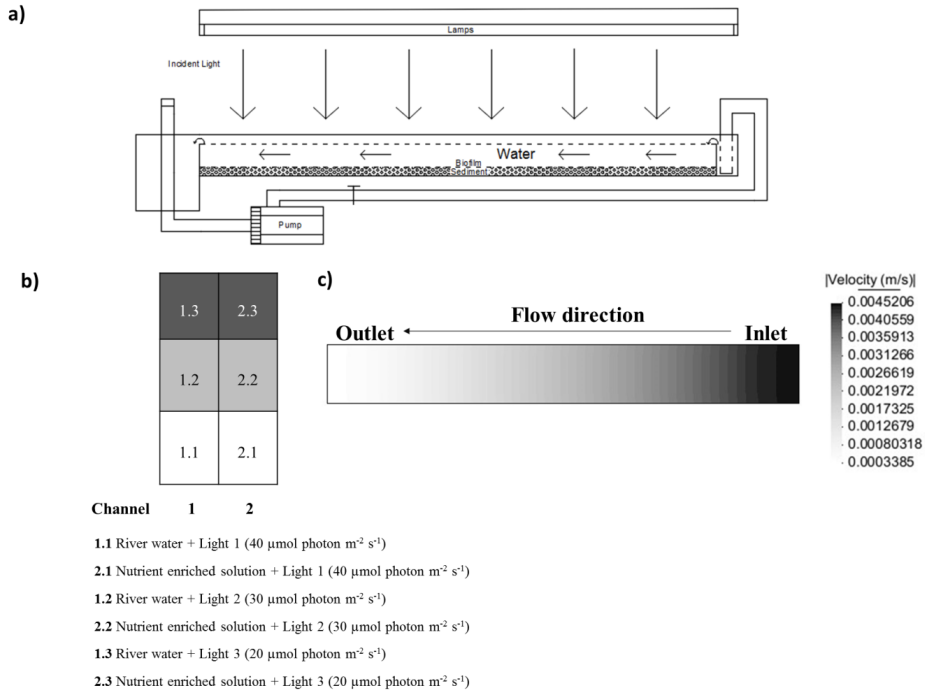

Figure C3. 1. a) Scheme of the experimental fluvial channel used in this study. b) Light and nutrient input fed to each channel and zone. c) Velocity profile along the experimental fluvial channel.

Experiments were carried out with laminar flow achieved using an individual pump system model 1260-210 (Eheim, Deizisau, Germany), with a maximum capacity of 40 L min^{-1}. The velocity profiles along the experimental channels have been modelled employing IBER v.2.3 (Bladé et al. 2014), a two dimensional depth averaged mathematical model for the simulation of free surface flow in rivers, estuaries and channels developed by Water and Environmental Engineering Group, GEAMA (University of A Coruña) and the Flumen Institute (UPC-CIMNE) (Fig. C3.1c). Selected Manning's Roughness Coefficients for bottom surface (sand/clay) and for channel walls (steel) were 0.023 and 0.011, respectively. Maximun water velocity reached was 4.5 10^{-3} m s^{-1} and Reynolds number max. was 178.1. Channel 1 was fed with river water (pH=7.47, electrical conductivity (EC)=105 µS cm^{-1}, total alkalinity=12.5 mg $CaCO_3$ L^{-1}, N=1.98 mg L^{-1}, TOC=1.25 mg L^{-1}, Cl^-=10.69 mg L^{-1}, NO_3^- =7.32 mg L^{-1}, PO_4^{3-}=0.10 mg L^{-1}, SO_4^{2-}=5.80 mg L^{-1}, Na^+=6.77 mg L^{-1}, K^+=0.81 mg L^{-1},

Ca^{2+}=7.41 mg L^{-1}, Mg^{2+}=2.02 mg L^{-1}, Al^{3+}<2.0 µg L^{-1}, Fe^{3+}<2.0 µg L^{-1}) and channel 2 with the following nutrient input: (pH=8.41, EC=28.80 mS cm^{-1}, N=5.69 g L^{-1}, TOC= 4.32 mg L^{-1}, Cl^-= 15.17 mg L^{-1}, NO_3^-=26.40 g L^{-1}, PO_4^{3-}= 1.90 g L^{-1}, Na^+= 9.67 g L^{-1}) prepared in distilled water, according to the composition previously optimized by Devesa-Rey et al. (2010c). Water loss by evaporation (6% of the circulating water), previously determined following the increase of concentration of 1 mM sodium chloride solutions incorporated to the experimental fluvial channels and corroborated by theoretical calculation by Stefan's law developed by Holman (1999), was corrected daily to keep constant the volume of the overlying water.

Biofilm development was monitored during 21 days. Every 3-4 days, approximately 50 g of the superficial (~0.5 cm) sediment was collected by taking samples at various points in each section. Aliquots were centrifuged at 2000 rpm to discard pore water and the solid sample used to determine phosphatase activity. The remainder of the samples were freeze-dried and stored in polyethylene bottles for later analyses. Water content (% w/w) was determined in order to calculate the results on a dry weight basis. All the determinations were carried out in triplicate.

2.3. Analytical procedures

Total organic carbon (OC) was determined by wet oxidation with $K_2Cr_2O_7$ and H_2SO_4, followed by titration with Mohr´s salt, according to the procedure proposed by Sauerlandt and modified by Guitián and Carballas (1976), using an automatic titration system. Biologically active organic carbon (BAOC) was determined as per Weill et al. (2003), based on the oxidation of the organic matter with 0.2 M $KMnO_4$.

Total nitrogen (N) was determined by wet digestion with H_2SO_4, following the Kjeldahl method as described in Guitián and Carballas (1976). Crude protein (PROT) contents were determined indirectly from total N content using an N-to-protein conversion multiplier. In this case, the classic factor of 6.25 (Jones 1931) may not be appropriate, because it could overestimate the actual protein content (Ezeagu et al. 2002) to include non-protein nitrogenaceous substances such as pigments, nucleic acids, free amino acids and inorganic nitrogen (Barbarino and Lourenço 2005). Hence, a factor of 4.78, proposed by Lourenço et al. (2004) for 12 marine microalgae species in different growth phases, was applied in this study.

Phytopigments, including chlorophyll-*a* (Chl a), chlorophyll-*b* (Chl b) and total carotenoids (CAR), were solubilised after 46 min of extraction with dimethylsulphoxide, using a extractant:sediment ratio of 3.6 mL g^{-1} at 57 ºC, following the method optimized by Devesa *et al.* (2007). In the extracts, Chl a, Chl b and CAR were determined spectrophotometrically (UV Visible Spectrophotometer, Cary 100 Conc., Varian Inc., Palo Alto, California) following the methodology proposed by Wellburn (1994). **Equations 1-3** were used to determine the concentrations of Chl a and b, as well as CAR, in $\mu g \cdot mL^{-1}$.

$$\text{Chl a}=12.47A_{665.1}-3.62A_{649.1} \quad \text{(Eq. 1)}$$

$$\text{Chl b}=25.06A_{649.1}-6.5A_{665.1} \quad \text{(Eq. 2)}$$

$$\text{CAR}=(1000A_{480}-1.29\text{Chl a}-53.78\text{Chl b})/220 \quad \text{(Eq. 3)}$$

Where Chl a and Chl b represent the concentration of chlorophyll-a and chlorophyll-b respectively, and CAR represents the concentration of total carotenoids which comprises the oxidized forms, the xantophylls, and the reduced forms, namely the carotenes. $A_{665.1}$, $A_{649.1}$ and A_{480} represent the absorbance of the extracts at 665.1, 649.1 and 480 nm, respectively. Each sample was submitted to several extractions until no phytopigments were quantified in the extracts, and the concentrations obtained were added to obtain the total concentration.

Soluble carbohydrates (SC) were extracted in water following the method described by Underwood et al. (1995) for the measurement of microbial carbohydrate exopolymers from intertidal estuarine sediments, and subsequently quantified in the aqueous extracts by the traditional phenol-sulphuric acid method (Dubois et al. 1956). D(+) glucose solutions were used as standards, carbohydrate absorbance was measured at 485 nm (UV Visible Spectrophotometer, Cary 100 Conc. Varian Inc., Palo Alto, California) and the results are given in mg glucose equivalents per g of DW sediment.

The activity of the enzyme acid phosphatase (APHOS) was determined using p-Nitrophenyl phosphate as a substrate, according to the procedure described by Dick et al. (1996). The intensity of the yellow colour due to p-nitrophenol in the filtrate was measured using a UV-Vis spectrophotometer at 410 nm and the results were expressed as mg p-nitrophenol per kg of DW sediment. Bioavailable phosphorous (BioP) was extracted with

$NaHCO_3$ 0.5 N (Olsen and Sommers, 1982), followed by colorimetric determination with molybdenum blue, as described by Murphy and Riley (1962).

3. Results and discussion

3.1. Influence of light availability on freshwater biofilm growth

Figure C3.2 shows the time evolution of OC (a) and BAOC (c) contents in channel 1, supplemented with river water. Different light intensity applied to each section (40, 30 and 20 μmol photon m^{-2} s^{-1} for sections 1, 2 and 3, respectively) are indicated by the grey scale, where the darkest grey indicates the least illuminated section. In section 1, receiving the highest light intensity, OC increased from an initial value of about 0.70 %, to 1.56 % at day 11. Then a subsequent decline until day 21 (1.09 %) was observed. In sections 2 and 3, the maximum OC contents were reached at day 18 (1.32 and 1.28 % respectively). Sections 1 and 3 (the most and the least illuminated respectively) always showed differences between their OC contents, proving the influence of light availability on biofilm growth. BAOC showed less variation and did not have a clear maximum throughout the experiment. Its values ranged between 405.3 and 724.0 mg kg^{-1}, representing between 3.4 and 11.1 % of OC. This active fraction of the organic matter has not been accurately defined in aquatic ecosystems. Some authors described BAOC as a mixture of simple carbohydrates, proteins and fatty acids (Fabiano and Danovaro 1994; Fabiano et al. 1995), or as the fraction of organic matter which is bioavailable to benthic organisms (Polymenakou et al. 2007). In any case, it has been observed that BAOC is closely related to substrate-induced respiration, basal respiration, microbial biomass and soluble carbohydrates, which are all evidence of biological activity (Weill et al. 2003). Thus, the highest BAOC contents observed in the most illuminated section 1 can be suggestive of a positive influence of light availability on biofilm growth.

Regarding the pigments, Chl a increased throughout the experiment up to day 14 in sections 1 and 2, reaching concentrations of 2.97 and 2.38 μg g^{-1}, respectively, and then it decreased slightly (Fig. C3.3a). In the darkest section 3, Chl a concentrations showed little variation or even decreased at intermediate times, not surpassing the initial concentrations of about 1.71 μg g^{-1}. Algal cells (together with the EPS) comprise the majority of the biofilm biomass in freshwater biofilms under light conditions (Romaní 2010). Thus, the increase in

Chl a, which is present in all algal groups, can be interpreted as an indicator of biofilm development, demonstrating the positive influence of light availability on the biofilm. The results of the present study are also consistent with those obtained by Romaní and Sabater (1999), and Sekar et al. (2002), in stream and lentic freshwaters, respectively, who observed that the concentration of Chl a was significantly higher in light-grown biofilms in comparison with dark-grown biofilms. Chl a concentrations in the experimental channels were in the range of those found by Devesa-Rey et al. (2009) and Sanmartín et al. (2011) for the riverbed sediments of the Anllóns River, which ranged between 3.4 and 83.8 µg g^{-1}, and 2.2 and 60.1 µg g^{-1}, respectively, but slightly lower than those obtained by Gerbersdorf et al. (2007) in the sediments of the Neckar River (Germany), where Chl a varied between 35 and 197 µg g^{-1}.

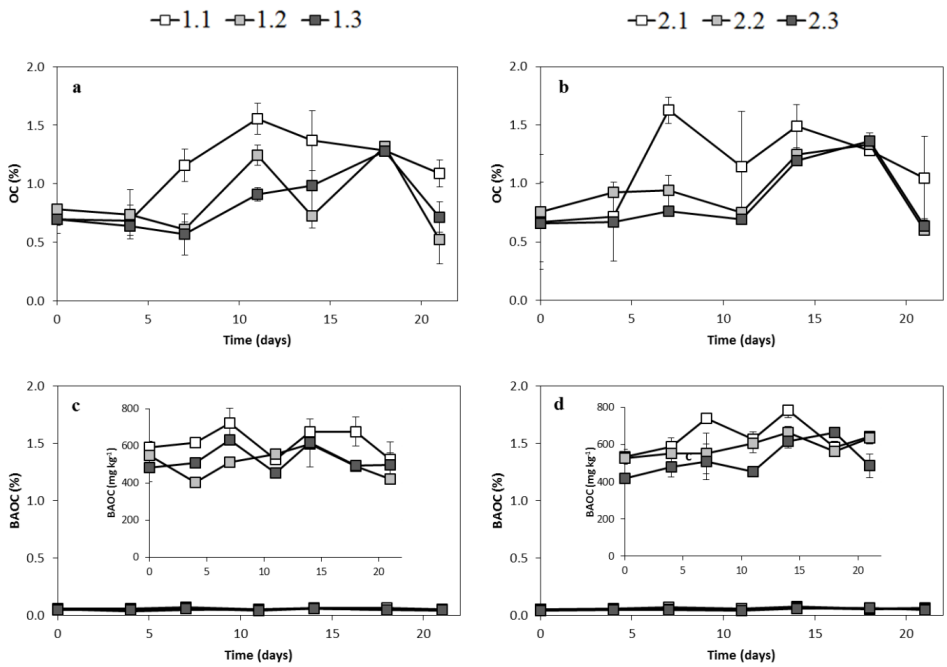

Figure C3.2. Analysis of total and biologically active organic carbon throughout the biofilm growth in channel 1 (fed with river water) (figures a and c, respectively) and channel 2 (fed with nutrient enriched solution) (figures b and d, respectively). The grey scale indicates the light intensity in the fluvial channel: 40, 30 and 20 µmol photon m^{-2} s^{-1} for sections 1 (the lightest grey), 2 and 3 (the darkest grey), respectively.

Chl b concentrations decreased with time in all three sections, the highest values corresponding to the most illuminated section 1, while CAR experienced a increase after day

7 (maximum values reached between 7-11) with a remarkable decline after reaching the maximun more pronunced for the least illuminated sections (Fig. C3.3c and e, respectively). The results may suggest a more cushioned decrease of Chl b for the most illuminated sections. Chl b and CAR concentrations are in the same range as those obtained by Devesa-Rey et al. (2009) and by Sanmartín et al. (2011) in the Anllóns riverbed sediments, with Chl b values of 0-31.0 and 2.7-55.6 µg g^{-1}, and CAR values of 0-44.5 and 1.0-24.2 µg g^{-1}, respectively.

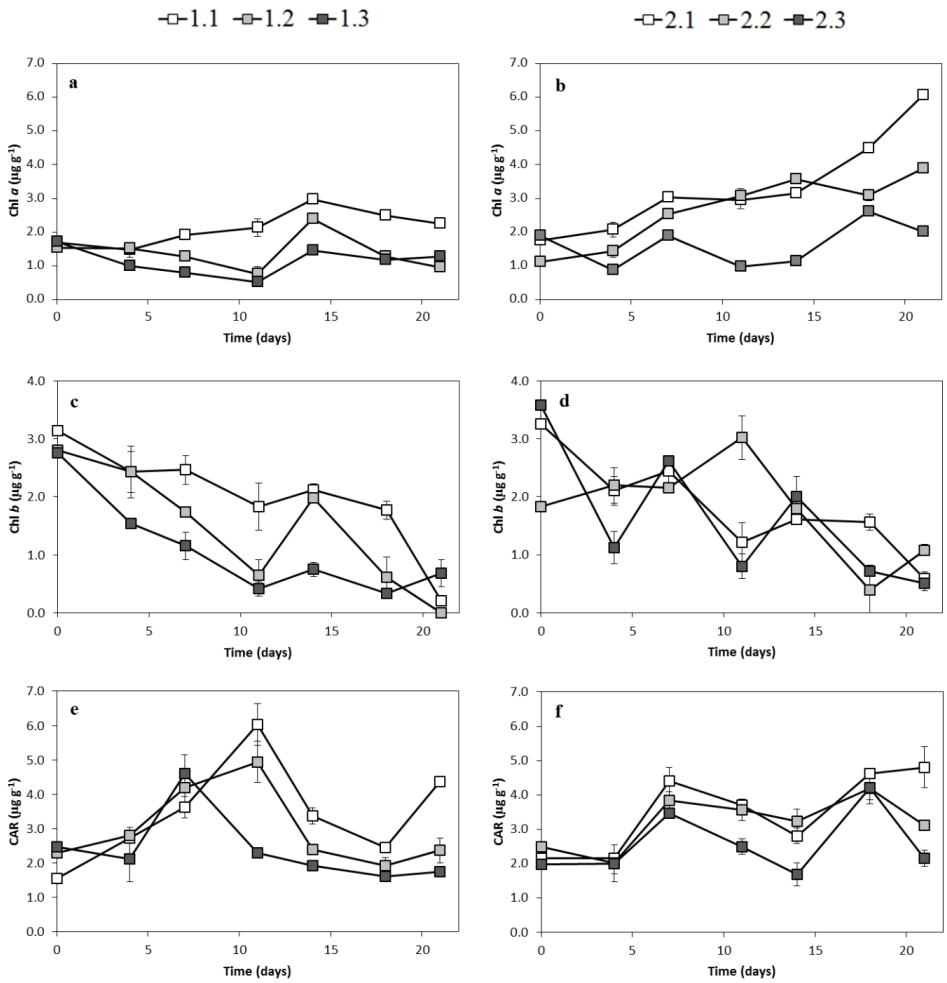

Figure C3.3. Analysis of phytopigments (chlorophyll a and b and total carotenoids) throughout the biofilm growth in channel 1 (fed with river water) (figures a, c and e, respectively) and channel 2 (fed with nutrient enriched solution) (figures b, d and f, respectively). The grey scale indicates the light intensity in the fluvial

channel: 40, 30 and 20 µmol photon m^{-2} s^{-1} for sections 1 (the lightest grey), 2 and 3 (the darkest grey), respectively.

Chl b and CAR act as light-harvesting pigments covering a region of the visible spectrum not accessible to Chl a (Colyer et al. 2005). Among the freshwater microalgae groups, Chl b is present in the Chlorophyta and Euglenophyta divisions, while carotenoids are ubiquitous, each algal group presenting its own particular carotenoid compounds (Leavitt and Hodgson 2001).

Previous studies carried out in the Anllóns River (chapter 2) showed that diatoms, belonging to the Heterokontophyta division, represent 90-98 % of the autotrophic population of the epipsammic biofilm. Diatoms present, as major photosynthetic pigments, chlorophyll a and c, as well as fucoxanthin (carotenoid) as an accessory pigment (Gómez et al. 2009). The possible predominance of diatoms in the biofilm growth in the experimental channel may explain the increase in Chl a throughout the experiment, without a concomitant increase in Chl b.

As mentioned above, soluble carbohydrates (SC) are considered a measure of the EPS (Sutherland et al. 1998) and thus they should increase with the development of the biofilm. SC increased up to day 11, 14 and 18, by about 250, 100 and 350 % of the initial values, for sections 1, 2 and 3, respectively (Fig. C3.4a), reaching maximum values between 0.32 and 0.50 mg glucose equivalents g^{-1}. SC values are in the range of the data reported by Underwood et al. (1995) for colloidal carbohydrates of four microbial assemblages in freshwater biofilms over sediments, with values between (1.57 and 5.57 mg glucose equivalents g^{-1}). Total C in the form of SC represented between <1-33 % of the BAOC. The influence of light availability was manifested in a delay of the SC maximum and in a more pronounced decline at the end of the experiment, for the darkest sections.

Crude protein (PROT) content did not exhibit a clear trend, but points to a decrease after day 18 (Fig. C3.4c). The influence of light is revealed by the highest PROT concentrations corresponding to the brightest section.

APHOS activity was variable, although the highest values were found for the two most illuminated sections at the end of the experiment (Fig. C3.5a). It has been observed that the synthesis of extracellular phosphatases by microorganisms is enhanced in a phosphate

depleted medium (Chróst and Overbeck 1987; Siuda and Chróst 1987; Romaní et al. 2004; Sabater et al. 2005; Proia et al. 2012). In fact, BioP inversely evolves in relation to APHOS activity (Fig. C3.5c), decreasing throughout the experiment as a consequence of this nutrient intake by the biofilm.

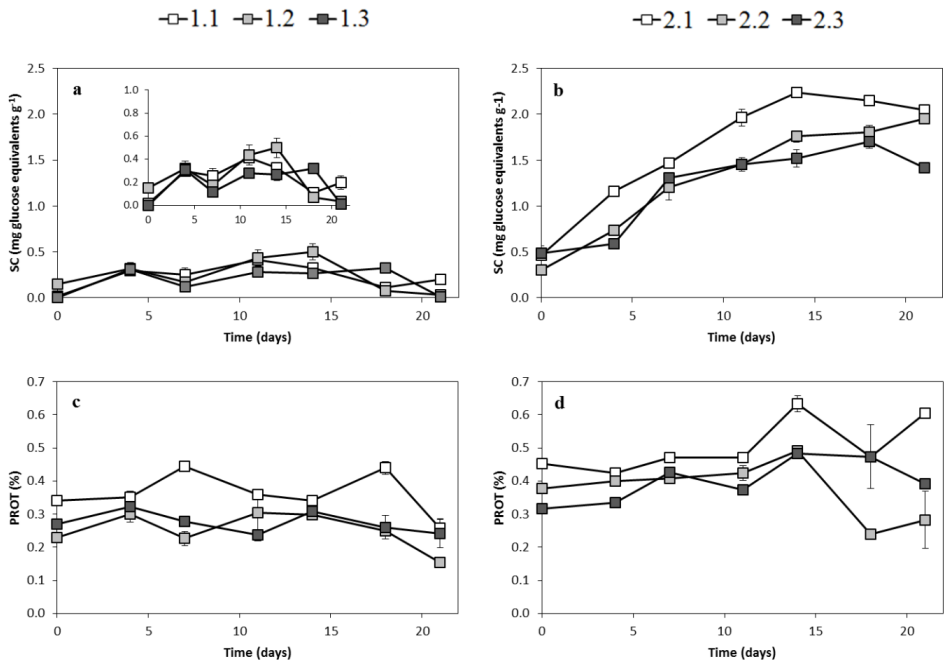

Figure C3.4. Analysis of soluble carbohydrates and crude proteins throughout the biofilm growth in channel 1 (fed with river water) (figures a and c, respectively) and channel 2 (fed with nutrient enriched solution) (figures b and d, respectively). The grey scale indicates the light intensity in the fluvial channel: 40, 30 and 20 µmol photon m^{-2} s^{-1} for sections 1 (the lightest grey), 2 and 3 (the darkest grey), respectively.

In summary, a clearly positive effect of light availability could only be observed for OC, BAOC, Chla a and CAR. The evolution in time of these parameters showed maximum Chl a and SC concentrations at days 14 and 11-18, respectively. Also, OC, CAR, PROT and APHOS reached maximum values at intermediate times (days 7-18), whereas Chl b showed a clear decrease over time. These results can be interpreted as light favouring the development of phototrophic organisms in the biofilm, namely Heterokontophyta algae, which have Chl a but not Chl b.

According to Chl a and SC concentrations, an incubation period of **11 to 18 days** is suitable for the development of biofilms, in the experimental conditions of this study. Biofilm development is possible in channel flumes fed with river water, which is of interest for further environmental and biotechnological studies.

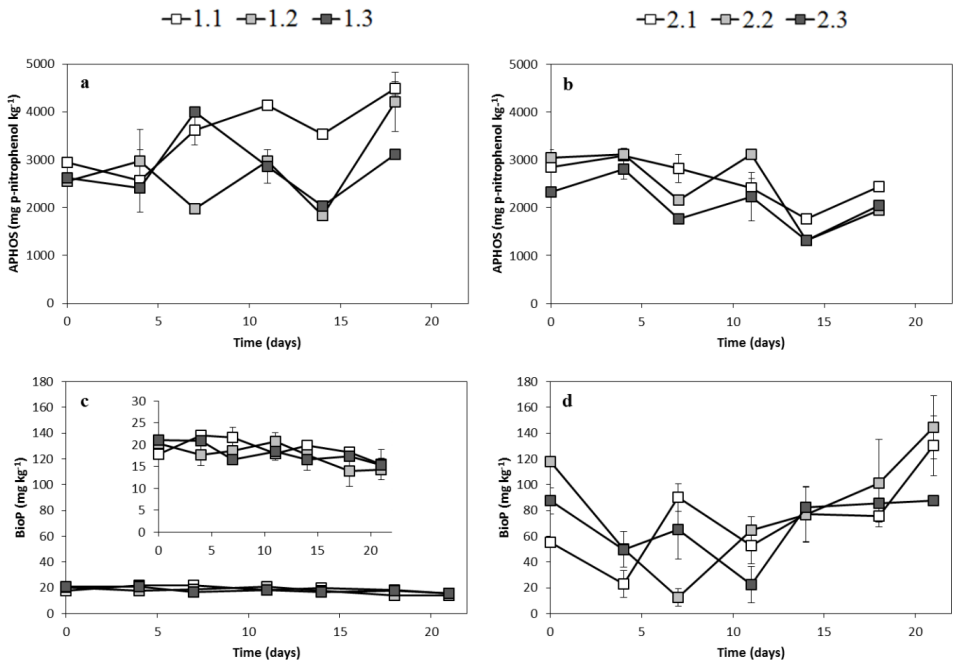

Figure C3.5. Analysis of acid phosphatase activity and bioavailable phosphorous throughout the biofilm growth in channel 1 (fed with river water) (figures a and c, respectively) and channel 2 (fed with nutrient enriched solution) (figures b and d, respectively). The grey scale indicates the light intensity in the fluvial channel: 40, 30 and 20 µmol photon m^{-2} s^{-1} for sections 1 (the lightest grey), 2 and 3 (the darkest grey), respectively.

3.2. Influence of nutrient input on biofilm growth

The second objective of this study was to test if nutrient inputs modify biofilm growth. The results for OC and BAOC for channel 2, fed with a nutrient enriched solution, are shown in Figure C3.2 (b and d, respectively**).** OC concentration increased over time to reach a maximum value at day 7-14 for the brightest section, and between days 14-18 for the other two, and then decreased towards the end of the experiment, most markedly for the darkest section. The highest OC values corresponded to the brightest section 1, but no clear difference attributable to nutrient input was observed in comparison with channel 1. BAOC, which

represented between 4.2-8.2 % of OC, showed similar profiles in both channels, the highest concentration corresponding to the brightest sections.

Chl a profiles showed clear differences between the two channels. Chl a steadily increased over time in the nutrient enriched channel 2, reaching levels 4 times higher than in channel 1 (Fig. C3.3b). The effect of light can be observed in the highest Chl a concentrations found in the brightest sections at days 18 and 21. The importance of nutrients (nitrate and phosphate) as key factors for algal growth was previously reported by Perrin et al. (1987), Borchardt (1996), Tank and Dodds (2003) and Dodds (2006). The combined effects of light and nutrients were put forward by Mosisch et al. (2001) who studied algal production in subtropical streams and revealed that nitrogen stimulated the production of periphyton in streams with sufficient light. Also Taulbee et al. (2005) observed an increase in Chl a concentrations with increasing light levels, for algae growing on substrata to which nitrogen was added. Our maximum values of Chl a were reached in the same period as those previously obtained by Proia et al. (2012), who studied the effect of light and nutrients on biofilms colonizing artificial glass substrata in a Mediterranean stream, and showed that the highest increase of Chl a was reached between days 16 and 22 in all conditions; they also observed a different Chl a temporal pattern under diverse light and nutrient conditions.

With regard to the other two analyzed pigments, Chl b showed a similar profile in the two channels, with an almost constant decline, whereas CAR exhibited once again a remarkable increase from day 7 and the maximum values were reached at days 18 and 21 (Fig. C3.3). The effect of light can be observed in the highest Car concentrations found in the brightest sections at days 7, 18 and 21.

SC were notably higher for channel 2 than for channel 1 (Fig. C3.4), reaching differences of up to 90 %, pointing to a clear influence of nutrient input on this parameter, which is related to EPS and thus to biofilm development. Total carbon in SC represented between 4-32% of OC and between 23-146% of BAOC. In the brightest section, SC increased up to day 14 and then levelled off. Similar time trends, but with lower SC concentrations, were found for the darkest sections.

PROT analysis revealed a noteworthy effect of nutrient input, as demonstrated by the higher concentrations in channel 2 (Fig. C3.4). Once again, for channel 2, the brightest section exhibited the highest PROT concentrations, with a maximum value at day 14.

APHOS activity was significantly affected by nutrient availability and was lower in channel 2. Moreover, it tended to decrease towards the end of the experiment, as opposed to the trend observed in channel 1 (Fig. C3.5). As mentioned above, the synthesis of extracellular phosphatases is enhanced in a phosphate depleted medium. Therefore, this behaviour can be inversely related to sediment BioP concentrations, which were initially higher in channel 2 than in channel 1, and again between days 7 to 14. In the interim, BioP decreased to similar concentrations in both channels, despite the addition of phosphate to channel 2 every two days to maintain P concentrations close to 20 mM. This fact suggests that organisms are taking available P from the overlying water and sediment in a very active growth phase. The subsequent increment in BioP may be explained by the repeated P input and the lower uptake by the organisms at later stages of growth.

3.3. General overview

This study attempts to assess the influence of light and nutrient inputs on biofilm development in mesocosm conditions. Nutrients may change in river waters in response to soil leaching and runoff and to inputs of cattle slurries and manure and wastewater to the river course. Light availability is mainly affected by the riparian cover and the turbidity. The results herein highlight the influence of nutrient input on biofilm growth, mostly under the non-light limiting conditions in which the highest algal growth occurred. Light availability also had direct effects on parameters indicative of biofilm development. Chl a, which can be considered a proxy of photosynthetic microorganisms, appears to be the most informative parameter related to biofilm growth under different light availability and trophic state. SC, which can be considered a proxy of the EPS embedding cells in the biofilm, is also a sensitive parameter which widely responds to the combination of brightness and high nutrient conditions.

Overall, the results confirmed that: a) light intensity and trophic state affects biofilm growth in experimental channels. Light is the key factor as limiting light reduces biofilm growth even in the presence of nutritive media; b) the designed experimental fluvial channels

can be employed to obtain epipsammic biofilm for use in environmental and biotechnological experiments; and c) the epipsammic biofilm can grow in channels fed only with river water, thus avoiding the affectation of competitor aninons or high ionic strength due to the presence of supplementary nutrients.

4. Conclusions

The present study demonstrates that biofilm growth is affected by the different conditions of light intensity and water composition set for the experimental fluvial channels. Increasing light availability favours the growth of the autotrophic component of the biofilm, and this effect is more noticeable under the highest nutrient conditions. The most sensitive parameters are chlorophyll a, total carotenoids, soluble carbohydrates and crude proteins, whereas phosphatase activity is conditioned by bioavailable P concentrations. Experimental indoor fluvial channels can be successfully used for the development of biofilm over riverbed sediments, which could be used in further environmental research and biotechnological applications.

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Chapter 4: Arsenate retention by epipsammic biofilms developed on streambed sediments. Influence of phosphate

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Chapter 4: Arsenate retention by epipsammic biofilms developed on streambed sediments. Influence of phosphate

Abstract

Natural geological conditions together with the impact of human activities could produce environmental problems due to high As concentrations. The aim of this study was to assess the role of epipsammic biofilm-sediment systems onto As^V sorption and to evaluate the effect of the presence of equimolar P concentrations on As retention. A natural biofilm was grown on sediment samples in the laboratory, using river water as nutrient supplier. Sorption experiments with initial As concentrations 0, 5, 25, 50, 100, 250 and 500 µg L^{-1} were performed. The average percentage of As sorbed was 78.9 ± 3.5 and 96.9 ± 6.6 %, for the sediment and biofilm-sediment systems, respectively. Phosphate decreased the As sorption capacity of the sediment devoid of biofilm in a 25 % whereas no significant negative effect was exhibited in the systems with biofilm and even a positive effect of phosphate was observed at the highest As concentration assayed. Freundlich, Sips and Toth models were the best to describe experimental data. The maximum As sorption capacity of the sediment and biofilm-sediment systems was respectively 6.6 and 6.8 µg g^{-1}, and in the presence of P 4.5 and 7.8 µg g^{-1}. It is concluded that epipsammic biofilms play an important role in the environmental quality of river systems, increasing As retention by the system, especially in environments where both As and P occur simultaneously.

Nomenclature

a_R	Redlich-Peterson model parameter (Eq. 6) (L μg^{-1})
a_S	Sips model parameter (Eq. 7) (L μg^{-1})
a_t	Toth model parameter (Eq. 8) ($\mu g\ g^{-1}$)
A	Linear model parameter (Eq. 1) (L g^{-1})
A_T	Temkin isotherm equilibrium binding constant (L μg^{-1}) (Eq. 5)
b	Langmuir model parameter (Eq. 3) (L μg^{-1})
b_S	Sips model parameter (Eq. 7) (-)
b_T	Temkin model parameter (Eq. 5)
B	Linear model parameter (Eq. 1) ($\mu g\ g^{-1}$)
B_D	Dubinin- Rabushkevich parameter (Eq. 4)
C_e	Solution pseudo-equilibrium concentration (mg L^{-1})
C_0	Solution initial concentration ($\mu g\ L^{-1}$)
k_f	Freundlich model parameter (Eq. 2) (L g^{-1})
K_S	Sips model parameter (Eq. 7) (L μg^{-1})
K_R	Redlich-Peterson model parameter (Eq. 6) (L μg^{-1})
K_t	Toth model parameter (Eq. 8) (L μg^{-1})
n	Freundlich model parameter (Eq. 2) (-)
q_D	Dubinin- Rabushkevich model parameter (Eq. 4) ($\mu g\ g^{-1}$)
Q_{ads}	Adsorbed concentration by sediment or biofilm-sediment interface ($\mu g\ g^{-1}$)
Q_{des}	Desorbed concentration by sediment or biofilm-sediment interface ($\mu g\ g^{-1}$)
Q_e	Solid-phase equilibrium concentration ($\mu g\ g^{-1}$)
Q_{Max}	Maximun adsorption capacity ($\mu g\ g^{-1}$) (Eq. 3)
R_L	Langmuir equilibrium parameter (Eq. 10) (-)
t	Toth model parameter (Eq. 8) (-)
T	Absolute temperature (K) (Eq. 4, 5, 11 and 12)
R	Gas Constant (J mol^{-1} K^{-1}) (Eq. 4, 5, 11 and 12)

Greek letters

β	Redlich-Peterson parameter (Eq. 6) (-)

1. Introduction

Arsenic (As) is a ubiquitous contaminant which is widely distributed in the environment. Due to its toxicity, its presence in soils, sediments and water, even at very low concentrations, may cause serious health hazards, increasing the incidence of cancer and dermatological, vascular and cerebrovascular diseases. For this reason it was one of the first chemicals recognized as carcinogens (Rosen 1971). It is estimated that 40 million people worldwide are at risk from drinking As-contaminated water (Nordstrom 2002). Several cases of people affected by As pollution have been reported; thus, for example, thousands of arsenic poisoned patients were identified in Bangladesh, suffering skin lesions, gangrene in legs as well as various types of cancer (Anawar et al. 2002). Consequently, the World Health Organization (WHO) has set the level of arsenic allowed at 10 $\mu g\ L^{-1}$ in drinking water (WHO 1993).

Environmental As problems are commonly the result of mobilization under natural conditions, such as weathering of As-bearing minerals and geothermal sources, but human activities have contributed to an important additional impact by means of mining processes, fossil fuel combustion and the use of As in pesticides, herbicides, crop desiccants and livestock feed (Smedley and Kinninburgh 2002).

Dissolved As can occur in aquatic systems in both organic and inorganic forms. Inorganic As species predominate in sediments and water, but, in contrast, organoarsenic compounds prevail in marine organisms (Francesconi et al. 1999).

The inorganic As can be present in natural aquatic systems in four oxidation states: + V (arsenate), + III (arsenite), 0 (elemental As), and –III (arsine). The oxidation state is determined by pH and Eh. As^{V} and As^{III} are the common valence states in natural waters. As^{V} is the thermodynamically stable form that generally predominates in oxic surface waters, whereas As^{III} is favoured in environments with low pH and low redox potential (Genç-Fuhrman et al. 2004). In natural waters and at normal pHs, arsenate and arsenite are present as oxyanions (such as $H_2AsO_4^-$ and $HAsO_4^{2-}$) and as neutral aqueous species (H_3AsO_3), respectively (Anderson and Bruland 1991).

As it was aforementioned, arsenic may also occur in organic forms due to biological transformation of inorganic As species. In the literature this fact has been widely reported, showing that microorganisms may methylate As species as monomethylarsonic acid

(MMA^V), dimethylarsinic acid (DMA^V) and trimetilarsine oxide (TMAO) (Duker et al. 2005; Páez-Espino et al. 2009). Additionally, arsenosugars could be produced by seaweed (Edmonds and Francesconi 1981), whereas arsenobetaine and arsenocholine could be produced by marine animals (Edmonds et al. 1977; Norin et al. 1983; Le et al. 1994).

Arsenic toxicity is dependent on the chemical form in which As is presented (inorganic or organic) and on its oxidation state. Traditionally, the inorganic forms of As have been considered more toxic than the organic forms (Ng 2005). Among inorganic forms, As^{III} is in general considered more toxic, soluble and mobile than As^V (Wang and Mulligan 2006).

In rivers, sediments act as a significant sink of As, although changes in the river flow or in other environmental conditions (Eh, pH, changes in water composition) may cause adsorption or desorption processes which should be controlled. In the last years studies based on As adsorption onto sediments were reported by Rubinos et al. (2003), Bostick et al. (2004), Stollenwerk et al. (2007), Borgnino et al. (2012) and Mandal et al. (2012). Arsenic adsorption capacity has been related to the content of metal oxides, particularly of Al, Fe and Mn (De Vitre et al. 1991; Sullivan and Aller 1996) and to the clay content of sediments (Smedley and Kinninburgh 2002).

A significant aspect to be taken into account when As^V adsorption is studied is the potential competition between arsenate and phosphate for surface sorption sites. Phosphate concentration has been considered a critical factor in the adsorption or release of As from solid phases (Liu et al. 2001), because arsenate and phosphate behave both as oxyanions and present striking similarities such as quasi-identical pK_a values and charged oxygen atoms (Larsen and Hansen 1992). Phosphate strongly competes with As^V for surface sites, inhibiting As^V adsorption by Fe and Al oxides (Manning and Goldberg 1996). In the literature, the mobilization of As by P from sediments has been widely reported by Kaplan and Knox (2004), Bauer and Blodau (2006), Stollenwerk et al. (2007), Rubinos et al. (2010) and Rubinos et al. (2011), amongst others.

The role of organisms that colonize the sediment water interface must also be taken into account. In recent years, several studies have treated the sorption and removal of arsenate by means of iron-oxidizing bacteria (Katsioyiannis and Zouboulis 2004), the seaweed *Lessonia nigrescens* (Hansen et al. 2006) and by sulphate-reducing bacteria (Teclu et al. 2008).

Therefore, we hypothesize that As adsorption capacity may be affected by the presence of biofilms in the water-sediment interface. Costerton (2007) defines a biofilm as a universal community of microorganisms (bacteria, fungi, cyanobacteria, algae, protozoa) linked to wet surfaces or interfaces and embedded in a polymeric matrix (EPS) which allows an efficient water, nutrients and gas exchange between the populations constituting the biofilm and the outside environment. Biofilms play an important role in rivers systems as they constitute the interface between the overlying water and the sediments and are the first to interact with dissolved substances such as nutrients, organic matter and toxicants (Sabater et al. 2007).

Literature and investigations on the behaviour epipsammic biofilms on the retention of heavy metals and metalloids are limited, so as in natural river ecosystems as at microcosm and mesocosm scale. Published researches, as it was aforementioned, are focused on As retention by sediments and by certain isolated organisms but not on the in the whole river bed system with the presence of multi-species biofilms, which will be one of the objectives of this study.

In this work, the effect of epipsammic biofilms developed over riverbed sediments on As retention is evaluated as well as their environmental role in river systems with presence of problematic As^V concentrations. The capacity of As^V retention of biofilm-sediment systems will be compared to that of the sediment without biofilm, as well as the potential remobilization produced after the retention. The effect of the biofilm on As retention in the presence of equimolar P concentrations will be also assessed.

2. Materials and Methods

2.1. Sediment sample

The sediment sample was obtained in the Anllóns River, in a non-contaminated area upstream of the town of Carballo. A complex sample was collected with a small plastic shovel from the top 5 cm at various points at the same site and taken to the laboratory in hermetic plastic containers topped up to prevent oxidation.

2.2. Sediment and river water characterization

The grain size distribution of the sediment was determined as per Guitián and Carballas (1976); the fractions were classified as coarse sand (2 - 0.2 mm), fine sand (0.2 - 0.05 mm), coarse silt (0.05 - 0.02 mm), fine silt (0.02 - 0.002 mm) and clay (< 0.002 mm). Total P (P_T) was determined by acid digestion (HF, H_2SO_4, HCl, 10:1:10), followed by colorimetric determination with molybdenum blue, as described by Murphy and Riley (1962).

Nitrogen was determined by wet digestion with H_2SO_4, using the Kjeldhal method as described in Guitián and Carballas (1976). The concentration of total organic carbon (TOC) of the samples was determined, according to the procedure proposed by Sauerlandt and modified by Guitián and Carballas (1976), in an automatic titration system.

Sediment native As concentration was determined by X-ray fluorescence spectrometry (custom-built equipped with a Philips high-voltage generator and a Mo anode a 2.2 kW as X-ray source), following the protocol described by Devesa-Rey et al. (2008a). The concentration of Al, Fe and Mn were also determined.

River water was collected and filtered by 0.45 μm to be employed as biofilm growth medium in the laboratory in order to better reproduce the natural conditions for biofilm growth. pH and conductivity were determined, as well as soluble P by means of an acid digestion with H_2SO_4 followed by colorimetric determination with ammonium molybdate (APHA 2005).

2.3. Native biofilm growth

A natural biofilm was grown in indoor systems during 15 days over 8 g of riverbed sediment, using 60 mL of natural river water as nutrient supplier, in small plastic containers of 100 mL. The samples were subjected to day-night cycles (12 h of light with 3,109 lux of intensity) to reproduce approximately the natural environmental conditions. The overlying river water was replaced each 5 days, together with the addition of 0.5 mL of inoculum (fresh river biofilm) in order to stimulate the biofilm growth. Once the biofilm was developed, the overlying water was removed and total P was measured by acid digestion with H_2SO_4.

2.4. Arsenate sorption experiments

To evaluate the sorption capacity and desorption behaviour of the biofilm-sediment system, batch experiments were conducted with 8 g sediment and their corresponding formed biofilm. In parallel, samples without biofilm following the same treatment of the biofilm-sediment samples were used as controls.

Aliquots of 60 mL of As^V solutions with initial concentrations (C_0) of 0, 5, 25, 50, 100, 250 and 500 μg L^{-1}, prepared in 0.01 M $CaCl_2$ solutions as background electrolyte, were added to the systems. All the experiments were carried out in triplicate. Arsenate solutions were prepared from a stock standard solution of 1000 mg L^{-1} (Panreac, Barcelona-Spain). All the samples were prepared in triplicate. The batch experiments were carried out at room temperature (20 ± 2 ºC). Eh and pH measurements were carried out with a Thermo Scientific Orion Dual Star meter with a combined Redox/ORP electrode and with a AQUAPRO pH electrode (Beverly, USA), respectively. After 24 h a pseudo-equilibrium state was reached and the overlying water was taken (pipetting without altering the system). Aliquots were filtered through a 0.45 μm Whatman filter and As concentration (Ce) of the samples was determined by Inductively Coupled Plasma Spectrometry (ICP-MS, Varian 820 MS) with collision reaction interface (CRI) technology to reduce polyatomic interferences. The adsorbed As^V concentrations (Qads) for the sediment or biofilm-sediment systems were obtained by the difference between C_0 and C_e, taking into account the water volume and sediment weight.

For the study of the desorption behaviour, 60 mL of 0.01 M $CaCl_2$ solutions were added to the previous loaded systems. After 24 h, aliquots of the overlying water were extracted by gently pipetting. Again, the samples were filtered and As concentration measured by ICP-MS. The weight of the samples was controlled in every moment to calculate the mass of As desorbed. All the experiments were carried out at pH 5.5 adjusted by addition of 0.1 M NaOH or HCl solutions.

2.5. Influence of phosphorous presence on arsenate sorption process

To assess the influence of P presence on arsenate sorption, experiments with solutions of equimolar As(V):P concentrations were carried out, using the aforementioned procedure and concentrations used for the As^V sorption experiments. P solutions were obtained by

dissolution of KH_2PO_4 (Panreac, Barcelona-Spain). As and P concentrations in the supernatants were determined by ICP-MS.

2.6. Sorption modelling

The adsorption experimental data were fitted using a linear equation, four two-parameters-models (Freundlich, Langmuir, Dubinin-Rabushkevich and Temkin) and three three-parameters-models (Redlich-Peterson, Sips and Toth).

Linear equation was given by Eq. 1.

$$Q_e = AC_e - B \qquad \text{Eq. 1.}$$

where Q_e is the adsorbed or desorbed As concentration for the sediment or biofilm-sediment system, A is the slope and B is the content of native arsenate.

The Freundlich equation (Eq. 2) is used to describe heterogenous systems characterized by a heterogenous factor 1/n.

$$Q_e = K_f C_e^{\frac{1}{n}} \qquad \text{Eq. 2.}$$

where K_f and n are empirical constants of Freundlich model which are referred to capacity and intensity of adsorption, respectively (Freundlich 1906).

The Langmuir equation (Eq. 3) assumes monolayer coverage of adsorbate over a homogenous adsorbent surface.

$$Q_e = \frac{(Q_{Max} b C_e)}{(1+bC_e)} \qquad \text{Eq. 3.}$$

where Q_{max} is the maximum adsorption capacity of the system and b is a constant related to the energy bonds As-sediment and As-biofilm sediment interface (Langmuir 1918).

The Dubinin-Rabushkevich model isotherm is generally given by Eq. 4 (Dubinin and Radushkevich 1947).

$$Q_e = q_D \exp\left(-B_D\left[RT\ln\left(1+\frac{1}{C_e}\right)\right]^2\right) \qquad \text{Eq. 4.}$$

B_D is related to the mean free energy of sorption per gram of the sorbate as it is transferred to the surface of the solid from infinite distance in the solution (Quintelas et al. 2009).

The Temkin isotherm model contains a factor which takes into the account of adsorbent–adsorbate interactions and has been generally used in the form of Eq. 5 (Temkin and Pyzhev 1940).

$$Q_e = \frac{RT}{b_T}\ln(A_T C_e) \qquad \text{Eq. 5.}$$

The Redlich-Peterson empirical equation (Eq. 6) incorporates features of both Langmuir and Freundlich equations (Redlich and Peterson 1959). It can be applied to represent adsorption equilibrium over a wide concentration range.

$$Q_e = \frac{(K_R C_e)}{\left(1+a_R C_e^{\beta}\right)} \qquad \text{Eq. 6.}$$

Sips model isotherm is also called Langmuir-Freundlich isotherm (Sips 1948). At low sorbate concentrations it reduces to a Freundlich isotherm and at high sorbate concentrations a monolayer sorption capacity is predicted [47].

$$Q_e = \frac{\left(K_S C_e^{\frac{1}{b_S}}\right)}{\left(1+a_S C_e^{\frac{1}{b_S}}\right)} \qquad \text{Eq. 7.}$$

The Toth isotherm model is an empirical equation useful in describing heterogeneous adsorption systems (Toth 1971). Eq. 8 exhibits the most general form of this model.

$$Q_e = \frac{(K_t C_e)}{\left[(a_t+C_e)^{\frac{1}{t}}\right]} \qquad \text{Eq. 8.}$$

The parameters of all studied models were estimated by non-linear regression procedure employing Table Curve software (Jandel Scientific).

2.7. Statistical analyses

Five error functions were tested in order to choose the best model to fit the experimental data. These error functions were the coefficient of determination (R^2), sum of absolute errors (EABS), hybrid fractional error function (HYBRYD), average relative error (ARE) and Marquardt's percent standard deviation (MPSD), and were calculated employing the equations described by Foo and Hameed (2010).

The adsorbed concentrations of the different studied systems were evaluated by one-factor analysis of variance (ANOVA). Critical F values ($\alpha = 0.05$) were used to evaluate if the factor is significant. In the case of positive significance, post hoc analyses using the Duncan comparison test ($\alpha = 0.05$) were performed to establish statistical differences between the means (SPSS 19.0 statistical package). Student's t-test was also employed to explore separately the differences between sediment and biofilm systems with and without the addition of P.

2.8. Theoretical aqueous speciation

Visual MINTEQ V 3.0 was employed to theoretically calculate As species in the solutions and to determine their saturation degree, expressed as saturation index (SI) with respect to mineral phases, by means of thermodynamic calculations and Eq. 9.

$$SI = \log\left(\frac{IAP}{K_C}\right) \qquad \text{Eq. 9.}$$

where IAP is the ionic activity product of the specific dissolution-precipitation reaction and K_C is equilibrium constant. Negative SI indicates a mineral which has potential to dissolve whereas positive SI shows a mineral which has thermodynamic potential to precipitate (Simms et al. 2000).

To study the influence of the pH and organic matter in arsenic speciation, sweeps with values between 4 and 10 and 1 and 10 ppm, respectively, were performed.

3. Results and Discussion

3.1. Sediment and river water characterization

The sediment sample collected for this study showed a predominance of the sandy fraction, with an average value of 86.3 %, and only 6.7 % of clayey fraction. Total organic matter content for the sediment was of 13.9 ± 0.6 g kg^{-1}. P and N concentrations presented average values of 471.9 ± 43.7 and 629.6 ± 98.3 mg kg^{-1}, respectively. The total As concentration of sediment determined by X-ray fluorescence spectrometry was 11.8 mg kg^{-1}, whereas the sediment content of Al, Fe and Mn was 50.4, 51.9 and 1.2 g kg^{-1}, respectively.

The values of pH, electrical conductivity and soluble P concentration in the river water were of 6.87, 71.80 µS cm^{-1} and 0.21 mg L^{-1}, respectively.

3.2. Arsenate retention

Fig. C4.1 shows the experimental data for As^V sorption *versus* the As equilibrium solution concentrations for the sediment and biofilm-sediment systems. Q_{ads} increased with increasing initial concentrations in all cases, thus indicating that the adsorption was not at its maximum. The values of Q_{ads} for sediment system without biofilm are in the range of the data reported by Stollenwerk et al. (2007) and Borgnino et al. (2012) but slightly lower which may be due to the higher ratios (solution:sediment), lower native As concentrations in sediments and higher added As concentrations.

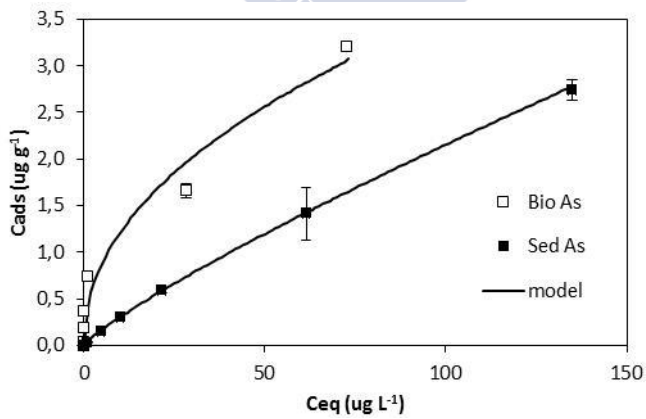

Figure C4.1. As^V retention by biofilm-sediment and sediment systems vs. dissolved As equilibrium concentration.

It is noteworthy that Q_{ads} values for the samples with biofilm were higher than for the samples without biofilms. The average percentage of As adsorbed with respect to C_0 for the sediment and biofilm-sediment system was 78.9 ± 3.5 and 96.9 ± 6.6 %, respectively. The difference between Q_{ads} for the biofilm-sediment system and for the sediment without biofilm increased, in the range of studied concentrations, from 6.81 10^{-3} up to 4.69 10^{-1} μg/g and was significant from As solutions from concentrations ≥ 50 μg L^{-1}. This may be explained by an increase in the specific surface area and the number of sorption sites and functional groups due to the presence of the biofilm, as well as by arsenate biouptake by microorganisms which constitute the biofilm. Arsenate could enter cells through phosphate-transporting systems (Zhao et al. 2009). Arsenate biouptake and bioaccumulation in green algae was studied by Karadjova et al. (2008) who reported that intracellular As increased linearly when As^V concentrations increased up to 50 μM, followed by a single saturation plateau.

Studies about As retention by epipsammic biofilm have not yet been reported. However, the removal of heavy metals by bacterial biofilm has been widely studied. For example, a biofilm of *Arthrobacter viscosus* was applied to remove Cr(VI), Cd(II) and Ni (II) (Quintelas et al. 2001, 2009, 2013; Pazos et al. 2010) whereas a biofilm of *Pseudomonas aeruginosa* was employed for the removal of Cr(III), Ni(II) and Co(II) (Kang et al. 2006).

These results highlight the important role that biofilms may play in river environments by increasing As^V retention. Moreover, if has been shown, biofilms promote As sequestration from the water column, they could be employed as a bioremediation tool for the treatment of contaminated waters, based on surface sorption and absorption of As by the biofilm.

Figure C4.2 presents Q_{ads} for As in the presence of equimolar P, for the sediment and biofilm-sediment systems. Again, Q_{ads} values for the biofilm-sediment system were higher than for sediment without biofilm. The difference between Q_{ads} for both systems increased, in the range of studied concentrations, from 0 up to 1.56 μg g^{-1} and was again significant from an initial concentration of 50 μg As L^{-1}. At the highest As concentration this difference was three times higher than in the experiments without P. This behaviour could be attributed to the presence of phosphate, which caused a significant reduction of the As^V adsorbed by the sediment without biofilm (a 25 % reduction at the highest added As concentrations) whereas no significant negative effect was detected in the systems with biofilm, and even a positive effect of phosphate was observed at the highest As concentration assayed.

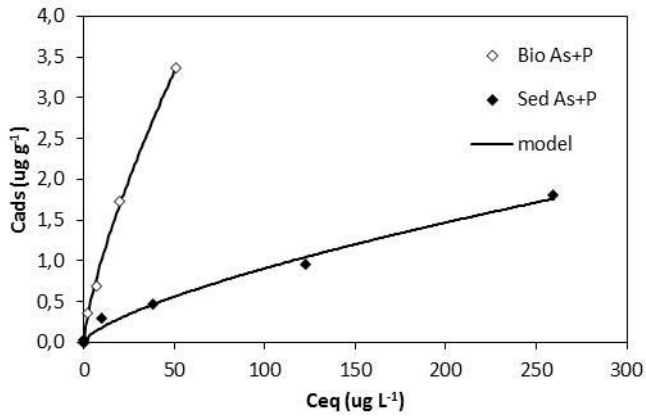

Figure C4.2. As^V retention for biofilm-sediment and sediment systems vs. dissolved As equilibrium concentration in the presence of equimolar P concentrations.

The competition between phosphate and arsenate for sorption sites in sediments has been widely reported. Thus for example, Stollenwerk et al. (2007) reported that, for concentrations of As^V of 100 µg L^{-1}, As^V adsorption was completely inhibited by 2 mg L^{-1} P. Rubinos et al. (2010) showed that the addition of increasing concentrations of phosphate enhanced the As^V release from sediments of the Anllóns River, and, in the same line, Rubinos et al. (2011) confirmed that increased As mobilization from these sediments in the presence of P occur in a wide range of pH (3-10).

Nevertheless, data have not yet been reported about the effect of P on As^V sorption in the presence of biofilms. In this study, phosphate did not affect As retention in the systems with biofim, which could be explained by the increase of sorption sites promoted by the biofilm and/or by the increase of intracellular arsenic uptake by microorganisms which constitute the biofilm.

As it regards desorption processes, they were negligible at the lowest and middle concentrations, in all the studied systems, and represented less than 0.5 % of the sorbed As concentrations at the highest initial As concentration.

3.3. Theoretical aqueous speciation

The calculations performed by Visual MINTEQ indicated that in the studied systems arsenic was present as inorganic As^V, with approximately 95 % of total species present as

$H_2AsO_4^-$. Negative values of SI were found in all studied cases, which indicated that the conditions are not favourable for the precipitation of As minerals. At pH 4 the monovalent $H_2AsO_4^-$ species prevails (approximately 98 % of total aqueous As), whereas at pH 10 the bivalent $HAsO_4^{2-}$ species prevails (approximately 96 % of the total aqueous As). In the studied conditions, the calculations did no predict significant complexation of As with the dissolved organic matter.

3.4. Sorption modelling

Figs. C4.1 and C4.2 showed the sorption curves for all the studied systems with their corresponding fits. Sorption isotherms were of type I according to Brunauer's classification (Brunauer et al. 1940). Table C4.1 shows the parameters for the different tested models. According to them, sorption data were satisfactorily adjusted by all the models. The values of the parameter A of the linear model were higher for the biofilm-sediment systems, especially in the presence of phosphate, while the lowest was obtained for sediment without biofilm in the presence of P.

Table C4.1. Parameters of the As^V sorption models

		Sediment		Biofilm	
		As^V	As^V + P	As^V	As^V + P
Linear	A	$2.02\ 10^{-2}$	$6.60\ 10^{-3}$	$4.15\ 10^{-2}$	$6.51\ 10^{-2}$
	B	$7.19\ 10^{-2}$	$1.21\ 10^{-1}$	$2.83\ 10^{-1}$	$1.71\ 10^{-1}$
Freundlich	k_f	$4.35\ 10^{-2}$	$3.71\ 10^{-2}$	$4.12\ 10^{-1}$	$1.91\ 10^{-1}$
	n	1.18	1.44	2.14	1.37
Langmuir	Q_{Max}	6.60	4.52	6.79	7.78
	b	$4.94\ 10^{-3}$	$2.50\ 10^{-3}$	$1.22\ 10^{-2}$	$1.49\ 10^{-2}$
Dubinin-	q_D	3.23	1.54	3.614	3.72
Rabushkevich	B_D	$5.07\ 10^{-4}$	$3.50\ 10^{-4}$	$1.04\ 10^{-4}$	$4.73\ 10^{-5}$
Temkin	A_T	2.48	$1.28\ 10^{-1}$	2.94	$4.79\ 10^{-1}$
	b_T	$4.93\ 10^{3}$	$5.68\ 10^{3}$	$4.84\ 10^{3}$	$2.66\ 10^{3}$
Redlich-	k_r	$6.00\ 10^{-2}$	$3.87\ 10^{5}$	$1.25\ 10^{6}$	$7.80\ 10^{5}$
Peterson	a_r	$5.59\ 10^{-1}$	$1.04\ 10^{7}$	$3.11\ 10^{6}$	$4.07\ 10^{6}$
	β	$2.56\ 10^{-1}$	$3.05\ 10^{-1}$	$5.32\ 10^{-1}$	$2.70\ 10^{-1}$
Toth	k_t	$4.69\ 10^{-2}$	$5.32\ 10^{-2}$	$2.59\ 10^{-1}$	$1.97\ 10^{-1}$
	a_t	3.91	$6.02\ 10^{-3}$	$3.62\ 10^{-2}$	$3.60\ 10^{-1}$
	t	5.89	2.68	2.43	3.62
Sips	k_s	$4.09\ 10^{-2}$	$1.04\ 10^{-1}$	$1.07\ 10^{-2}$	$3.45\ 10^{-2}$
	a_s	1.15	4.28	330.52	66.13
	b_s	$7.18\ 10^{-4}$	$-2.15\ 10^{-1}$	$-9.84\ 10^{-1}$	$-9.32\ 10^{-1}$

The Equilibrium Arsenic Concentration (EAC) can be defined, analogously to the Equilibrium Phosphorous Concentration EPC, as the concentration of As that is supported by

the sediment when in contact with an ambient solution such that no arsenate is either gained or lost by the sediment (Rubinos et al. 2003). When As concentrations in water are higher than EAC the sediment would act as a sink for As, whereas for water As concentrations lower than EAC, the sediment would act as a source of As. Calculated EAC values obtained in this study ranged from 2.62 to 18.28 μg L^{-1}; the lowest EAC corresponded to the biofilm-sediment system with P, and the highest to the sediment system with P, thus pointing to a higher risk of As transfer towards the water column in these conditions.

Table C4.2. Values of error functions for each model and for each analysed system.

		Sediment		Native Biofilm	
		As^V	As^V + P	As^V	As^V + P
Freundlich	R^2	0.999	0.990	0.953	0.998
	EABS	$7.83\ 10^{-2}$	$2.89\ 10^{-1}$	1.36	$2.26\ 10^{-1}$
	HYBRYD	8.31	$3.77\ 10^{1}$	$7.29\ 10^{1}$	$3.13\ 10^{1}$
	ARE	5.94	$2.51\ 10^{1}$	$5.21\ 10^{1}$	$2.09\ 10^{1}$
	MPSD	$1.40\ 10^{2}$	$5.37\ 10^{2}$	$8.00\ 10^{2}$	$5.06\ 10^{2}$
Langmuir	R^2	0.996	0.977	0.927	0.998
	EABS	$2.85\ 10^{-1}$	$4.31\ 10^{-1}$	1.34	$2.09\ 10^{-1}$
	HYBRYD	6.01	$4.78\ 10^{1}$	$7.86\ 10^{1}$	$3.49\ 10^{1}$
	ARE	4.29	$3.19\ 10^{1}$	$5.61\ 10^{1}$	$2.33\ 10^{1}$
	MPSD	$6.18\ 10^{1}$	$5.98\ 10^{2}$	$8.68\ 10^{2}$	$5.30\ 10^{2}$
Dubinin-Rabushkevich	R^2	0.925	0.854	0.912	0.930
	EABS	$1.24\ 10^{1}$	3.15	4.87	8.65
	HYBRYD	$1.73\ 10^{3}$	$1.79\ 10^{2}$	$9.36\ 10^{1}$	$3.68\ 10^{2}$
	ARE	$1.24\ 10^{3}$	$1.19\ 10^{2}$	$6.69\ 10^{1}$	$2.46\ 10^{2}$
	MPSD	$2.51\ 10^{4}$	$2.37\ 10^{3}$	$1.60\ 10^{3}$	$5.10\ 10^{3}$
Temkin	R^2	0.760	0.839	0.810	0.895
	EABS	5.48	$9.30\ 10^{-1}$	1.19	1.51
	HYBRYD	$5.41\ 10^{2}$	$3.77\ 10^{1}$	$1.37\ 10^{1}$	$4.80\ 10^{1}$
	ARE	$3.86\ 10^{2}$	$2.52\ 10^{1}$	9.79	$3.20\ 10^{1}$
	MPSD	$6.74\ 10^{3}$	$4.20\ 10^{2}$	$1.91\ 10^{2}$	$5.98\ 10^{2}$
Redlich-Peterson	R^2	0.999	0.990	0.953	0.998
	EABS	$1.74\ 10^{-2}$	$2.44\ 10^{-1}$	1.40	$1.97\ 10^{-1}$
	HYBRYD	3.46	$1.25\ 10^{1}$	$7.31\ 10^{1}$	6.36
	ARE	2.47	8.35	$5.22\ 10^{1}$	4.24
	MPSD	$6.70\ 10^{1}$	$1.96\ 10^{2}$	$8.02\ 10^{2}$	$7.87\ 10^{1}$
Toth	R^2	0.999	0.987	0.948	0.998
	EABS	$1.06\ 10^{-2}$	$3.60\ 10^{-1}$	1.27	$2.39\ 10^{-1}$
	HYBRYD	1.57	$3.81\ 10^{1}$	$7.50\ 10^{1}$	$3.18\ 10^{1}$
	ARE	1.12	$2.54\ 10^{1}$	$5.35\ 10^{1}$	$2.12\ 10^{1}$
	MPSD	$2.83\ 10^{1}$	$5.22\ 10^{2}$	$8.26\ 10^{2}$	$5.07\ 10^{2}$
Sips	R^2	0.999	0.999	0.984	0.978
	EABS	$1.12\ 10^{-2}$	$3.34\ 10^{-1}$	0.99	$2.69\ 10^{-1}$
	HYBRYD	1.62	$3.68\ 10^{1}$	$6.80\ 10^{1}$	$3.30\ 10^{1}$
	ARE	1.23	$2.27\ 10^{1}$	$4.97\ 10^{1}$	$2.31\ 10^{1}$
	MPSD	$2.89\ 10^{1}$	$5.01\ 10^{2}$	$7.98\ 10^{2}$	$5.29\ 10^{2}$

Among the analysed two-parameters-models, the Freundlich model showed the best fit to experimental data in all cases, as indicated by the highest R^2 values and the lowest values of

other error functions (Table C4.2), and is suggestive of adsorption on a heterogeneous surface of the solid phases. The Langmuir model also successfully fitted the experimental data, with R^2 values above 0.92 in all cases, whereas the Dubinin- Rabushkevich and Temkim models were not completely satisfactory.

The estimated maximum adsorption capacity of the sediment and biofilm-sediment systems, obtained from the Langmuir model, was 6.6 and 6.8 µg/g, respectively, and 4.5 and 7.8 µg/g, respectively, in the presence of P. These values fall within the range, although slightly lower, than those reported by Stollenwerk et al. (2007), studying As adsorption on oxidized aquifer sediments. Again, these results highlight the key effect that the presence of biofilm has in the fate of As in the river system, mainly in the presence of P.

The essential characteristics of the Langmuir isotherm can be expressed in terms of an equilibrium parameter, R_L, which allows to determine if the adsorption process is favourable or unfavourable (Weber and Chakarvoti 1947). Eq. 10 shows the relationship between R_L and C_0.

$$R_L = \frac{1}{(1+bC_0)} \qquad \text{Eq. 10.}$$

The values of R_L ranged between 0 and 1 for all the analyzed concentrations, which corresponds to a high affinity favorable adsorption process, being more favorable at the highest the initial As concentrations. As it could be seen in Figure C4.3, the biofilm-sediment systems in the presence of phosphate present the highest affinity for As (the lowest R_L) whereas the sediments in the presence of phosphate show the lowest (the highest R_L).

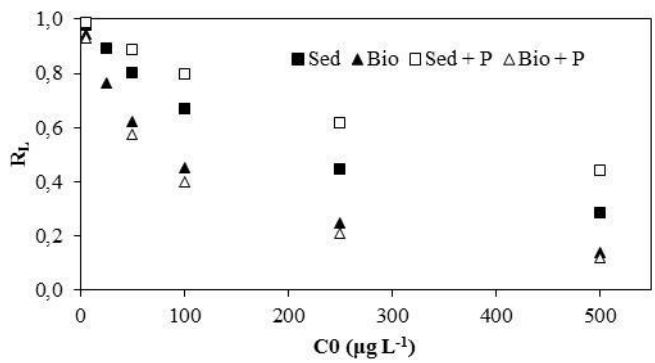

Figure C4.3. R_L values for all studied systems vs. initial As concentrations.

Gibbs free energy of adsorption process could be obtained from Langmuir and Temkin models by means of Eqs. 11 and 12, respectively.

$$\ln\left(\frac{1}{b}\right) = \frac{\Delta G^0}{RT} \qquad \text{Eq. 11.}$$

$$\ln\left(\frac{1}{A_T}\right) = \frac{\Delta G^0}{RT} \qquad \text{Eq. 12.}$$

The values of Gibbs free energy yield negative values in all cases, which indicate that the adsorption process was always spontaneous. The biofilm-sediment system in the presence of P showed the most negative value by applying Eq. 11 (-34.50 kJ mol^{-1}) and Eq. 12 (-43.10 kJ mol^{-1}), whereas the sediment system with P presented the least negative (-30.08 kJ mol^{-1} by Eq. 11 and -39.83 kJ mol^{-1} by Eq. 12). The systems without P showed an intermediate behaviour, being more spontaneous As^V retention in biofilm-sediment system. Therefore, the presence of biofilms jointly with P presence suggests that As retention process was more favoured.

Table C4.2 shows also that the three-parameters-models, especially Sips and Toth models, also satisfactorily fit the experimental data based on the high R^2 values (>0.95) and low values of the error functions.

4. Conclusions

The biofilm increased the As^V sorption capacity of the studied sediment. An input of P at equimolar P:As concentrations reduces the sorption of As^V onto the sediment, whereas no negative effect is exhibited by the systems with biofilm, and even a positive effect of phosphate was observed at the highest As concentration assayed. The Freundlich model showed the best fit, amongst the two parameters models, to the As retention data, which is indicative of their heterogeneous surface. The sorption of As is spontaneous and favourable in all cases, especially under the combined effect of biofilm and P, whereas the desorption of this As retained is not significant. Overall, is can be concluded that epipsammic biofilms play a key role in the fate and mobility of As in riverine environments, particularly in the As transfer from the water column towards the sediment, as they enhance the sorption capacity of the sediments for As, especially in environments where both As and P occur simultaneously.

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Chapter 5: Influence of epipsammic biofilms on arsenic retention and transformation in freshwater environments

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Abstract

The influence of epipsammic biofilms developed on riverbed sediments on the sorption, uptake, mobility and transformation of As^V was studied. A native biofilm was incubated on sediment samples at microcosm level. Once the biofilm was developed, 500 μg L^{-1} As^V were spiked in two systems designated BAS and BASP, without P and with equimolar As:P concentrations, respectively, and compared with identical control sterilized systems (CAS and CASP). The evolution and speciation of As concentrations in the overlying water were followed during two weeks. The biofilm enhanced the removal of As^V from water up to 91% of its initial concentration in BAS, while the removal only attained ~70% in CAS. The presence of equimolar P concentrations enhanced up to 97 % the amount of As removal in BASP, but had no effect in CASP. In the systems with biofilm As was mostly (~97%) As^V, whilst As^{III} only accounted for ~1% of total aqueous As, and the organic species, MMA^V and DMA^V, represented 0.6 and 0.7% of total As, respectively. In contrast, in the systems devoid of biofilm As^{III} accounted for up to 39% of aqueous As and methylated aqueous species were negligible. The distribution of As in the biofilm showed that ~71% of the retained As was extracellular, most of it (>99.5%) in the form of As^V. Volatile As forms were only detected in the systems incorporating the biofilms. It is concluded that biofilms covering sediments increase the As retention, inhibit the reduction of As^V to As^{III} and methylate inorganic As, thus playing a key role in the biogeochemistry of As in river environments.

1. Introduction

Arsenic (As) is a highly toxic metalloid which is widespread in the environment and causes severe and numerous health problems worldwide. The presence of As in soils, sediments and water is attributed to natural sources, such as the weathering of minerals with high As contents, and to human activities (the use of arsenical fertilizers and pesticides, as well as industrial and mining activities) (Smedley and Kinniburgh 2002). The mobility and toxicity of As strongly depend on its chemical form (Oremland and Stolz 2003). As^{III}, which is the predominant form in reduced environments, is more mobile and is considered more toxic than As^{V}, which is the predominant form in oxic environments. In turn, inorganic As (iAs) is generally recognized as more toxic than the organic forms (Sharma and Sohn 2009), with the exception of the methylated As^{III} species (Petrick et al. 2000; Styblo et al. 2000).

In aquatic environments, As may undergo transformations in its chemical form as a consequence of changes in the environmental physico-chemical conditions and interaction with mineral surfaces (oxidation/reduction, surface complexation), but also through biologically mediated reactions (bio-transformation) which can strongly affect its mobility and bioavailability (Oremland and Stolz 2005). Studying the effect of algae on As speciation, Hellweger and Lall (2004) proposed a model where algae absorb As^{V} (mistaking it for phosphate). Inside the cell, an As^{V} detoxification mechanism takes place which consists in reducing As^{V} to As^{III}, methylating it to monomethylarsonic acid (MMA^{V}) and then MMA^{V} to dimethylarsinic acid (DMA^{V}). Finally, As is excreted as As^{III} and/or DMA^{V}, depending on algae growth rate and on phosphate conditions (Hellweger and Lall 2004). Besides MMA^{V} and DMA^{V}, the products of methylation include trimetilarsine oxide (TMAO) (Duker et al. 2005) and the volatile trimethylarsine ($TMAs^{III}$) which is the final product of the methylation pathway (Yin et al. 2011a).

In water-sediment interfaces, biofilms consisting of microorganisms (bacteria, fungi, cyanobacteria, algae and protozoa), embedded in an extracellular polymeric matrix (EPS) mainly composed of polysaccharides, are commonly found (Costerton 2007). Consequently, biofilms are the first that interact with dissolved substances such as nutrients, organic matter, metals and metalloids, as well as other toxicants, in aqueous systems (Sabater et al. 2007).

It has been demonstrated that biofilms play a key role in the retention of metals and metalloids from the overlying waters (Nelson et al. 1996, 1999; Friese et al. 1997; Headley et al. 1998; Decho 2000; Dong et al. 2000; Haack and Warren 2003; Morris et al. 2005; Dong et al. 2007; Serra el al. 2009a; Beck et al. 2011; Drahota et al. 2014). Pollutants are removed by the biofilm by a variety of mechanisms, including (bio-)sorption, precipitation as sulfides or phosphates and microbial reductive precipitation. Biosorption consists of several mechanisms, including ion exchange, chelation, adsorption and diffusion through cell walls and membranes (van Hullebusch et al. 2003).

The interaction between As and biofilms in aquatic systems can be approached from two perspectives: the influence of biofilm on As biogeochemistry, and the effect of As on biofilm development, composition and functionality. Firstly, As biogeochemistry may be modified by the presence of biofilms; the most immediate effect is its removal from water. The potential of As enrichment in biofilms has been reported by Drewniak et al. (2008) who found As concentrations up to 60 mg kg^{-1} in rock biofilms. Yang et al. (2011) demonstrated that multi-species biofilms, inoculated from a source receiving coal mining effluent, can sequester and detoxify As. Drahota et al. (2014), studying As adsorption from natural As sources onto natural surface coatings growing on glass slides, confirmed that dissolved As was sorbed and retained by the biofilm. Tuulaikhuu et al. (2015) investigated the fate and the toxicity of As on periphytic and epipsammic biofilms, using a simplified fluvial system including fish, biofilms and sediment, and reported that periphytic biofilms accumulated As, although it was predominantly retained by the sediment. With regard to As retention by epipsammic biofilms, Prieto et al. (2013) observed that they increased As^{V} sorption with respect to biofilm-devoid river sediments, and a more noticeable effect was observed in the presence of phosphate. Secondly, the presence of As in aquatic systems can affect periphyton communities. Arsenate is responsible for inhibiting algal growth and photosynthetic capacity, as well as for decreasing total biofilm biomass, changing community composition (selecting tolerant species, reducing species richness and making biofilms more heterotrophic), reducing diatom cell sizes and the ability of the community to use phosphorus (Blanck and Wangberg 1988; Wangberg et al. 1991; Blanck and Wangberg 1991; Rodriguez Castro et al. 2015; Tuulaikhuu et al. 2015; Barral-Fraga et al. 2016).

Despite this evidences, literature on the (bio-)sorption, speciation and detoxification of As by epipsammic biofilms is still scarce. Hence, the present work aims to contribute to the understanding of the role played by epipsammic biofilms in As biogeochemistry. The main objective is to study the influence of biofilms developed on riverbed sediments on the adsorption and/or uptake, mobility, transformation and detoxification of As^V at a microcosm scale, using specifically designed systems to control As concentration and speciation over time. The kinetics of the removal process are evaluated, along with changes in As aqueous speciation, As volatilization, the distribution of As species within biofilms and the remobilization of the previously retained As. The effect of phosphate as a potential competitor for As sorption and biouptake and as a nutrient for biofilm growth is also explored.

2. Materials and Methods

2.1. Sediment characterization

The sediment was sampled in the Anllóns River, where As contamination is a problem in some sections and where gold mining activities carried out during the Roman Empire and more recently in the early twentieth century, brought about the remobilization of associated arsenic and its accumulation in sediments (Devesa-Rey et al. 2008a, 2011; Costas et al. 2011). It has been shown that As mobilization in the Anllóns riverbed sediments is enhanced under conditions of high salinity, extreme pH or high P concentrations, and low solid:liquid ratios as those expected during high-flow resuspension events (Rubinos et al. 2010, 2011). Conversely, the favourable effect of the biofilm on As retention has been demonstrated in the previous chapter.

For this study, the sediment was sampled in an uncontaminated area upstream the Au-As mineralized area, known as Ponte de Eguas (43° 13’ 24.46’’ N 8° 45’ 44.61’’ W), which is located 8 km downstream from the town of Carballo. A complex sample was collected with a small plastic shovel from the top 5 cm at various points at this site and taken to the laboratory in hermetic plastic containers topped up to prevent oxidation. Eh and pH in the sediment were measured *in situ* with a portable device (HANNA instruments, HI 9025 microcomputer). Eh values obtained with the Pt-Ag/AgCl electrodes were corrected to refer them to the standard hydrogen electrode (SHE) by adding 245 mV.

Once in the laboratory, solid sediment samples were freeze-dried and sieved (<2 mm) for characterization. Only some organic debris was eliminated by sieving, so that the <2 mm fraction used for the experiment practically represented the bulk sediment. Grain size distribution was determined using wet sieving and the pipette method as described in Guitián and Carballas (1976). Total phosphorus (P_T) was determined by means of acid digestion (HF: H_2SO_4: HCl 10:1:10) followed by colorimetric determination (Murphy and Riley 1962). Total organic carbon, nitrogen and sulphur content were analysed using a LECO TruSpec CHNS analyzer. Major and trace constituents were determined by X-ray fluorescence spectrometry. An X-ray power diffraction method was used for the semiquantitative mineralogical analysis of the mineral phases present in the sediment.

2.2. River water characterization

River water was collected and filtered by 0.45 µm cellulose nitrate membrane filters NCS 045 47 BC (Albet LabScience, Dassel, Germany) to be used as biofilm growth medium in the laboratory, in order to mimic the natural conditions for biofilm development. Water pH, Eh and electrical conductivity (EC) were measured *in situ* with portable electrodes (HANNA instruments, HI 9025 and HI 9033, respectively). Soluble P was measured by means of acid digestion with H_2SO_4 followed by colorimetric determination with ammonium molybdate (APHA 2005). Total N was determined by segmented flow analysis and colorimetry with Futura console (AMS Alliance) after filtration through a 0.45 µm-membrane Millex-HM (Millipore). Nitrate was measured by ion chromatograph model Metrohm 850 Professional IC and ammonia was determined by ion-selective electrode (ISE) (Thermo Scientific 9512BNWP). Alkalinity was measured by colorimetric determination using an AquaKem 250 Analyzer (Thermo Scientific, Waltham). Dissolved organic carbon (DOC) was measured using a Total Organic Carbon Analyzer Model TOC-5000 (Shimadzu, Kyoto). With this equipment, the DOC concentration is obtained by subtracting the inorganic carbon (IC) concentration from the total carbon (TC) concentration. TC is determined by the 680 °C combustion catalytic oxidation method, whereas IC is determined by acidification and sparging. The carbon dioxide generated in both determinations is detected using a non-dispersive infrared gas analyzer (NDIR). Na and K concentrations were measured by atomic emission spectrometry, whereas Ca and Mg by atomic absorption spectrometry (SPECTRA AA 220 FS, Varian Inc., Palo Alto). Total Fe, Mn and As concentrations were measured by

Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 820MS Varian Inc., Palo Alto), and As species were analysed using HPLC-ICP-MS, as described below in the arsenic analysis section.

2.3. Biofilm growth

The sediment sample (500 g, 31% water content) was incubated in bioreactors filled with 2 L of filtered As-free water, equipped with systems for air-supply, sample collection and for volatilized arsenic trapping (Fig. C5.1). All the materials were previously sterilized by autoclaving at 121 ºC for 30 min. The flasks were maintained for 3 weeks in an incubation chamber under optimal controlled conditions of light (day-night cycles, 12 h of light, intensity ca. 40 µmol photon m^{-2} s^{-1}), temperature (20 ºC) and air-supply (ca. 1 L min^{-1}). The water was replaced weekly to provide the necessary nutrients for biofilm growth. After 3 weeks an appreciable biofilm layer was clearly perceived on the sediment surface. Although taxonomic identification of the components of the biofilm was not performed, we can take into account that epipsammic biofilms, covering the bed sediments in the Anllóns River, are mainly constituted by Bacillariophyceae, which represent >86 % of the total abundances in the superficial sediments (Martiñá Prieto et al., 2016; Chapter 2). At Eguas sampling site *Cocconeis*, *Navicula* and *Karayevia* were identified as the predominant genera. Also, epipsammic biofilm inocula from this river have been satisfactory incubated at lab scale in experimental fluvial channels, reaching the maximum growth at 2-3 weeks, as indicated by chlorophyll-a and soluble carbohydrates contents (Prieto et al. 2016; Chapter 3).

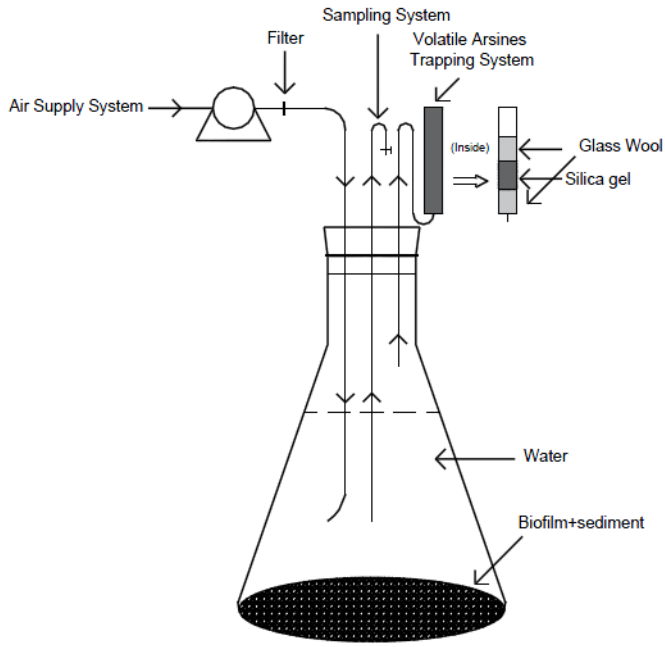

Figure 5.1. Scheme of the experimental setup.

2.4. Arsenic retention and speciation

A stock solution of 1000 mg L^{-1} As^{V} was prepared by dissolving Na_2HAsO_4 $7H_2O$ (Panreac, Spain) in MilliQ water. Once the biofilm was developed, an aliquot of the As^{V} solution was spiked resulting in an initial 500 µg L^{-1} (6.67 µmol L^{-1}) As exposure concentration. This As concentration, which exceeds the USEPA's Aquatic Life Criteria Maximum Concentration (CMC) (acute exposure) for As in freshwater, set at 340 µg L^{-1} (USEPA 2014), was selected because it was below the reported EC_{20} (that represents a measurable threshold of As toxicity) for As^{V} (2.0±0.6 mg L^{-1}) for *Aliivibrio fischeri* model bacteria (Microtox® acute toxicity screening bioassay) (Rubinos et al. 2014), while still enabling the detection of quantifiable changes in the As species concentration in the water column. The effect of phosphate on As^{V} retention and speciation was also studied in identical systems, incorporating equimolar As:P concentrations. P was added to the systems from a stock solution of 1000 mg L^{-1} P, prepared by dissolving $Na_2HPO_4 \cdot 7H_2O$ (Panreac, Spain) in

MilliQ water. The systems incorporating the biofilms were labelled BAS and BASP for the systems with and without added phosphate, respectively. In parallel, identical systems without biofilms (sediment and river water sterilized by 3 cycles of autoclaving at 120 ºC for 30 min) were developed as reference systems (controls) for experiments with and without phosphate (named CAS and CASP, respectively).

The systems were maintained under the conditions indicated above for two additional weeks, during which aliquots (5 ml) of the water column were sampled daily, using single use sterile PP syringes (Braun Inkjet, B Braun AG, Melsungen). The samples were immediately filtered (sterile 0.45-µm Whatman Puradisc 25AS™ syringe filters, GE Healthcare Europe GmbH, Barcelona) and stored frozen (−80 ºC), until analysis of total As and its species (As^{III}, As^{V}, MMA^{V}, DMA^{V}, arsenobetaine AsB) by ICP-MS and HPLC-ICP-MS, respectively. Relative standard deviations for total As analysis by ICP-MS were < 3%. Additionally, dissolved P, Fe and Mn concentrations were measured (determined by ICP-MS) as well as dissolved organic matter (DOC) (as previously determined by TOC-Analyzer). At the end of the experiment, the As speciation in the sediment interstitial waters of the systems was also determined. 10 mL samples of the interstitial water were filtered, frozen at −80ºC and analysed for As species as described above. The pH, Eh and sulphate concentration were also measured in the overlying waters of the different systems at the end of the experiment, as previously described in the section 'Sediment and river water characterization'.

Analyses of sediment and biofilm solid samples were performed by triplicate, in addition to three analytical replicates. The concentration of As in water (and other elements included in the study) was analysed in triplicate by ICP-MS to ensure the quality of the analysis (RSD<3%). The experiment was conducted in large volume (2 L) bioreactors tempting at simulating at a laboratory scale the conditions of reactor for water purification, with the purpose of evaluating a possible biotechnological application. Water samples (5 mL at each of 18 sampling times) are considered representative to the whole solution due to the effect of agitation and to diffusion processes.

2.5. Arsenic analyses

The analysis of the dissolved As species was carried out using High-Performance Liquid Chromatography together with Inductively Coupled Plasma Spectrometry (HPLC-ICP-MS).

A Varian Prostar 230 HPLC, equipped with a guard column and a Hamilton PRP-X100 anion exchange column (4.1 x 250 mm and 10 µm), was used to separate five primary As species (As^V, As^{III}, MMA^V, DMA^V and AsB) using a 13 minute gradient LC method with 12.5 mM and 30 mM (pH 9) $(NH_4)_2CO_3$ as the mobile phase, a flow rate of 1 mL min^{-1} and an injection volume of 50 µL. For the quantification, a Varian 820-MS ICP-MS, equipped with collision reaction interface (CRI) technology to reduce polyatomic interferences, was used. Relative standard deviations for total As analysis by ICP-MS were < 3%. The detection limits under the experimental conditions were 2.8, 4.1, 2.9, 4.6 and 2.5 ng L^{-1} for As^V, As^{III}, MMA^V, DMA^V and AsB, respectively.

2.6. Volatilized arsenic

To quantify volatilized As, arsines were trapped using the $AgNO_3$ based chemo-trapping approach described by Mestrot et al. (2009) and Yin et al. (2011a). In this method, arsine (AsH_3), monomethyl arsine ($MeAsH_2$), dimethyl arsine (Me_2AsH), and trimethyl arsine (TMAs or Me_3As), react with $AgNO_3$ and are preserved by oxidation to their pentavalent oxy-species (As^V, MMA^V, DMA^V and TMAO) (Mestrot et al. 2009).

To prepare the traps, silica gel (2.5-5 mm) was submersed overnight in a 5% HNO_3 (w/v) solution and washed with Milli-Q water (18.2 MΩ.cm^{-1} resistivity), then impregnated with a 10% $AgNO_3$ (w/v) solution and placed overnight in an oven at 70 ºC (covered with aluminium foil to avoid the photodecomposition of $AgNO_3$) (Yin et al. 2011a). Subsequently, the silica gel (~1 g) was loaded in the trap tubes (10-mL sterilized syringe) and held in place by a small quantity of glass wool washed QP (Panreac, Barcelona) at both ends. Trap tubes were again covered with aluminium foil to avoid the photodecomposition of $AgNO_3$ and coupled to the flask systems.

At the end of the experiment, 5 mL of 1% (v/v) hot boiling HNO_3 was used to elute the collected As in the trap tubes (Yin et al. 2011b). Eluates were filtered (0.45 µm) and kept frozen (-80 ºC) until measurement of total As by ICP-MS.

2.7. Remobilization and bioavailability of arsenic

Immediately after the retention experiment, the overlying water was removed and the potential remobilization of the previously retained As was assessed by washing the As-

loaded sediment-biofilm or sediment with As-free filtered (0.45 µM) river water (1:10 solid:liquid ratio). The aqueous extracts were filtered (0.45 µm) and kept frozen (-80 ºC) until analyzed for As by HPLC-ICP-MS.

To evaluate As bioavailability, the diffusion gradient in thin films (DGT) technique was used. DGT devices, purchased from DGT Research Ltd. (Lancaster), accumulate metals on a binding agent after they have passed through a well-defined diffusive layer (Davison and Zhang 1994; Zhang and Davison 1995; Zhang et al. 1995). DGT incorporating Fe-oxide gels have been specifically developed for assessing As biovailability (Stockdale et al. 2008, 2010; Luo et al. 2010).

DGT devices specific for As were placed onto the sediment surface of the studied systems for 24 h, to afford an operationally defined measure of the "bioavailable" fraction of As. Once removed from the systems, the devices were rinsed with Milli-Q water and opened for the removal of the resin gels, which were then eluted with 1mL of 7.2 M HNO_3 for at least 24h to allow the complete extraction of the As from the resin. An aliquot from the sample tube was pipetted and diluted 6 times with Milli-Q water prior to analysis by ICP-MS. The mass of As in the resin gel (M), the time-averaged DGT concentrations (C_{DGT}) and the flux (F) of As measured by DGT were calculated according to Zhang and Davison (1995) and DGT® technical documentation.

To determine time-averaged DGT concentrations, the diffusion coefficient of total As was calculated from the diffusion coefficient of individual species using Eq. 1:

$$D_{AsT} = \sum_{i=1}^{5} x_i D_i \qquad \text{Eq. 1}$$

Where x_i and D_i are the fraction of individual As species over the total As and the diffusion coefficient of the individual As species, respectively.

Values for the diffusion coefficient of As^{III} and As^{V} have been reported in the ranges 5.9-10.1 $\times 10^{-6}$ cm^2 s^{-1} and 4.9-6.8 $\times 10^{-6}$ cm^2 s^{-1}, respectively (Fitz et al. 2003; Panther et al. 2008; Österlund et al. 2010; Bennet et al. 2010; Luo et al. 2010, Österlund et al. 2012; Moreno-Jiménez et al. 2013), whereas diffusion coefficients values of 6.10 and 6.30 $\times 10^{-6}$ cm^2 s^{-1} have been reported for MMA^{V} and DMA^{V}, respectively (Österlund et al. 2012).

2.8. Extracellular and intracellular arsenic

Extracellular As in the biofilms was determined using the extraction procedure described by Levy et al. (2005), consisting in a phosphate extraction, and was followed by the extraction of intracellular As by the method described by Miyashita et al. (2009), consisting in an As extraction from lysed cells with methanol-water. In detail, after exposure to As^V, biofilm samples (ca. 1 g) were gently harvested and rinsed with 10 mL of filtered river water (1:10 solid:liquid ratio). Then, the solid phases were submitted to two washing cycles with 10 mL of 0.1M KH_2PO_4/K_2HPO_4 buffer solution (pH 5.95) in order to extract the extracellular As. The suspensions were shaken for 30 s and allowed to stand for 20 min; then they were centrifuged (3,000 rpm, 15 min), the supernatant filtered (0.45 μm syringe filters) and the extraction cycle repeated. The eluates of two washes were combined and kept frozen (-80 ºC) until analyzed for arsenic species (HPLC-ICP-MS). For the determination of intracellular As the remaining solid phases were gently washed with Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity), centrifuged (3,000 rpm, 15 min) and frozen (-20 ºC).Then, the solid phases were thawed and intracellular As was extracted with 10 mL of methanol/H_2O (1:1, v/v) solution. After standing for 10 min, the suspensions were sonicated for 10 min and centrifuged at 3000 rpm for 15 min. The extraction was repeated twice with 5 mL of methanol/ H_2O (1:1, v/v) solution.

The extracts were combined and evaporated under vacuum to dryness using a rotavapor (Büchi Rotavapor R-200, BÜCHI Labortechnik GmbH, Essen). The dried extracts were redissolved with Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity), filtered (0.45 μm) and kept frozen (-80 ºC) until analyzed for As species (HPLC-ICP-MS). At the end of the sequential extractions, solid phases were dried at 105 ºC up to constant weight to determine the dry weight of the analyzed samples. All the determinations have been carried out in triplicate.

3. Results and Discussion

3.1. Sediment and river water characterization

The main physico-chemical properties of the bed sediments and river water are presented in Table C5.1. The superficial sediment had a slightly acidic pH (6.3) and Eh of 235 mV, which correspond to a suboxic state (ranging between 100-400 mV at around neutral pH), and

had a predominance of the sandy fraction. Semiquantitative mineralogical analysis of the mineral phases showed quartz as the predominant mineral, with about half of the total abundances, followed by microcline and hornblende. The presence of this highly weatherable mineral can be explained by the bedrock composition at this sampling site, where the Anllóns River runs over basic rocks, namely peridotite, piroxenite, anphibolite and serpentinite, and it is also indicative of a short transport distance of the sediments. Total organic matter content was 1.5%, whereas P and N concentrations were 370 and 253 mg kg^{-1}, respectively. C/N ratio was 35.4, which is indicative of OM rich in lignin and cellulose as well as poor in N, attributable to terrestrial origin (Lamb et al. 2006). Total P concentration was lower than the Lowest Effect Level (LEL) established by the Ontario Sediment Quality Guidelines (Persaud et al. 1993), set at 600 mg kg^{-1}. This level of pollution is expected to have no effect on the majority of sediment-dwelling organisms, and the sediment is considered clean to marginally polluted. The total As concentration of the sediment was 15 mg kg^{-1}, which is lower than the values detected in the sampling campaign performed by Devesa-Rey et al. (2008a), falling within the range 33-264 mg kg^{-1}. The As concentration slightly exceeded the Effects Range-Low (ERL) fixed by Long et al. (1995) at 8.2 mg kg^{-1}, which represents the upper end of a range of concentrations at which effects rarely would be observed.

The river water exhibited a neutral pH (7.0), low alkalinity (23.3 mg L^{-1}) and low concentrations of P (0.05 mg L^{-1}), nitrate (1.59 mg L^{-1} as NO_3^--N) and ammonia (0.09 mg L^{-1} as NH_4^+-N). These parameters classify the river water as high ecological status as they are below the limits of this category for Spanish rivers of the Atlantic and Cantabrian watersheds, fixed at 0.07, 2.26 and 0.16 mg L^{-1} for P, nitrate (as NO_3^--N) and ammonia (as NH_4^+-N), respectively (BOE 2015). Arsenic concentration in the river water was 3.6 µg L^{-1} and was mainly in the form As^V (92%), the remainder being As^{III}. This As concentration was in the range of those previously detected in the Anllóns River freshwater by Costas et al. (2011) (0.16-3.96 µg L^{-1}) and lower than the recommended permissible As level in drinking water set at 10 µg L^{-1} by the World Health Organization (WHO 1993).

Table C5.1. Physico-chemical properties of river water and bed sediments.

River waters	**pH**	7.0	**Na^+ (mg L^{-1})**	21.5
	TA[a] (mg $CaCO_3$ L^{-1})	23.3	**K^+ (mg L^{-1})**	2.0
	EC (µS cm^{-1})	101.4	**Ca^{2+} (mg L^{-1})**	6.3
	Eh (mV)	368.4	**Mg^{2+} (mg L^{-1})**	3.4
	DOC[c] (mg L^{-1})	2.4	**Fe (µg L^{-1})**	13.8
	N_t[b] (mg L^{-1})	2.1	**Mn (µg L^{-1})**	8.1
	NO_3^--N (mg L^{-1})	1.59	**Total As (µg L^{-1})**	3.6
	NH_4^+-N (mg L^{-1})	0.09	**As^V (µg L^{-1})**	3.3
	P (mg L^{-1})	0.05	**As^{III} (µg L^{-1})**	0.3
Bed sediments	**pH**	6.3	**Eh (mV)**	235
	Particle size (%)			
	Clay	5.0		
	Silt	6.1		
	Sand	88.9		
	X[d] (kg H_2O/ kg d.s.)	0.31	**Ti (%)**	2.1
	P_T (mg kg^{-1})	370.0	**Mg (%)**	1.5
	C (%)	0.89	**Ca (%)**	1.3
	N (%)	0.025	**K (%)**	1.1
	C/N	35.4	**Mn (ppm)**	1186
	S (%)	0.013	**Cr (ppm)**	180
	Si (%)	29.1	**Cl (ppm)**	157
	Al (%)	5.5	**Zn (ppm)**	79
	Fe (%)	5.5	**As (ppm)**	15

a) TA: Total Alkalinity b) N_t: Total Nitrogen c) DOC: Dissolved Organic Carbon d) X: Moisture Content

3.2. Arsenic retention

The As concentration in the overlying water decreased during the course of the experiment, to a nearly constant final value. The removal of As was higher in the presence of the biofilm (Fig. C5.2). After 14 days exposure, As retention in the BAS system was 91% ($\{As\}_{max}$=32.5 µmol kg^{-1}) of the initial concentration in water (500 µg L^{-1}), but only reached 70% in CAS ($\{As\}_{max}$=27.8 µmol kg^{-1}). An empirical exponential equation given by Eq. 2 satisfactorily fitted the experimental data (R^2>0.94).

$$[As] = a + b\, e^{(-t/c)} \qquad \text{Eq. 2.}$$

where [As] is the As concentration (µg L^{-1}) at time t (d) and a, b and c are the adjustment parameters. The equilibrium aqueous As concentration in BAS, defined by the "a" parameter of the fitting, was 46.71 µg L^{-1}, which is 3.6 times lower than in the system without biofilm (CAS) (167.4 µg L^{-1}). These results are in agreement with those obtained by Prieto et al. (2013) (Chapter 4 of this document), who studied As removal by biofilms in batch experiments and reported an increase of 18% in the average As retention for systems incorporating biofilms in comparison with sediments without biofilm.

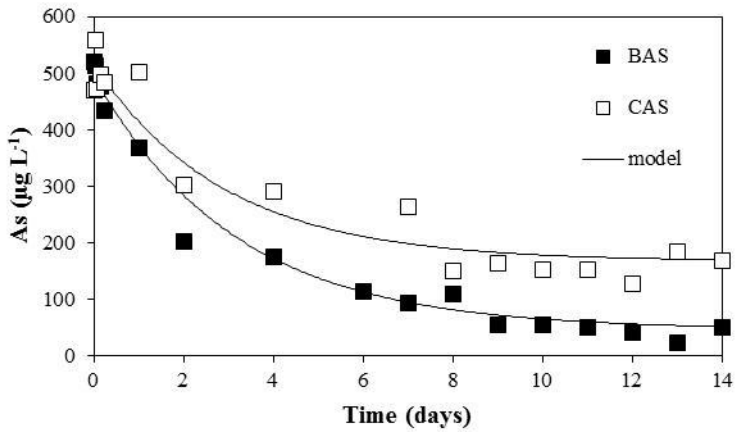

Figure C5.2. Time evolution of As concentrations in the overlying water in BAS and CAS systems, with and without biofilm, respectively. BAS and CAS represent systems with and without biofilms, respectively.

In the environmental conditions of this study, a significant reduction of the As concentration has been achieved in the presence of the biofilm, which highlights its importance in natural aquatic systems and supports their potential application in biotechnological systems for water purification. Thus, the final concentrations in the BAS and CAS systems were lower than USEPA's Aquatic Life Criteria Maximum Concentration (CMC), fixed at 340 µg L^{-1} in surface waters (USEPA 2014), but only the BAS value was lower than the Criteria Continuous Concentration (CCC), fixed at 150 µg L^{-1}. CMC and CCC are the highest concentrations in surface waters to which an aquatic community can be exposed briefly or indefinitely, respectively, without resulting in an unacceptable effect. The BAS final concentration was also lower than the permissible limit for irrigation water (100 µg L^{-1}) (FAO 1985), although slightly higher than the Environmental Quality Standards (EQS) for As in inland surface waters, set at 25 µg L^{-1} by the Priority Substances Directive in

Surface Waters (S.I. No. 272/2009-European Communities Environmental Objectives (Surface Waters) Regulations 2009). This EQS represents a threshold for annual average concentration of As in surface waters to ensure protection against long-term exposure to pollutants in an aquatic environment.

The epipsammic biofilms not only increased the amount of retained As but also the retention rate. Thus, BAS exhibited higher initial retention rates (3.84 µmol As kg^{-1} d^{-1} at 7 d) than CAS (2.97 µmol As kg^{-1} d^{-1} at 7 d) up to 7 d, after which the values were similar.

The presence of equimolar P concentrations in the BASP system slightly enhanced the amount and rate of As removal, reaching 97 % of its initial concentration ($\{As\}_{max}$=36.3 µmol kg^{-1}). The effect was lower in the absence of the biofilm (CASP), where As removal only reached 69 % ($\{As\}_{max}$=26.1 µmol kg^{-1}) (Fig. C5.3). In terms of water quality, the equilibrium As concentration in BASP, determined according to the exponential equation, was 18.85 µg L^{-1}, which is lower than the CMC and CCC values, and also that the FAO permissible limit for irrigation water, but still higher than EQS. The equilibrium As concentration in CASP was the same as for CAS (167.4 µg L^{-1}). With respect to the retention rates, BASP also exhibited higher values (4.92 µmol As kg^{-1} d^{-1}) than CASP (3.28 µmol As kg^{-1} d^{-1}) up to 7 d, after which the values were similar for both systems.

The soluble P decreased from 50 to 12 µg L^{-1} in BAS, and from 220 to 23 µg L^{-1} in BASP to which P was added (Fig. C5.4). This reduction in P concentration was attributed to its consumption by the microorganisms composing the biofilm. In CAS, the soluble P concentration remained almost constant between 100 and 150 µg L^{-1} throughout the experiment, while in CASP it decreased in the first 3 days from an initial concentration of 312 µg L^{-1} to 100-150 µg L^{-1} which can be considered the equilibrium concentration in both systems.

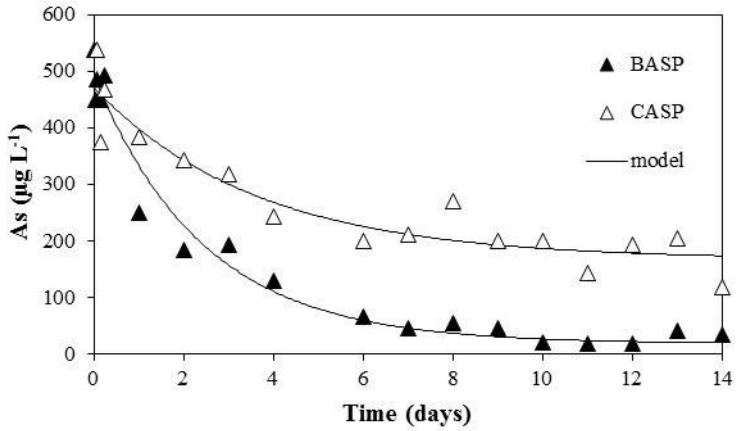

Figure C5.3. Time evolution of As concentrations in the overlying water in BASP and CASP systems. BASP and CASP represent systems with and without biofilms, respectively, with the incorporation of equimolar As:P concentrations.

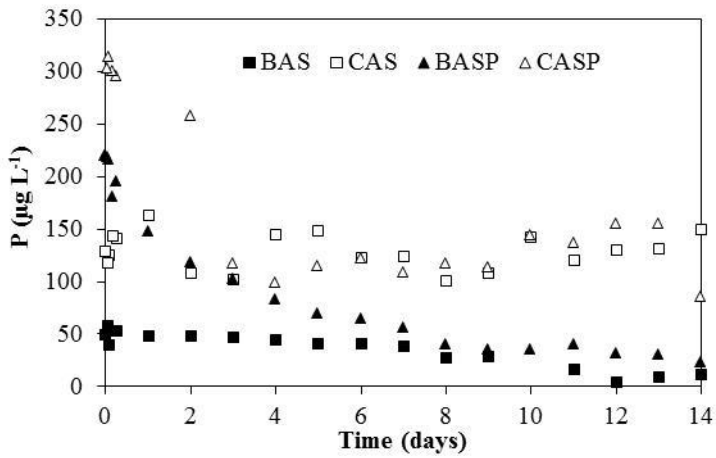

Figure C5.4. Time evolution of P concentrations in the overlying water in BAS, CAS, BASP and CASP systems.

3.3. Arsenic speciation

Five soluble As species were detected in the overlying water in the systems incorporating biofilm (BAS) exposed to the As-enriched solution. During the two weeks of the experiment, their concentrations followed the order: As^{V} >>As^{III}> MMA^{V}≈ DMA^{V} > AsB. Arsenic in

solution was mostly (~98%) As^{V}, whereas As^{III} only reached 1.2% of total As, and MMA^{V} and DMA^{V} only represented 0.6 and 0.7% of total As, respectively, indicating that there was a slight (bio-)methylation by the epipsammic biofilms during the incubation period. In the absence of the biofilm (CAS), the speciation of As in the water column changed significantly. In this system, As^{III} represented up to 39% (80 µg/L) of the final As in solution (Fig. C5.5a), the remainder being As^{V}, and no methylated forms were present.

The concentration of As^{III} in the systems incorporating phosphate (BASP and CASP) was similar to that observed for the systems without phosphate (Fig. C5.5b), and methylated aqueous species were, also in this case, only detected in the presence of biofilm (Fig. C5.6a, b), indicating that some biomethylation occurred in the presence of the biofilm, even during the short time of the study.

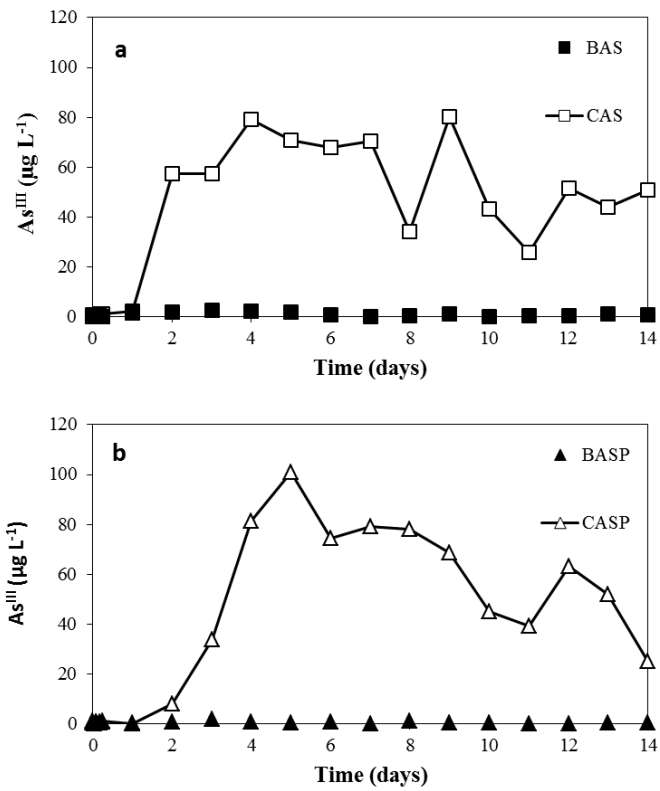

Figure C5.5. Time evolution of As^{III} concentrations in the overlying water in BAS and CAS systems (a) and in BASP and CASP systems (b).

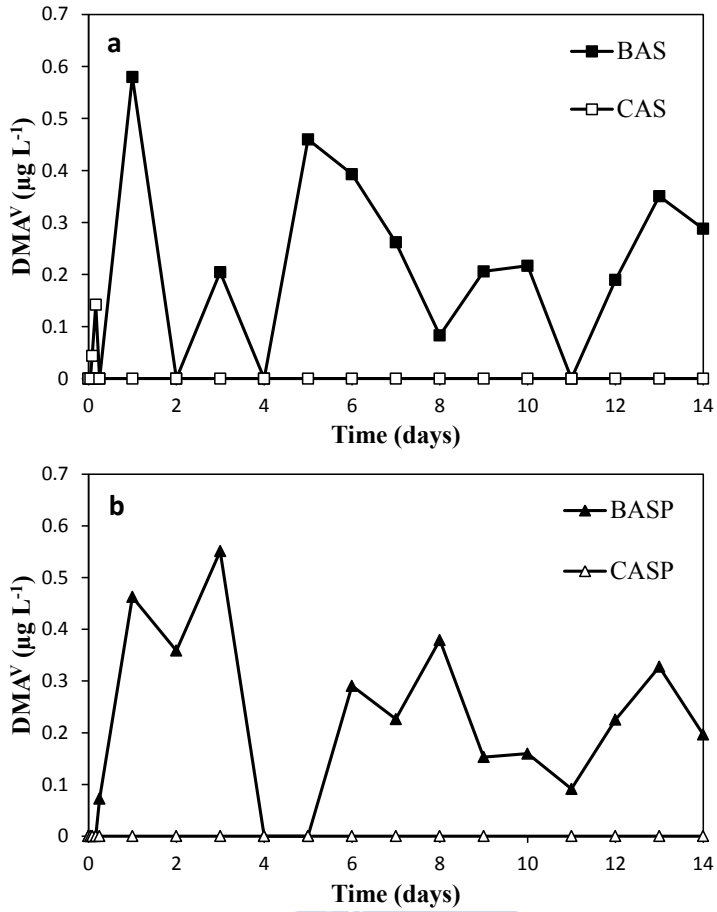

Figure C5.6. Time evolution of DMAV concentrations in the overlying water in BAS and CAS systems (a) and in BASP and CASP systems (b).

In control systems CAS and CASP, As^{III} was detected in the overlying water after 48 h exposure, reaching similar concentrations in both systems. To give explanation to this fact, physico-chemical water conditions were analyzed, as well as the behaviour of compounds susceptible to promote As^V reduction. The pH and Eh values in the overlying water at the end of the experiment indicated that both sediment-biofilm and control systems were under near neutral and oxic conditions (Table C5.2). There was no evidence of sulphur oxidation, as sulphate concentrations were similar at the end of the experiment for all the systems and similar to the initial concentrations in the river water. Also, Fe concentrations were similar throughout the experiment in all systems (Fig. C5.7a and b).

Table C5.2. Values of pH, Eh and sulphates at the end of the experiment.

	pH	Eh (mV)	SO_4^{2-} (mg L^{-1})
BAS	6.3	543	14.6
BASP	6.1	565	33.9
CAS	7.1	501	25.0
CASP	6.9	491	28.6

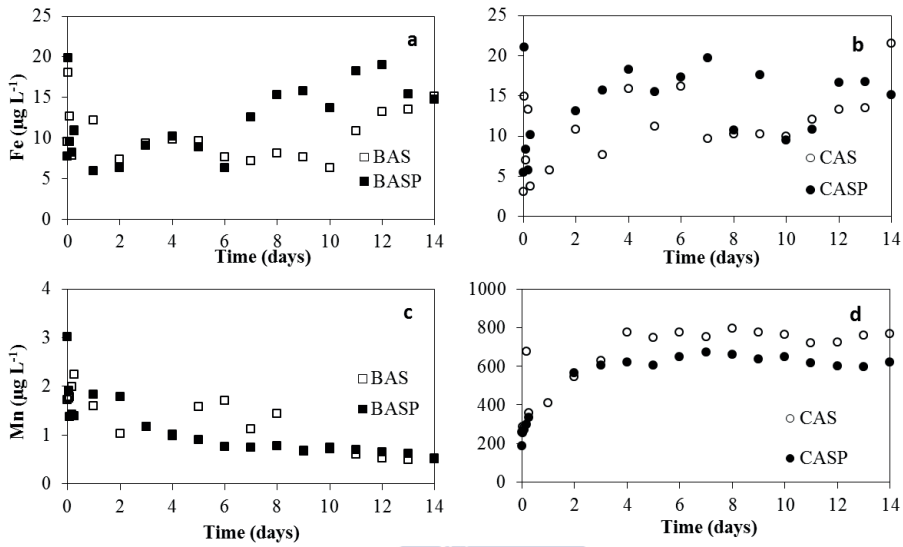

Figure C5.7. Time evolution of Fe and Mn total concentrations in the overlying water in BAS and BASP (a and c, respectively) and for CAS and CASP (b and d, respectively).

It is interesting to note that, in the control systems, Mn concentrations increased up to day 4 and then remained constant at values much higher (up to 1,500-fold) than those observed in the systems with the biofilm, where Mn concentrations decreased over time (Fig. C5.7c and d). Similarly, higher DOC concentrations were measured in CAS (mean value 55.3±4. mg L^{-1}) than in BAS (mean value of 14.0±3.8 mg L^{-1}). These results suggest that oxidation-reduction processes are occurring in the systems without biofilm, which will be explained in the general discussion. Biofilms seem to inhibit the reduction of added As^V, by covering the mineral surfaces of the sediments and thus hindering the interaction of soluble As with sediment

components susceptible to promote As reduction, and also maintaining an oxygenated biofilm-sediment interface which favours the oxidized forms.

3.4. Arsenic in interstitial waters

Similarly to what occurred in the overlying water, As concentrations in interstitial waters were notably higher (at least 9 times) in the CAS and CASP systems, with a higher percentage of As^{III}, than in the BAS and BASP systems, where detectable concentrations of DMA^{V} were also found (Table C5.3).

Table C5.3. Total As and As species concentrations of interstitials waters at the end of the experiment.

	As^{V} (µg L^{-1})	As^{III} (µg L^{-1})	MMA^{V} (µg L^{-1})	DMA^{V} (µg L^{-1})	AsB (µg L^{-1})	As^{T} (µg L^{-1})
BAS	27.50	0.64	-	0.10	-	28.24
BASP	6.70	0.99	-	0.06	-	7.75
CAS	188.06	68.08	-	-	-	256.14
CASP	187.88	34.68	-	-	-	222.56

3.5. Volatilized arsenic

Volatile As forms were only detected in systems incorporating biofilms. This fact, together with the detection of methylated As species, suggests the occurrence of detoxification processes. It has been reported that some microorganisms (bacteria, fungi and algae) form arsine in order to decrease intracellular As, as a (bio-)transformation pathway to cope with As toxicity (Wang et al. 2014). The mean amount of volatilized As after 14 d of exposure was 17.0 ng and the volatilization rate was 2.4 ng As mg^{-1} added As^{V} day^{-1}. This value was higher than those calculated from data (0.28-0.54 ng mg^{-1} d^{-1}) reported by Yin et al. (2011a) for *Microcystis*, *Nostoc* and *Synechocystis* cyanobacteria, treated with either 7.5 or 30 mg L^{-1} As^{V} for 6 weeks. They are also within the range (1.3-9.3 ng mg^{-1} d^{-1}) of those reported by Yin et al. (2011b) for As biotransformation by the protozoan *Tetrahymena thermophile* after 48 h and 72 h of exposure to 150-3000 µg L^{-1} As^{V} concentrations. With respect to the As initial concentration, the maximum percentage of volatilized As was only 0.002 %. This value was slightly lower than the percentage (<0.1 %) calculated by Yin et al. (2011a) and those

(0.006-1%) reported by Yin et al. (2011b). In summary, the results suggest that epipsammic biofilms carried out detoxification processes leading to the formation of volatile As, although its contribution was of minor relevance in relation to the overall As balance.

3.6. Remobilization and bioavailability of arsenic

In regards to As remobilization, the release to water of the previously retained As was low and varied between 113.0 ± 9.4, 166.8 ± 10.0, 181.1 ± 9.1 and 176.5 ± 7.9 µg kg^{-1} for BAS, CAS, BASP and CASP, respectively, which represent a 4.7, 6.1, 8.7 and 9.0 % of the retained As, respectively.

In the presence of biofilm, 94 % of the released As was As^{V} and only 3% was As^{III}. Conversely, As^{III} accounted for 21 % of the released As in the systems without biofilm, and was 10 times higher than in the systems with biofilm. MMA^{V} was only detected (3.3 % of total As released) in the extracts from the systems with the biofilm, while DMA^{V} was not found in any case.

To estimate As bioavailability, the concentrations of As species were determined in the eluates from DGT devices and are presented in Table C5.4, jointly with the parameters calculated from these concentrations. Once again, the values obtained were similar for the different systems. The speciation of the DGT extracts revealed that the percentage of As^{V} was higher than 77 % in all cases, and DMA^{V} was only detected in the BAS samples.

Table C5.4. Total As and As species concentrations in the extracts of DGT and DGT parameters

	As^{V} (µg L^{-1})	As^{III} (µg L^{-1})	MMA^{V} (µg L^{-1})	DMA^{V} (µg L^{-1})	AsB (µg L^{-1})	M[a] (ng)	C_{DGT}[b] (µg L^{-1})	F[c] (ng m^{-2} s^{-1})
BAS	11.56	2.58	-	0.77	-	17.30	0.96	0.64
BASP	9.74	0.52	-	-	0.20	12.51	0.71	0.46
CAS	12.27	1.04	-	-	0.24	16.10	0.93	0.59
CASP	14.01	3.08	-	-	-	19.83	1.11	0.73

a) M: mass of As in the resin gel b) C_{DGT}: time-averaged DGT concentrations c) F: flux of As measured by DGT

3.7. Extracellular and intracellular arsenic

In biofilm-enriched samples taken from BAS and BASP systems, 71.1±1.5 % of the retained As was extractable in phosphate buffer, most of it as the oxidized species As^V (>99.6 %). This fraction represents an estimation of the easily-leachable As (Gleyzes et al. 2002) and can be attributed to extracellular As (Levy et al. 2005). DMA^V was also identified in phosphate extracts, revealing once again the occurrence of a (bio-)methylation process. In turn, intracellular As, extractable in methanol-H_2O solutions (Miyashita et al. 2009), represented 28.9±1.5% of the retained As and is almost exclusively in the form of As^V (>99.8 %).

3.8. General Discussion

In this study, the influence of epipsammic biofilms on the behaviour of As^V in freshwater environments has been evaluated at a microcosm scale. The results revealed that biofilms increase the amount and rate of As^V retention by the sediment. In chapter 4 (Prieto et al. 2013) it has been also observed, in batch experiments with short-term (24 h) As exposure, that epipsammic biofilms increased As^V sorption by sediments from the Anllóns River and that this effect was more noticeable in the presence of phosphate.

Sediments may retain As by sorption on Fe, Al and Mn (oxy)hydroxides (Oscarson et al. 1981b; Jiang et al. 2005), clay minerals (Manning and Goldberg 1997a) and organic matter (Thanabalasingam and Pickering 1986). The additional favourable effect of the biofilm on As removal may be attributed to the combined effects of (bio-)accumulation and (bio-)sorption. With respect to As (bio-)accumulation, it takes place when As^V enters the cells via phosphate transporters (Páez-Espino et al. 2009). As regards As (bio-)sorption, it may be improved by the biofilm as it increases the specific surface area, the number of sorption sites and consequently the number of potential As sinks. It has been observed that sorption of metals by biofilms is governed by their interactions with the biofilm matrix, constituted by cells and extracellular polymeric substances (EPS), containing ionisable functional groups which can sequester toxic compounds (van Hullebusch et al. 2003). Moreover, Fe and Mn oxides precipitated as biominerals in biofilms have been identified as responsible for the retention of trace elements (Dong et al. 2000; Warren and Haack 2001). Drahota et al. (2014) reported the enhancement of As retention by the precipitation of poorly crystalline biogenic Mn oxides, in

turn induced by the growth and accumulation of biofilms. In our study, the extracellular fraction which corresponds to As retained by biosorption represents the main compartment of the retained As in the systems with the biofilm.

The addition of equimolar phosphate concentrations had no effect on As adsorption in the control system CASP. Although phosphate usually competes with arsenate for sorption sites in soils and sediments due to their chemical similarities (Manning and Goldberg 1996; Hongshao and Stanforth 2001), there was not competition between As and P in this system, and the reason may be that phosphate rapidly decreased in solution (63 % in the first 3 days) while As concentrations did not decrease so abruptly and the retention continued up to day 10. The As retained in control systems (2.0 μg g^{-1}) was only slightly lower than the maximum adsorption capacity estimated with the Langmuir model (Prieto et al. 2013-chapter 4) for sediments from the same site (6.6 μg g^{-1}), which could explain the high As concentration that remained in the overlying water of CAS and CASP control systems.

The presence of phosphate had a slight positive effect on the retention of As by the epipsammic biofilm. This effect could either be due to the stimulative effect of phosphate on the growth of the microorganisms, which is proved by an almost complete absorption of phosphate by the biofilm, which is supposed to contribute to its growth. Other possible explanations are the increase in the efficiency and/or number of phosphate/arsenate cellular transporters or even the alleviative effect of phosphate against As^V toxicity, as was observed by Karadjova et al. (2008) for the green microalga *Chlorella salina* in seawater, by Wang et al. (2013) for two freshwater green algae, and by Rubinos et al. (2014) for *Aliivibrio fischeri* bacteria.

The biofilms inhibited the reduction of added As^V. Several key factors are reported in the literature that control As speciation, such as pH and Eh (Smedley and Kinniburgh 2002), the presence of redox pairs, such as Fe^{3+}/Fe^{2+}, Mn^{4+}/Mn^{2+}, SO_4^{2-}/HS^- (Cherry et al. 1979) and the effect of natural organic matter (NOM) (Sharma and Sohn 2009). Among all the conditions evaluated, DOC and Mn concentrations were notably higher in the systems devoid of the biofilm. Lower DOC concentrations in BAS and BASP could be attributed to inhibition of DOC release from the sediment due to the presence of biofilm, or to DOC consumption by biofilm heterotrophs. This fact is relevant because an important role is attributed to natural organic matter (NOM) in As geochemistry (Buschmann et al. 2006; Klitzke and Lang 2009).

Thus, it has recently been demonstrated that the addition of As to DOM solutions may result in arsenate reduction (Redman et al. 2002). In turn, the release of Mn^{2+} is attributed to the reduction and dissolution of the Mn oxyhydroxides in the sediment. Therefore, the results of this study indicate that dissolution and reduction processes occurred in CAS and CASP, involving As^{V} reduction and Mn and NOM,mobilization, which were inhibited in BAS and BASP systems. These effects may be due to the covering of the mineral surfaces of the sediments, preventing their interaction with aqueous As, and/or to the maintenance of a more oxygenated interface due to photosynthesis, hence avoiding reductive processes. The inhibition of As^{V} reduction by the biofilms has relevant geochemical and toxicological implications, since As^{III} is usually considered more mobile and toxic than As^{V} (Sharma and Sohn 2009; Huang 2014).

Arsenic methylation has been demonstrated in different aerobic and anaerobic microorganisms (Kuehnelt and Goessler 2003). The presence of methylated As species in the overlying water, in the interstitial waters and within the biofilm in BAS and BASP are indicative that (bio-)methylation processes are taking place. Among the methylated As compounds, DMA^{V} was the main species detected in the overlying and interstitial waters, while MMA^{V} was detected at lower concentrations. This fact could be explained because MMA^{V} is an intermediate in the As methylation process, with a rapid intracellular metabolism and ten times lower permeability to the membranes than DMA^{V} (Cullen et al. 1994a, b, c). Higher DMA^{V} concentrations compared to MMA^{V} have also been found in natural environments such as in a German forested catchment by Huang and Matzner (2007), in eutrophic and mesotrophic lakes by Hasegawa et al. (2009) and in marine sediments by Fauser et al. (2013).

In summary, epipsammic biofilms play a key role in the biogeochemistry of As in river environments, by enhancing the removal of As^{V} from water and affecting As speciation. Biofilms inhibit the occurrence of aqueous As^{III} in the water column and carry out detoxification processes inside the cells via the production of methylated and volatilized species.

4. Conclusions

Epipsammic biofilms covering riverbed sediments enhance the removal of As^{V} from the water column and strongly affect the speciation of As in solution, by inhibiting the occurrence of aqueous As^{III}, which has noteworthy toxicological and geochemical relevance and for remediation purposes, considering the usually greater toxicity and mobility of As^{III} species. The detoxification of As driven by epipsammic biofilms is supported by the presence of methylated arsenic species such as MMA^{V} and DMA^{V} in the overlying waters and within the biofilms, as well by the detection of volatilized arsenic. The enhancement of the As retention and potential toxicity of its toxicity in the presence of biofilms highlights their importance in natural aquatic systems and their potential application in biotechnological water purification systems.

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Chapter 6: Influence of epipsammic biofilms on arsenic transfer from As-rich sediments to water systems

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Abstract

The influence of epipsammic biofilm on arsenic (As) release from contaminated river sediments was evaluated in a microcosm experiment where biofilms were grown on sediments containing 106 mg kg^{-1} As, collected in the Anllóns River, and compared with control systems without biofilm. The As transfer to the water column was low (< 0.11 % of total As in the sediment) and was further reduced by 64 % in the presence of biofilm. As^{V} was the predominant species in the overlying water in both systems. As^{III} concentration was higher (up to 12 % of total dissolved As) in the control systems than in the systems with biofilm, where this species was almost absent. This fact is of toxicological relevance due to the usually higher mobility and toxicity of the reduced As^{III} species. Control systems exhibited higher As mobility in water, in sulphate solution and in weak acid medium, as well as higher bioavalability in diffusive gradients in thin films (DGT) devices. Arsenic retained by the biofilm was equally distributed between extracellular and intracellular compartments. Inside the cells significant concentrations of As^{III}, monomethyl-arsenate (MMA^{V}) and dimethyl-arsenate (DMA^{V}) were detected, suggesting that active methylation (detoxification) processes are occurring in the intracellular compartment.

1. Introduction

Arsenic (As) is a toxic element widely distributed in aquatic environments. Its presence is often attributed to lithogenic origin exacerbated by human activities (Smedley and Kinninburg 2002). Arsenic pollution has a negative impact on water quality, constituting a risk for the environment and human health when it is incorporated to water or food (Fendorf et al. 2010). Arsenic mobilization from soils and sediments into the aqueous phase may be caused by chemical and biological processes which can be classified into four categories: (a) ion displacement, (b) desorption (or limited sorption) at pH values > 8.5, (c) reduction of arsenate to arsenite, and (d) mineral dissolution, particularly reductive dissolution of Fe and Mn (hydr)oxides (Fendorf et al. 2008). Microorganisms play also a key role in As transfer from sediments, mainly in the reduction of As^{V} to As^{III} and in the dissolution of Fe and Mn oxides acting as arsenic carriers.

In the Anllóns River basin, high As concentrations of natural lithogenic origin have been detected in rocks and soils. Gold mining activities, carried out through history, resulted in the removal of As associated with Au mineralizations and its accumulation in sediments in the lower reaches of the river (Devesa-Rey et al. 2008a; Costas et al. 2011). Arsenic concentrations in these sediments reached up to 264 mg kg^{-1} (Devesa-Rey et al. 2008a), which exceed up to 5 times the reference levels of Galician soils defined at 50 mg kg^{-1} (Macías Vázquez and Calvo de Anta 2009). Arsenic in the sediments is mainly present as low solubility forms (Rubinos et al. 2011), mainly bound to Fe oxides and in the residual phase. Despite this low mobility, it has been shown that As solubility in these sediments increase in conditions of high salinity, extreme pH or high P concentrations, as well as during high-flow resuspension events (Rubinos et al. 2010; 2011).

Because microorganisms play an important role in As geochemistry, their influence has to be explored in the fluvial ecosystems. In fact, multi-species communities forming biofilms are ubiquitous over the wet surfaces of plants, rocks or sediments. Biofilms are complex systems of microorganisms, mainly constituted by algae, bacteria, fungi and protozoa, embedded in a polymeric matrix. They are crucial in aquatic ecosystems because they are involved in primary production, carbon and nutrient cycling, retention of inorganic and organic nutrients, and support of food webs (Mora-Gómez et al. 2016). Epipsammic biofilms growing over granular surface sediments have been identified in the Anllóns River, and were

deeply characterized in recent field and lab studies (Martiñá Prieto et al. 2016-chapter 2; Prieto et al. 2016-chapter 3). The main taxa identified in the Anllóns River belonged to Chlorophyta, Cyanophyta, Euglenophyta and Heterokontophyta. The most abundant class was diatoms (Bacillariophyceae), which represent >86 % of the total abundances in the superficial sediments, and specifically, the most abundant genus was *Navicula*.

Previous studies have demonstrated that, despite the apparent low As mobility of As in the Anllóns sediments, it could be mobilized by changes in the environmental conditions (Rubinos et al. 2010; 2011). Also, the role of epipsammic biofilm in the biogeochemistry of As has been studied for sediments of the Anllóns River, observing that the biofilms increased the sorption capacity of As, an effect which is enhanced by the presence of phosphate (chapter 4-Prieto et al. 2013), and inhibited the reduction of the aqueous As^{III} (chapter 5). Therefore, at this point, it is necessary to elucidate the effect of the epipsammic biofilms on As transfer from As-rich sediments to the river water, an aspect that, to our knowledge, has not been previously investigated. This objective is addressed in this work, with the aim of improving the knowledge of the role of epipsammic biofilm on As cycling in the riverine ecosystem and, more precisely, to investigate its role on As mobilization from As-rich sediments to the aqueous phase. With this purpose, a complete study was carried out at a microcosm scale, where epipsammic biofilm were grown on natural As-rich sediments, and As leaching and speciation was studied in comparison with sterilized sediments. Particular objectives were:

1) To evaluate the effect of biofilm on As release from contaminated sediments, determining the changes in the concentration and speciation of aqueous As released to the water column, and establishing the kinetics of the leaching process.

2) To determine the distribution of the As retained by the biofilm, among the cells and the EPS matrix.

3) To measure the volatilization of As and quantify its importance during the incubation experiment.

4) To evaluate the leaching and bioavailability of As at the end of the incubation experiment in biofilm-rich sediments in comparison with sediment devoid of biofilm.

2. Materials and Methods

2.1. Sampling site

The river water sample and sediment samples used in this study were collected in the Anllóns River (Galicia, NW Spain), at a point known as Xavarido, just downstream an area where gold mining operations have been performed throughout history and where high As concentrations have been previously detected in the riverbed sediments (Devesa-Rey et al. 2008a). The coordinates of the sampling point were: Latitude 43º 13' 48.82'' N. Longitude 8º 49' 54.29'' W. The geological substrate of the sampling site mainly consists of alkaline gneiss.

2.2. Sediment and river water sampling

The experimental procedure outlined in Figure C6.1 was followed. A complex sample of sediment was collected with a small plastic shovel from the top 5 cm at various points at this site and taken to the laboratory in hermetic plastic containers topped up to prevent oxidation. Sediment < 2 mm was air dried before analysis. Total organic carbon, nitrogen and sulphur content were determined in a LECO TruSpec CHNS analyzer. Arsenic concentration and other major and trace constituents were determined by X-ray fluorescence spectrometry.

The river water was collected at the same site and transported cooled to the laboratory. Once in the lab, water was filtered by 0.45 μm to be employed as biofilm growth medium in order to better reproduce the natural conditions for biofilm development. Water analysis was conducted using the following methods: pH and electrical conductivity (EC) were measured using a Hamilton electrode and a Metrohm 712 conductivity meter, respectively, coupled to a Metrohm Titrando 808. Alkalinity was measured by colorimetric determination using a AQUAKEM 250 Analyzer (Labmedics). Cations were measured by ICP-MS whereas anions were measured using a 850 Professional IC ion chromatograph (Metrohm).Total P was measured using a ICP-MS, and Total N was determined by segmented flow analysis and colorimetry with Futura console (AMS Alliance) after filtration through a 0.45 μm-membrane Millex-HM (Millipore). Dissolved organic carbon (DOC) was measured using a Total Organic Carbon Analyzer Model TOC-V CSN (Shimadzu, Kyoto). With this equipment,

DOC concentration is obtained by subtracting the inorganic carbon (IC) concentration from the total carbon (TC) concentration. TC is determined by the 680 °C combustion catalytic oxidation method, whereas IC is determined by acidification and sparging. The CO_2 generated in both determinations is detected using a non-dispersive infrared gas analyzer (NDIR).

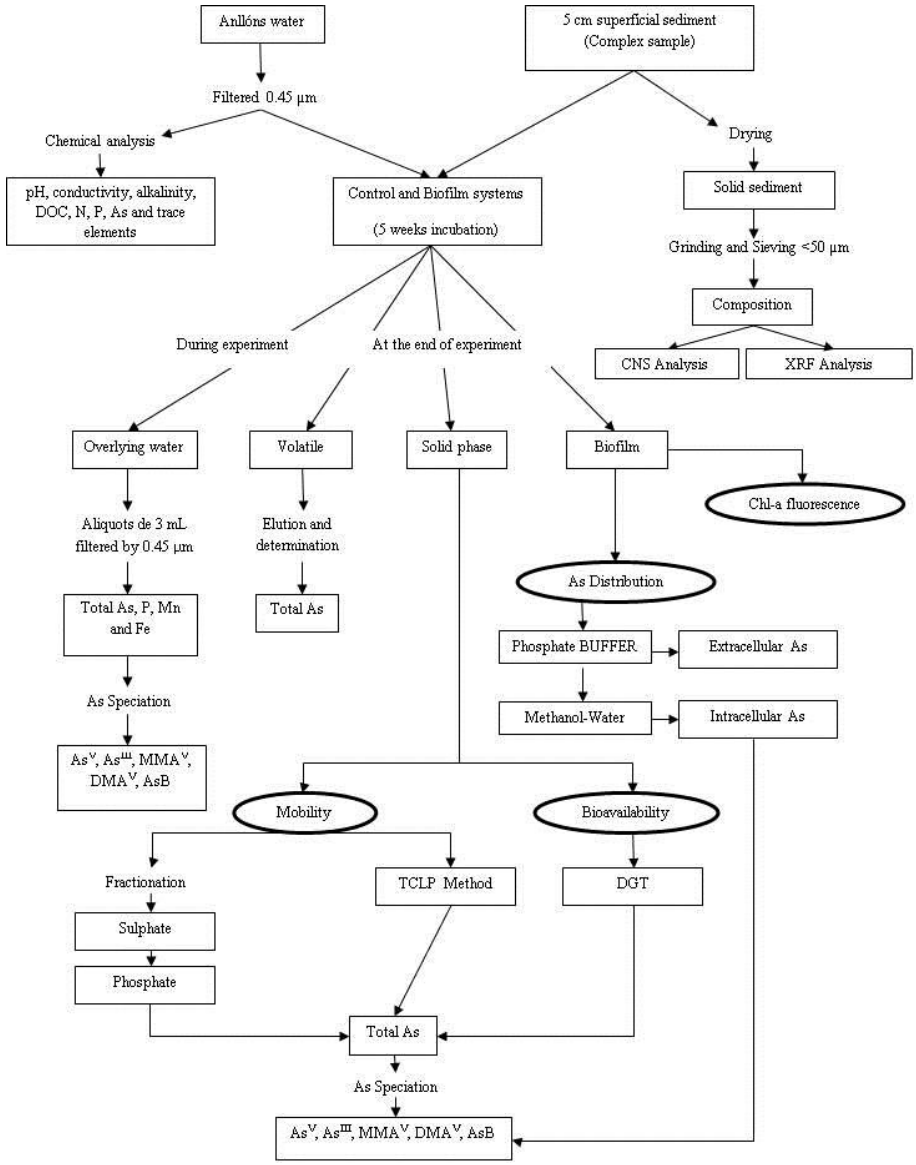

Figure C6.1. Experimental procedure.

2.3. Effect of biofilm on As transfer from sediments to water

Arsenic transfer from sediment to water was evaluated in laboratory experiments conducted at microcosm level. Sediment samples (125 g, 20 % water content) were incubated in Erlenmeyer flasks (two replicates per sample) filled with 500 mL of filtered (<0.45 µm) river water. The flasks were equipped with systems for air-supply, sample collection and trapping of volatilized arsenic. All the materials were previously sterilized by autoclaving at 120 ºC for 30 min. The biofilm was grown in an incubation chamber under optimal controlled conditions of light (day-night cycles, 12 h of light with intensity ca. 40 µmol photon m^{-2} s^{-1}), temperature (20 ºC) and air-supply (ca. 1 L min^{-1}). Control systems were prepared similarly to those explained above, but in this case sediment samples were previously submitted to 3 cycles of autoclaving at 120 ºC for 30 min to avoid microbial activity and growth. Hereafter the control and biofilm systems are referred to as C and B systems, respectively.

The systems were maintained under these conditions for 5 weeks, during which aliquots (3 mL) of the water column were sampled daily using single use sterile PP syringes (Braun Inkjet, B Braun AG, Melsungen). The samples were immediately filtered (sterile 0.45-µm Whatman Puradisc 25AS™ syringe filters, GE Healthcare Europe GmbH, Barcelona) and stored frozen (−80 ºC) until analysis of total As by ICP-MS and of As species (arsenite -As^{III}-, arsenate-As^{V}-, monomethyl-arsenate -MMA^{V}-, dimethyl-arsenate -DMA^{V}-, arsenobetaine – AsB-) by HPLC-ICP-MS. Additionally, the dissolved P, Fe and Mn concentrations were determined by ICP-MS. At the end of this experiment the volatilized As and the potential leaching and bioavailability of As have been evaluated.

2.3.1. Arsenic volatilization

To quantify the As volatilized during the experiment, arsines (the volatile As species) were trapped using the $AgNO_3$ based chemo-trapping approach described by Mestrot et al. (2009) and Yin et al. (2011a). In this method, the arsine (AsH_3), monomethyl arsine ($MeAsH_2$), dimethyl arsine (Me_2AsH) and trimethyl arsine ($TMAsorMe_3As$) react with $AgNO_3$ and are preserved by oxidation to their pentavalent oxy-species (As^{V}, MMA^{V}, DMA^{V} and trimethylarsine oxide (TMAO), respectively) (Mestrot et al. 2009).

To prepare the traps, silica gel (2.5-5 mm) was submersed in 5 % (w/v) HNO_3 solution overnight and washed with Milli-Q water (18.2 MΩ.cm^{-1} resistivity), then impregnated with

10 % (w/v) $AgNO_3$ solution and placed overnight in an oven at 70 ºC (covered with aluminum foil to avoid the photodecomposition of $AgNO_3$) (Yin et al. 2011a). Subsequently, to prepare trap tubes, silica gel (~1 g) was loaded into a 10 mL sterilized syringe and held at both ends with a small quantity of washed QP glass wool (Panreac, Barcelona). Trap tubes were again covered with aluminum foil to avoid photodecomposition of $AgNO_3$ and coupled to microcosm systems. At the end of the experiment, 5 mL of 1 % (v/v) hot boiling HNO_3 was employed to eluate the collected As in the trap tubes (Yin et al. 2011b). Eluates were filtered (0.45 µm) and stored frozen (-80 ºC) until total As was measured by ICP-MS.

2.3.2. Leachability and bioavailability of As

At the end of the experiment, the overlying water was removed and As mobility in the substrate was evaluated in three ways:

1) by washing 0.5 g solid sample (sediment or sediment + biofilm) with As-free filtered (0.45 µm) river water (1:10 solid:liquid ratio) (soluble As) (DIN 38414-S4 1984).

2) by applying the first two steps (exchangeable and specifically sorbed As) of the sequential extraction procedure described by Lombi et al. (1999) for As fractionation. 0.5 g samples (sediment or sediment + biofilm) were subjected to sequential extractions with 12.5 mL extractant (0.05 M $(NH_4)_2SO_4$ for F1 fraction and 0.05 M $NH_4H_2PO_4$ for F2 fraction) and shaken during 1 h. The extracts resulting in each phase were centrifuged at 5,000 rpm for 15 min.

3) by using the Toxicity Characteristic Leaching Procedure (TCLP) according to EPA Method 1311 (USEPA 1992) (As mobilized in a weak acid medium), consisting in a 24 h extraction in Milli-Q water at pH 4.5 adjusted with acetic acid, using a 1:20 soil:water ratio. After the extraction step, the suspensions were centrifuged at 2,000 rpm for 15 min.

The aqueous extracts from the determinations of soluble, exchangeable and specifically sorbed As and TCLP were filtered (0.45 µm) and stored frozen (-80 ºC) until analyzed for total As and As speciation by ICP-MS and HPLC-ICP-MS, respectively. All the experiments were carried out in triplicate and were done within the quality requirements.

To evaluate As bioavailability, DGT devices (DGT Research Ltd., Lancaster) incorporating Fe oxide gels were employed. These devices accumulate metals and metalloids

on a binding agent after passing a well-defined diffusive layer (Davison and Zhang 1994). DGT devices were placed onto the sediment surface during 24 h to afford an operationally defined measure of the As "bioavailable" fraction. After that exposure time, the devices were rinsed with Milli-Q water and the resin gels were removed and then eluted with 1 mL of 7.2 M (32.5 %) HNO_3 for 24 h to allow a complete extraction of As. The extracts were filtered (0.45 μm) and diluted 6 times with Milli-Q water prior to analysis by ICP-MS. The mass of As in the resin gel (M), the time-averaged DGT concentrations (C_{DGT}) and the flux (F) of As measured by DGT were calculated according to Zhang and Davison (1995) and DGT® technical documentation. The mean ($5.85\ 10^{-6}\ cm^2\ s^{-1}$) of the values found in the literature for the diffusion coefficient of As^V in the DGT® gel was used in the calculations of C_{DGT}.

2.4. Determinations in the biofilm

At the end of the experiment chorophyll-a fluorescence was determined to confirm the development of a mature biofilm, and the distribution of As in extracellular and intracellular compartments within the biofilm was evaluated.

2.4.1. Chlorophyll-a fluorescence measurements

The FIBER version of the Phyto-PAM fluorometer (Walz, Effeltrich, Germany) was used for the determination of the content of active chlorophyll in the surface of the sediments developing biofilm and to differentiate between pigmented groups of algae (green algae, diatoms and cyanobacteria). For this instrument, fluorescence is excited alternatingly at high repetition rates by μsec pulses of 470, 520, 645 and 665 nm light originating from light emitting diodes (LED) and is detected by an extremely sensitive miniature photomultiplier detector.

At the end of the experiment, in *vivo* chlorophyll-a fluorescence was determined. Biofilm samples were previously incubated for 20 minutes in dark conditions to ensure that all reaction centers were open, and then the photosynthetic activity was assessed using red actinic light and saturation pulses. As a result of this analysis, the minimal fluorescence yield (F_0) of a dark adapted cell, which is proportional to its chlorophyll-a concentration and can be used as an estimation of algal biomass (Corcoll et al. 2012), and the maximum PII quantum yield

(Y_{max}), calculated as $Y_{max}=(F_m-F_0)/F_m$ according to Schreiber et al. (Schreiber et al. 1986), which is defined as a measure of the photosynthetic capacity of the community (Corcoll et al. 2012), were obtained. All calculations were done using the fluorescence signal recorded at 665 nm and are given as relative units of fluorescence.

The relative abundance of each phototrophic group composing the biofilm was estimated from the fluorescence signals recorded at 470 (F_{01}), 520 (F_{02}), 645 (F_{03}) and 665 (F_0) nm. The ratio F_{01}/F_{03} was used as an indication of dominance of green algae (high values) *vs* dominance of cyanobacteria (low values) (Izaguirre et al. 2009).

2.4.2. Distribution of As within the biofilm

For the extraction of extracellular As the procedure of Levy et al. (2005) was followed. To this end, biofilm samples (0.5 g) were gently taken from 5 different points of the sediment and mixed in a Falcon 15 mL conical tube, rinsed with 10 mL of filtered river water (1:10 solid: liquid ratio) and allowed to stand for 20 min. Then, the solid phases were submitted to two washing cycles with 10 mL of 0.1M KH_2PO_4/K_2HPO_4 buffer solution (pH 5.95) to extract the extracellular As. The suspensions were shaken for 30 s and allowed to stand for 20 min, before they were centrifuged (3000 rpm, 15 min). The supernatants were filtered (0.45 µm syringe filters) and the extraction cycle was repeated again. The eluates of the two washes were combined and stored frozen (-80 ºC) until analyzed for total As in the extracellular fraction by ICP-MS. The remaining solid phases were gently washed with Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity), centrifuged (3000 rpm, 15 min) and stored frozen (-80 ºC) until further analysis of intracellular As. For this purpose the procedure of Miyashita et al. (2009) was followed. The solid phases of the previous steps were thawed and As was extracted with 10 mL of 1:1 (v:v) methanol:H_2O solution. After standing for 10 min, the suspensions were sonicated for 10 min and centrifuged at 3000 rpm for 15 min. The extraction was repeated twice with 5 mL of methanol/ H_2O solutions. The extracts were combined and evaporated using a rotavapor (Büchi Rotavapor R-200, BÜCHI Labortechnik GmbH, Essen). The dried extracts were redissolved with Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity), filtered (0.45 µm), and stored frozen (-80 ºC) until analyzed for total As and As species by ICP-MS and HPLC-ICP-MS, respectively. At the end of the sequential extractions, solid phases were dried at 105 ºC to constant weight to determine the dry weight of the analyzed samples.

2.5. Arsenic analysis

For the quantification of total As concentrations, a Varian 820-MS ICP-MS was employed, equipped with collision reaction interface (CRI) technology to reduce polyatomic interferences. The total concentration of P, Fe and Mn were also determined by ICP-MS. The detection limits for As, P, Fe and Mn were 3.4, 77.6, 31.0, 1.2 ng L^{-1}.

Speciation analysis of dissolved As was carried out by High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Spectrometry (HPLC-ICP-MS). With this purpose, a Varian Prostar 230 HPLC equipped with a guard column and an anion exchange column Hamilton PRP-X100 (4.1 x 250 mm and 10 μm) was employed. Separation of the five arsenic species was performed using a 13 minutes gradient LC method with 12.5 mM and 30 mM (pH 9) $(NH_4)_2CO_3$ as mobile phase, a flow rate of 1 mL min^{-1} and an injection volume of 50 μL. For quantification, the Varian 820-MS ICP-MS was employed. The detection limits under the experimental conditions were 2.8, 4.1, 2.9, 4.6 and 2.5 ng L^{-1} for As^{V}, As^{III}, MMA^{V}, DMA^{V} and AsB, respectively.

2.6. Statistical analysis

All the statistical analyses were performed using the SPSS 19 package (IBM SPSS, 2010). Two-Way Repeated Measures ANOVA was carried out with total As, P, Fe and Mn concentrations throughout the experiment. Time was the within-subject continuous variable, whereas the type of sample (Control, C, and Biofilm, B) was the between-subject variable. Finally, post hoc Bonferroni's tests was applied to check significant differences ($p<0.05$; $\alpha = 0.05$).

The Student's t-test was carried out to analyze the significant differences ($p<0.05$; $\alpha=0.05$) between control and biofilm systems, in extracellular and intracellular As concentrations, As extracted with sulfate, phosphate and TCLP, and As bioavailability by DGT, as well as arsenic speciation. As a first step, data were checked for normal distribution. Levene contrast was employed to evaluate the homogeneity or equality of variances.

3. Results and Discussion

3.1. Sediment and River water characterization

The main characteristics of the river water and the sediment from Xavarido site are shown in Table C6.1. The river water had pH of 7.45, EC of 145 µS cm^{-1} and DOC of 2.05 mg L^{-1}. Total P and nitrate (as NO_3^--N) concentrations were low (0.02 and 1.87 mg L^{-1}, respectively) and classified the Anllóns River water as of high ecological status as they were below the limits of this status for Spanish rivers of the Atlantic and Cantabrian watersheds, fixed at 0.07 and 2.26 mg L^{-1} for P and nitrate (as NO_3^--N), respectively (BOE 2015). P concentration was also lower than the maximum acceptable concentration to avoid accelerated eutrophication or to promote algal blooms, fixed at 0.1 mg L^{-1} (USEPA 1986). The total As concentration was also low (0.98 µg L^{-1}), in the range of those previously detected in freshwaters from the Anllóns River by Costas et al. (2011) (0.16-3.96 µg L^{-1}), and well below the maximum concentration level recommended by WHO for drinking water, fixed at 10 µg L^{-1} (WHO 1993).

Table C6.1. Characteristics of the Anllóns river water and sediment.

River water							
pH (25 °C)	**EC (25 °C) (µS cm^{-1})**	**Alkalinity (mg L^{-1})**	**TC (mg L^{-1})**	**IC (mg L^{-1})**	**DOC (mg L^{-1})**	**N_{total} (mg L^{-1})**	**NO_3^--N (mg L^{-1})**
7.45	145	27	7.76	5.71	2.05	2.28	1.87
NO_2^--N (mg L^{-1})	**P_{total} (µg L^{-1})**	**PO_4^{3-} mg L^{-1}**	**SO_4^{2-} (mg L^{-1})**	**F^- (mg L^{-1})**	**Cl^- (mg L^{-1})**	**Br^- (mg L^{-1})**	**Na (mg L^{-1})**
0.02	22.82	<0.04	8.63	<0.05	17.20	0.07	13.60
K (mg L^{-1})	**Ca (mg L^{-1})**	**Mg (mg L^{-1})**	**Al (µg L^{-1})**	**Fe (µg L^{-1})**	**Mn (µg L^{-1})**	**As (µg L^{-1})**	
1.12	6.90	4.20	14.70	7.16	0.04	0.98	
Sediment							
C (%)	**N (%)**	**C/N**	**S (%)**	**Mg (%)**	**Ca (%)**	**K (%)**	**Fe (%)**
1.57	0.13	12	0.03	0.70	1.20	2.10	3.90
Ti (%)	**Mn (ppm)**	**Cr (ppm)**	**As (ppm)**	**Zn (ppm)**	**Ni (ppm)**	**Cu (ppm)**	
1.00	892	138	106	69	40	27	

The sediment exhibited a C/N ratio of 12, which is the limit of OM associated to algal biomass, and therefore from autochthonous origin (Müller 1977), whereas ratios >12 are indicative of OM rich in lignin and cellulose as well as poor in N, attributable to terrestrial

origin (Lamb et al. 2006). This value is in the range of those reported by Devesa-Rey et al. (2009) for riverbed sediments from 14 sampling sites in the Anllóns River, with values varying from 5 to 36 and with a mean value of 13, and slightly lower than those reported by Barral et al. (2012), who found C/N values from 13 to 35, with a mean value of 18, for 10 sediments from the same river.

The content of As in the sediment was 106 mg kg^{-1}, which highly exceeds the general reference level for As in soils in Galicia, fixed at 50 mg kg^{-1} (140 mg kg^{-1} [6], and was higher than the threshold of the European Water Framework Directive for suspended matter and sediment (40 mg kg^{-1}) (EU-WFD 2000)). The value was also higher than the Effects Range Median (ERM) (the level at which half of the studies reported harmful effects) set for As at 70 mg kg^{-1} by Long et al. (Long et al. 1995).

As the geological substrate of the Xavarido site mainly consists of alkaline gneiss, the high total concentrations of Fe, Ti and Mn found in the sediment are indicative of the transport of solid particles from basic rocks located upstream of the sampling site.

3.2. Effect of biofilm on As transfer from sediment to water

The As concentrations in the overlying water during the incubation experiment increased in the early days of the experiment (up to days 7 and 14 in B and C systems, respectively), and then maintained an almost constant value (Fig. C6.2). In both cases, the percentage of As released from the As-rich sediments was low, representing at most 0.11 and 0.04 % of the total As content in the sediments, for C and B systems, respectively. Notwithstanding, repeated measures ANOVA indicate that As release from sediment to water was significantly lower ($p<0.01$) in B systems, where As concentrations only reached 13±2 µg L^{-1}, whereas in C systems As concentrations reached up to 30±10 µg L^{-1}. This latter value highly exceeds the maximum concentration level recommended by WHO for drinking water (10 µg L^{-1}) and is slightly higher than the Environmental Quality Standard (EQS) for As in inland surface waters (a threshold for annual average concentration of As in surface waters to ensure protection against long-term exposure to pollutants in an aquatic environment) set at 25 µg L^{-1} by the Priority Substances Directive in Surface Waters (S.I. No. 272/2009 2009).

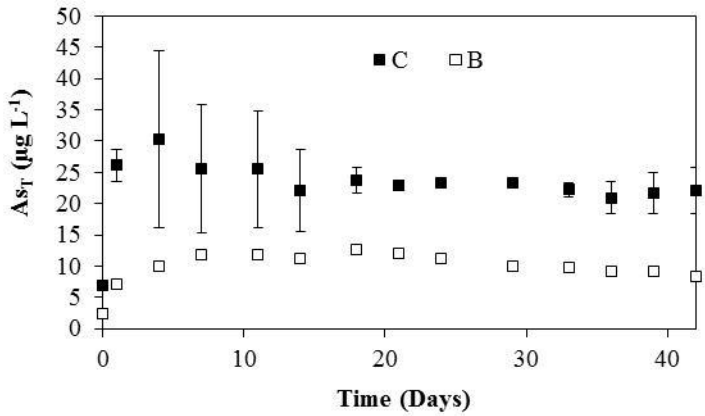

Figure C6.2. Arsenic concentration in the overlying water during the incubation experiments in control (C) and biofilm (B) systems.

The behaviour of P and Mn was similar to that of As, with significant higher concentrations in C systems throughout the experiment ($p<0.05$ and $p<0.01$, respectively), but, unlike As, they exhibited a clear maximum on the first day in C systems (Fig. C6.3). It is noticeable that Mn reached 2090 µg L^{-1} in C systems, whereas it did not exceed 4 µg L^{-1} in B systems. Also P concentrations were about 7 times higher in C than in B samples; in the latter systems P concentration decreased with time, which is attributed to its uptake by the biofilm. Fe concentrations only showed significant differences at the beginning and at the end of the experiment between both systems.

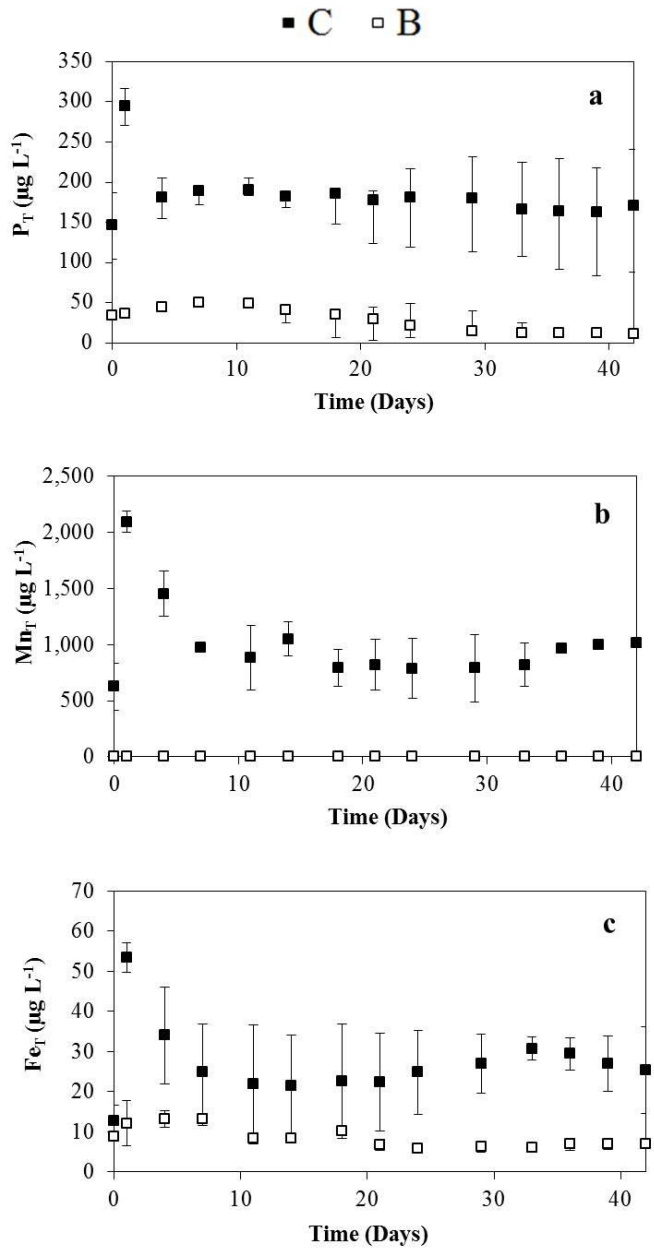

Figure C6.3. Total P (a), Mn (b) and Fe (c) concentrations in the overlying water during the incubation of control (C, black squares) and biofilm (B, white squares) systems.

3.2.1. Effect of biofilm on As speciation in the overlying water

The concentration of aqueous As species is shown in Table C6.2. The oxidized species As^{V} predominated in both systems at all times, with percentages above 78 % of the total dissolved As. In C systems the As^{V} values ranged from 5.52 µg L^{-1} at time zero to a maximum of 24.80 µg L^{-1} on the 4th day, whereas in B systems it ranged from 1.73 µg L^{-1} to 13.28 µg L^{-1} on the 11th day. The maximum As^{III} concentration (2.47 µg L^{-1} accounting for 10.5 % of the total As) was found in C systems, where the highest concentrations of this reduced species were observed in the first four days, but it was practically undetectable in B systems. This different behavior of B and C systems can be explained because the autotrophic components of the biofilm generate a more oxygenated interface, due to photosynthesis, promoting As oxidation and avoiding As reduction. Considered together, the lower As concentrations in B samples and the higher proportion of As^{V} found in these systems have important environmental and toxicological relevance, because As^{V} is considered less toxic than As^{III} (Sharma and Sohn 2009).

Contrary to what might be expected, organic As forms (MMA^{V}, DMA^{V} and AsB) appeared in both systems, and DMA^{V} and AsB even exhibited higher concentrations in C systems. The highest DMA^{V} and AsB concentrations reached in C systems were 0.67 and 0.94 µg L^{-1}, respectively, whereas in B systems they were 0.41 and 0.10 µg L^{-1}. In the case of methylated species, this fact could be explained because methylated compounds had already been produced by the microorganisms in the sediments *in situ*, taken with samples and transferred from the sediment to the water column during the incubation. Cell lysis during sterilization could favor the release of methylated species from inside the cells to the surrounding medium. Additionally, AsB (fish-As) may have originated in a seafood canning industry and a sewage treatment plant (AsB may be excreted in urine) located upstream the sampling point.

Table C6.2. As speciation in the overlying water for C and B systems.

System	Time (Day)	As^{V} ($\mu g\ L^{-1}$)	As^{III} ($\mu g\ L^{-1}$)	MMA^{V} ($\mu g\ L^{-1}$)	DMA^{V} ($\mu g\ L^{-1}$)	AsB ($\mu g\ L^{-1}$)	Σ As sp. ($\mu g\ L^{-1}$)	Total As ($\mu g\ L^{-1}$)	% Recovery
B	**0**	1.73	n.d.	0.47	n.d.	n.d.	2.19	2.50	87.94
	1	4.28	n.d.	0.52	n.d.	n.d.	4.79	7.19	66.64
	4	9.92	n.d.	n.d.	0.03	0.04	9.98	9.92	100.70
	11	13.28	0.03	n.d.	0.02	0.01	13.34	11.90	112.86
	18	12.69	n.d.	n.d.	n.d.	0.02	12.71	12.75	99.36
	24	10.67	n.d.	n.d.	0.12	0.10	10.89	11.21	96.90
	33	7.58	n.d.	n.d.	0.19	n.d.	7.77	9.69	80.04
	39	7.30	0.13	n.d.	0.41	0.07	7.91	9.17	86.34
C	**0**	5.52	0.47	0.29	n.d.	0.12	6.40	6.88	92.64
	1	20.52	2.47	0.12	0.08	0.39	23.58	26.14	89.80
	4	24.80	1.54	n.d.	0.59	0.61	27.54	30.30	91.09
	11	24.62	0.47	n.d.	0.43	0.89	26.42	25.51	103.35
	18	22.66	0.47	n.d.	0.59	0.92	24.63	23.73	103.98
	24	22.07	0.08	n.d.	0.60	0.94	23.69	23.34	101.50
	33	17.56	0.29	0.09	0.61	0.73	19.29	22.20	86.83
	39	16.74	0.34	0.18	0.67	0.68	18.62	21.67	85.64

n.d. not detectable

3.2.2. Volatilized As

The mean value for As volatilized from B samples was 1.44 ng (representing only 1.1 10^{-5} % of the total As content in sediments), whereas 0.92 ng (representing only 6.9 10^{-6} % of the total As content) were retained in traps of the C samples, with no significant differences between both systems. Therefore, the volatilization of As from the Anllóns river bed sediments by the microorganisms composing the biofilm is not a key factor in the global balance of As.

3.2.3. Leachability and bioavailability of As

Both in the control and biofilm systems, As mobilized in various extractants decreased in the following order: specifically sorbed > extracted in TCLP ≥ exchangeable > water soluble (Table C6.3). The latter three were significantly higher ($p<0.05$) in C systems whereas the specifically sorbed As was significantly higher ($p<0.01$) in the presence of biofilm. The

bioavailability of As measured by DGT devices was also significantly higher ($p<0.05$) in the control systems than in the sediments incorporating biofilm.

Table C6.3. As mobilized in various extractants, and time-averaged bioavailable concentration in DGT devices (C_{DGT}). Percentages in brackets represent the As mobilized with respect to the total As in sediments.

Sample	Water soluble As	Exchangeable As	Specifically sorbed As	TCLP As	C_{DGT}
	($\mu g\ g^{-1}$)				($\mu g\ L^{-1}$)
C	0.051 (0.05 %)	0.18 (0.17 %)	1.39 (1.31 %)	0.28 (0.26 %)	2.25
B	0.045 (0.04 %)	0.13 (0.12 %)	2.58 (2.43 %)	0.11 (0.10 %)	1.44

As^V was the predominant As species in all the extracts (>80 % of total As species) (Table C6.4). The highest concentrations of As^{III} (50.5 $\mu g\ kg^{-1}$) and MMA^V (15.3 $\mu g\ kg^{-1}$) were detected in TCLP extracts for C samples, while the highest concentration of DMA^V was detected in sulphate extracts for B samples (12.4 $\mu g\ kg^{-1}$).

Table C6.4. Speciation of As mobilized by the different extractants in C and B samples.

Extractant	Sample	As^V	As^{III}	MMA^V	DMA^V	AsB	Σ As sp.	% Recovery
As-WS ($\mu g\ kg^{-1}$)	C	36.8	n.d.	n.d.	2.4	1.1	40.3	80
	B	35.1	1.9	n.d.	2.0	0.2	39.2	87
As-Ex ($\mu g\ kg^{-1}$)	C	193.4	4.9	n.d.	4.7	n.d.	203.0	104
	B	121.6	n.d.	n.d.	12.4	n.d.	134.0	89
As-SS ($\mu g\ kg^{-1}$)	C	1400.9	20.7	5.5	8.9	3.4	1439.4	104
	B	2163.1	20.0	8.4	1.9	n.d.	2193.4	86
As-TCLP ($\mu g\ kg^{-1}$)	C	276.1	50.5	15.3	4.4	n.d.	346.3	118
	B	125.9	4.6	n.d.	3.4	0.9	134.8	109

n.d. not detectable. Detection limits: 4.1, 2.9, 4.6 and 2.5 ng L^{-1} for As^{III}, MMA^V, DMA^V and AsB, respectively.

3.3 Biofilm measurements

3.3.1. Chlorophyll-a fluorescence measurements

The development of a mature biofilm after 5 weeks was corroborated by the measurement of in *vivo* chlorophyll-a fluorescence using the Phyto-PAM device. The minimal fluorescence yield of a dark adapted cell (F_0) increased in B systems up to a mean value of 674 at the end of the incubation period with respect to sterilized sediment used as blank, whereas this parameter did not change throughout the experiment in the sterilized C systems. This result evidences that no phototrophic activity occurred in C systems, and confirms the effectiveness of the sterilization process.

The maximum PII quantum yield, which measures the photosynthetic capacity of the autotrophic microorganisms composing the epipsammic biofilm, exhibited a mean value of 0.52 in B systems. This value is in agreement with the typical values found for freshwater periphyton developed in unpolluted waters (Serra et al. 2009b, Serra et al. 2010, Ricart et al. 2009, Barral-Fraga et al. 2016).

Based on the computer-aided deconvolution of fluorescence measurements using the PhytoWin software, the relative contents of phytoplankton groups, composing biofilm at the end of the experiment, followed the order: cyanobacteria (52 %) > green algae (33 %) > diatoms (15 %). The low value (0.29) found for the ratio F_{01}/F_{03} corroborates the dominance of cyanobacteria with respect to green algae.

3.3.2. Distribution and speciation of As in the biofilm

Arsenic was uniformly distributed in the biofilm, because no significant difference was found between the extracellular (1.03 ± 0.05 µg g^{-1} of As) and the intracellular (1.38 ± 0.35 µg g^{-1}) compartments. The speciation of intracellular As (Table C6.5) shows that As^V was the predominant species, although, interestingly, As^{III}, MMA^V and DMA^V were also detected inside the cells. This may be indicative of processes of detoxification performed by the epipsammic biofilm, by reduction of As^V to As^{III} and subsequent methylation to MMA^V and DMA^V.

Table C6.5. Speciation of intracellular As.

As^{V} (µg kg^{-1})	As^{III} (µg kg^{-1})	MMA^{V} (µg kg^{-1})	DMA^{V} (µg kg^{-1})	AsB (µg kg^{-1})	Σ As sp. (µg kg^{-1})
1025.8	14.7	2.6	8.7	n.d.	1051.8

n.d. not detectable. Detection limit for AsB: 2.5 ng L^{-1}.

3.4. Discussion

The present chapter investigates the role of epipsammic biofilms in the As transfer from As-rich sediments to the river water and their effect on As speciation. The literature on epipsammic biofilms and As is limited and mainly focused on As toxicity (Tuulaikhuu et al. 2015) and on the effect of biofilms on the retention and speciation of As in the sediments (Prieto et al. 2013-chapter 4, Prieto et al. 2014-chapter 5]. Previous studies conducted with sediments of the Anllóns River indicated that epipsammic biofilms increased As^{V} sorption by the sediments, particularly in the presence of phosphate (chapter 4), and that they strongly affected the speciation of As in the water column by decreasing the proportion of As^{III} (chapter 5). However, further research was required to elucidate the effect of the epipsammic biofilm on As transfer from As-rich sediments, with the objective of assessing their potential environmental risk and contributing to the knowledge of the role of biofilms in the As biogeochemistry in riverine systems.

The results of this study revealed that As transfer from a sediment containing 106 mg kg^{-1} As was low, representing at most 0.11 and 0.04 % of the total As content in sediments for C and B systems, respectively. The low mobility of As found in this study is in agreement with the results previously reported by Rubinos et al. (2011) who found a low As solubility in the bed sediments of the Anllóns River using the DIN 38414-S4 standard procedure. This behaviour is related to the As chemical forms in the sediments which is mainly associated to low-mobility phases, predominantly to Fe oxides and in the residual phase (Devesa-Rey et al. 2008a; Rubinos et al. 2011).

Despite the low percentages of As mobilized, concentrations in water are relevant for human health, as the highest As concentrations measured in C samples exceeded 3 times the

maximum concentration level recommended by WHO (1993) for drinking water, fixed at 10 $\mu g\ L^{-1}$, whereas the As concentrations measured in B samples were close to this limit value. This fact is of broad interest for the population of the sampled area consuming water from the Anllóns River, which could be at risk if As solubility increases.

Arsenic transfer to the water column was reduced by 64 % in the presence of the biofilm. This fact could be explained because this complex community of microorganisms immersed in a EPS matrix constitutes a new sediment-water interface which modifies the exchange of solutes between the two phases, increasing As retention from the As-rich sediments and preventing its transfer to water. This behavior was also shown for the retention of As from As-polluted waters in chapters 4 and 5, and may be attributed to the sum of two combined effects: (bio-) accumulation and (bio-) sorption. As^V (bio-)accumulation takes place when As^V enters the cells via phosphate transporters (Páez-Espino et al. 2009), while (bio-)sorption may be improved because the biofilm increases the surface area, the number of sorption sites and consequently As sinks, as was indicated by van Hullebusch et al. (2003) studying metal sequestration by biofilms.

The lower concentrations of As observed in the presence of epipsammic biofilm were accompanied by lower concentrations of P, Fe and Mn. This behavior could be explained because microorganisms composing the biofilm are able to use P as an essential nutrient, thus reducing its concentration in solution; also biofilms oxygenate the sediment-water interface, thus avoiding the reductive dissolution of Fe and Mn oxides (and the associated As) and promoting the precipitation of biominerals of Fe and Mn (Dong et al. 2000; Warren and Hack 2001), which may contribute to As sequestration (Drahota et al. 2014). Higher As mobilization in gamma-sterilized sediments, together with higher leaching of DOC, Fe and Mn, has been reported by Schaller et al. (2011), who attributed this effect to cell lysis by sterilization and the release of the cellular content to the overlying water. This effect can also be envisaged for autoclaved control systems as an additional mechanism contributing to the higher As, Fe and Mn concentrations in C systems.

Arsenic speciation in the overlying waters indicates that As^V was the predominant As form in both systems, what would be expected because As^V is the most stable species in aerobic environments (Sharma and Sohn 2009). As^{III} was detected in the sterilized sediments; its occurrence could be inhibited by the biofilms because they maintain a more oxygenated

sediment-water interface due to photosynthesis. This inhibition of As^{V} reduction to As^{III} by the biofilms has relevant geochemical and toxicological implications, since As^{III} is usually considered more mobile and toxic than As^{V} (Sharma and Sohn 2009; Huang 2014).

The volatilization of As from As-rich sediments from Anllóns River is not relevant in the global balance of As in this river, as the percentage of As volatilized during the incubation experiment only accounted for 1.1 10^{-5} % of the total As content in sediments. This percentage is three orders of magnitude lower than the reported by Mestrot et al. (2009) from Bangladesh paddy soils containing 24.2 mg kg^{-1} of total As.

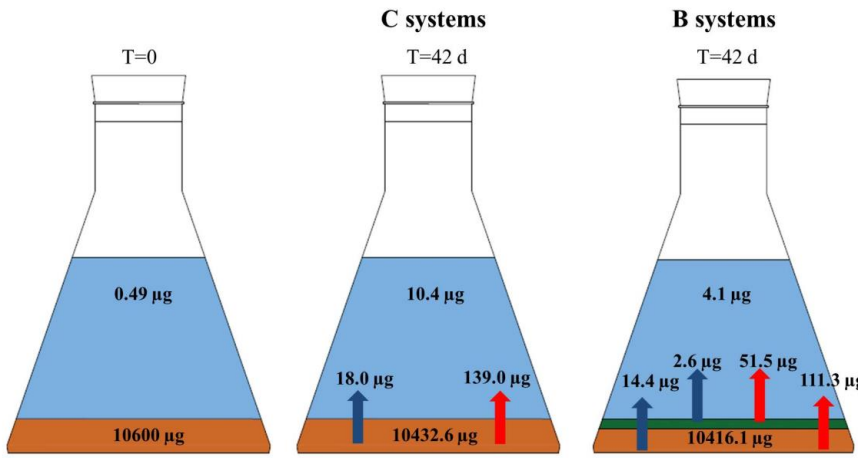

Figure C6.4. Global balance of As in C and B systems. Blue arrows indicate As extractable with 0.05 M $(NH_4)_2SO_4$ while red arrows show the As extractable with 0.05 M $NH_4H_2PO_4$. For the calculation of this balance, the following parameters have been employed: the density of the sediment (1.5 g cm^{-3}), the area occupied by sediment (55.9 cm^2), the depth of sediment (1.5 cm), the mass of sediment (125 g), the sediment water content (0.2 kg H_2O/kg wet solid), the depth of the layer of biofilm-enriched sediment (0.3 cm), together with the total As content in sediments, the As concentrations detected in the overlying waters at the end of the experiment and the As mobilized by 0.05 M $(NH_4)_2SO_4$ and 0.05 M $NH_4H_2PO_4$.

The epipsammic biofilm not only inhibited the As transfer into the water column but also the water-soluble, exchangeable and TCLP-extractable As, as well as bioavailable As measured as the time-averaged DGT concentrations. However, the specifically sorbed As, desorbable with phosphate, was approximately twice in the presence of biofilm, which can be explained by the higher retention of As coming from the sediment. This extractant solubilizes

As specifically sorbed to cells and EPS matrix, as well as to sediment particles. The lower As concentration in water along the experiment in the systems with biofilm may be related with the higher retention of As in a form which can be mobilized with phosphate. In fact, the global balance of As in C and B systems shown in Figure C6.4 indicates that the sum of dissolved As at the end of the experiment, plus sulphate-extractable As and phosphate-extractable As in B systems is in the order of the total As mobilizable in C systems.

The effect of phosphate on As desorption is of interest because in the Anllóns River catchment both diffuse and point sources of P pollution have been identified, coming from urban and industrial sewage treatment plants, and from fertilizers leached or eroded from agricultural soils in the river catchment, and high As concentrations detected in the riverbed sediments in the As-Au mineralized area coincide with P concentrations up to 2324 mg kg^{-1} (Devesa-Rey et al. 2009), thus aggravating the risk of As mobility in the presence of biofilm.

Arsenic was equally distributed between the extracellular and intracellular compartments of the biofilm. These results do not coincide with those found in chapter 5, studying the influence of epipsammic biofilm in the retention and speciation of As from As-polluted water, where 70 % of the As taken from solution was retained in the extracellular compartment. This different behavior could be attributed to the limited intracellular As uptake capacity of the biofilm at high As^V dissolved concentrations (as those tested in chapter 5, 500 µg L^{-1}), so that As concentrations exceeding saturation tend to accumulate in the extracellular compartment. This fact has been previously described by Wang et al. (2013), studying the toxicity and bioaccumulation kinetics of arsenate in two freshwater algae (*Chlamydomonas reinhardtii* and *Scenedesmus obliquus*), and by Karadjova et al. (2008), studying the biouptake of As species by *Chlorella salina,* which reported that intracellular As increased linearly when As^V concentration increased between 10 µM and 50 µM As, followed by a saturation plateau above this concentration.

The occurrence of As^{III} jointly with methylated As species (MMA^V and DMA^V) inside the biofilm cells indicate that methylation processes are occurring. As^V is reduced to As^{III} inside the cells, via various reductases, using glutaredoxin, glutathione or thioredoxin as an electron donor (Zhao et al. 2009; Yin et al. 2011c). Methylation of As^{III} is slow and it is more likely to occur in the stationary phase of algae growth (Hellweger et al. 2003). Our findings are in agreement with the model proposed by Cullen et al. (1994a, 1994b) in which As^V is

taken up by algal cells using a phosphate transport system. Subsequently As^{V} is reduced to As^{III} in the cell by thiols and/or dithiols and then excreted into the growth medium. As^{III} may further be methylated to MMA^{V}, then to DMA^{V} and to trimethylated As species.

In summary, the risk of As transfer from As-rich sediments from the Anllóns River can be considered low and is further reduced in the presence of the biofilm. These results are interesting from a human health perspective, as they contribute to the assessment of the risks affecting the quality of water supplies; they are also relevant from an environmental point of view, because the Anllóns Basin is partly considered a Site of Community Importance, as defined in the European Commission Habitats Directive (92/43/EEC).

4. Conclusions

1) Arsenic transfer to water from As-rich sediments from the Anllóns River is very low. Even so, the presence of epipsammic biofilm reduces by 64 % the transfer of As from As-polluted sediments to the water column. The mobility of P, Mn and Fe is also lower in the presence of biofilm. As^{V} is the predominant As species in the overlying water of the systems with and without biofilm.

2) The concentrations of As^{III} are higher in the systems devoid of biofilm. This fact has an important toxicological relevance due to the usually higher toxicity of As^{III} compared to As^{V}.

3) The arsenic retained by the biofilm is similarly distributed in the extracellular and intracellular compartments. In the intracellular fraction significant concentrations of As^{III}, MMA^{V} and DMA^{V} are detected, indicative of the occurrence of methylation (detoxification) processes.

4) The volatilization of As from As-rich sediments from the Anllóns River is very low and must not play a relevant role in the global balance of As in this river.

5) The biofilm decreases As leaching by water (water soluble As), by sulphate (exchangeable As) and by TCLP, as well as As bioavalability measured by DGT devices, but it increased the extractability in phosphate (specifically sorbed As).

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5. GENERAL OVERVIEW

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5. GENERAL OVERVIEW

An integrated analysis of the As transfer from soils to aquatic systems, the retention by sediments and biofilms and the transformation of As species in fluvial environments is developed in this section.

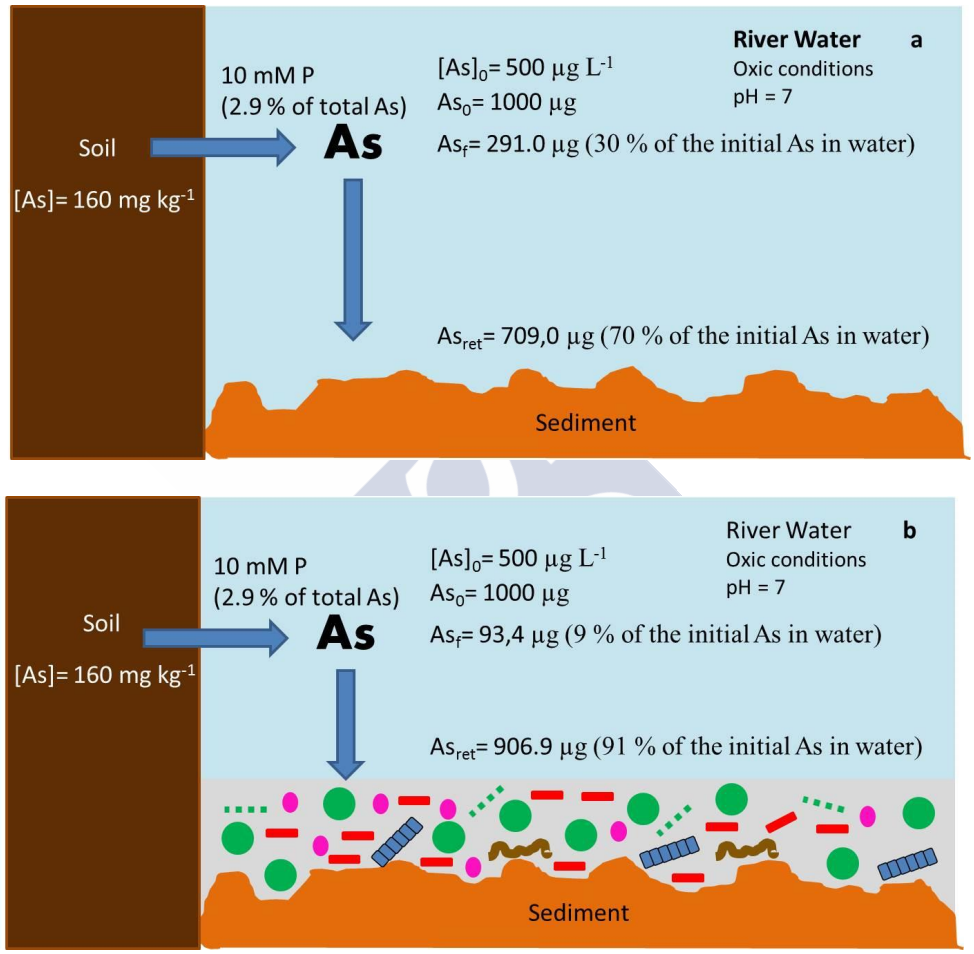

Figure 5.1. Retention of As by sediments (a) and by sediments with epipsammic biofilm (b) previouslty mobilized by 10 mM P from soils located along the Anllóns River basin.

In Figure 5.1 a global balance of As between the different compartments studied is shown. Considering an approximate total As concentration of 160 mg kg^{-1} in the As-rich soils in the basin, as is the case of those studied in chapter 1, and a percentage of soluble As of 2.9

%, As transferred to water approximately represents 500 µg L^{-1}. Considering the water volume of experiments in chapter 5, the total amount of As is 1000 µg. Of this amount, 70 % is retained by the sediments, while, in the presence of biofilms, 91 % is retained, which rose to 97 % in the presence of equimolar P.

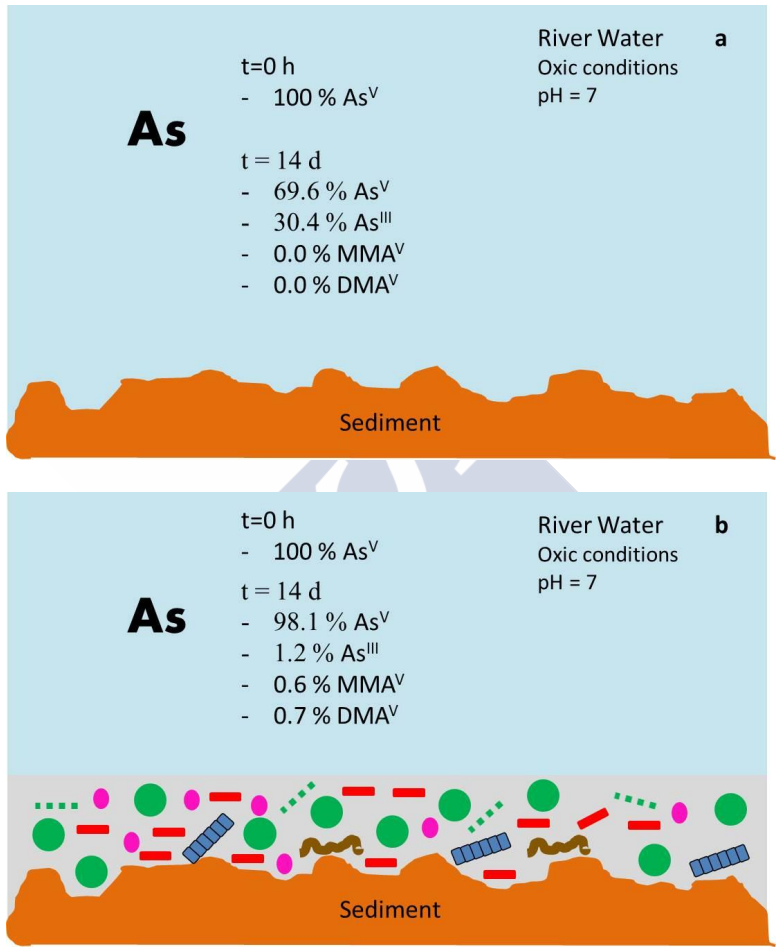

Figure 5.2. Speciation of As in the overlying water in systems devoid and incorporating biofilms.

The effect of biofilm on speciation of As added as As^V is shown is Figure 5.2, after two weeks of exposition to As solution. In the absence of biofilm, As^V was partly transformed to As^{III} (~30 %), while in the presence of biofilm 98 % was As^V and methylated species were detected.

The mobilization of As from a polluted sediment containing 106 mg kg^{-1} (total amount in 100 g sediment = 10600 µg) represented 0.11 % (10.4 µg) for sediment without biofilm and only 0.04 % (4.1 µg) for the system with biofilm (Fig. 5.3.). The effect of biofilm on the speciation of As released from the sediment is shown in Figure 5.3, after two weeks of incubation. In both systems As^{V} was the predominant species, but in the system without biofilm As^{III} reached up to 10.5 % and then levelled around 1.9 %. Methylated species were considered to be present in the sediment.

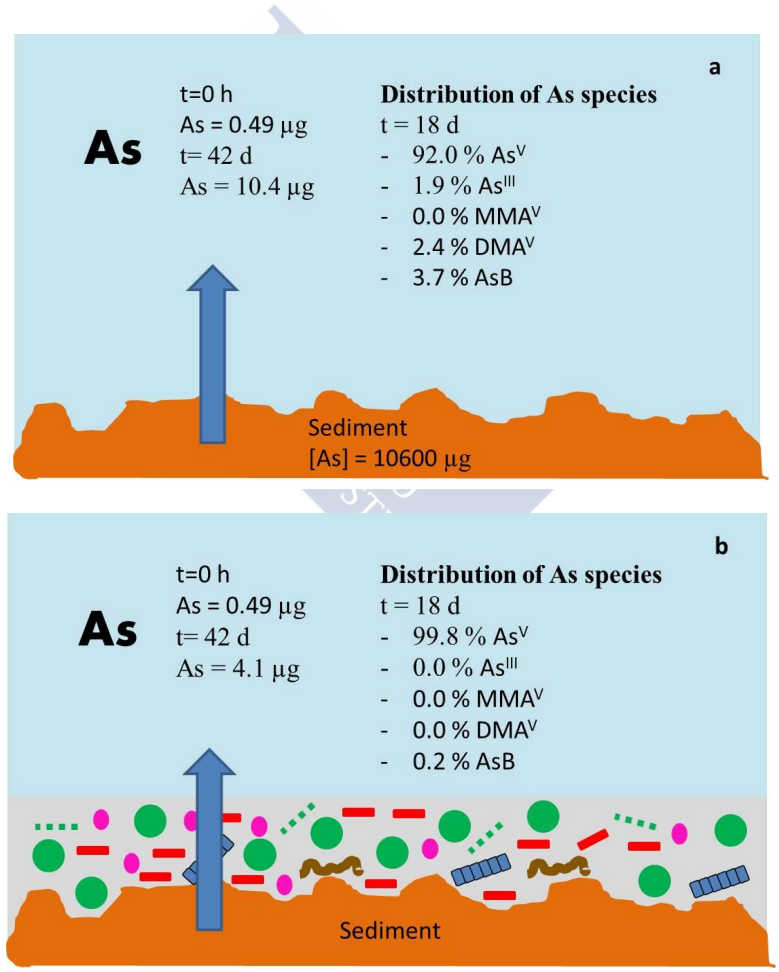

Figure 5.3. Mobilization of As in the overlying water in systems devoid and incorporating biofilms.

When As retention from polluted waters is studied it is mostly sorbed in the extracellular compartment. In both compartments the predominant species is As^{V} (Fig. 5.4a). When As release from polluted sediment is studied it is uniformly distributed between extra and intracellular compartments. The predominant species inside cells is As^{V} but the products of the biological transformation As^{III}, MMA^{V} and DMA^{V} are also detected (Fig. 5.4b).

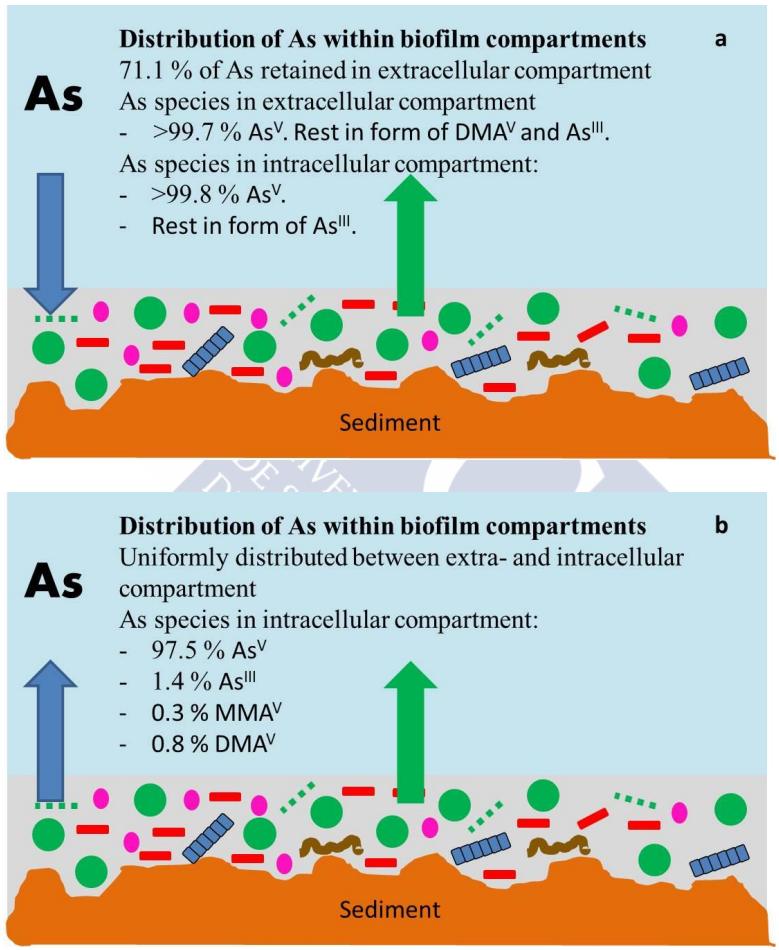

Figure 5.4. Distribution of total and As within biofilm compartments and As speciation. a) As retention from As polluted waters. b) As retention from As-rich sediments.

6. FINAL CONCLUSION

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6. FINAL CONCLUSION

The results of this study support the previous hypothesis that biofilms developed on river bed sediments actively retain As and cause changes in its chemical species, leading to a decrease of its mobility and/or toxicity, thus confirming the role of biofilm in the the immobilization and transformation of As^{V} and their importance on the environmental quality of fluvial ecosystems.

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