ARSENIC AND FLUVIAL BIOFILMS: BIOGEOCHEMISTRY, TOXICITY AND BIOTIC INTERACTIONS

Laura Barral Fraga

DOCTORAL THESIS

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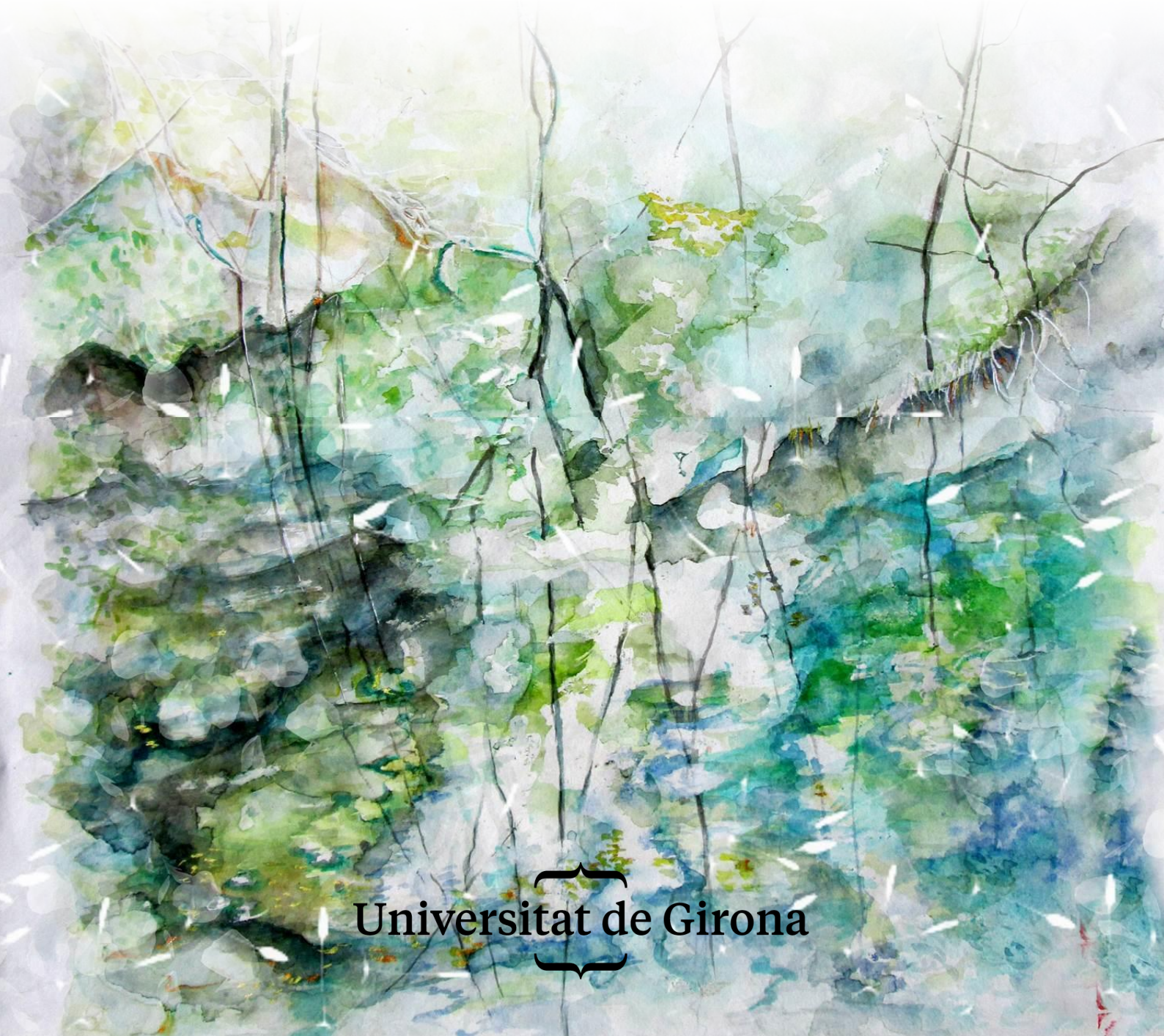

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2017

DOCTORAL PROGRAM IN WATER SCIENCE AND TECHNOLOGY

Supervised by:

Dr. HELENA GUASCH

Dr. MARÍA TERESA BARRAL SILVA

Dr. SOIZIC MORIN

Thesis submitted in fulfillment of the requirements for the Degree of Doctor at the University of Girona

Dr. HELENA GUASCH, from the *Universitat de Girona*

Dr. MARÍA TERESA BARRAL SILVA, from the *Universidade de Santiago de Compostela*

Dr. SOIZIC MORIN, from the *Institut national de recherche en sciences et technologies pour l'environnement et l'agriculture (IRSTEA- Bordeaux)*

WE DECLARE:

That the thesis entitled "**Arsenic and Fluvial Biofilms: Biogeochemistry, Toxicity and Biotic Interactions**", presented by **LAURA BARRAL FRAGA** to obtain a Doctoral degree, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Signature

Dr. **HELENA GUASCH**	Dr. **MARÍA TERESA BARRAL SILVA**	Dr. **SOIZIC MORIN**

Girona, June 2017

A miña nai

A meu pai

E ós meus avós Manuel e Carmen

Pasa, río, pasa, río,

co teu maino rebulir;

pasa, pasa antre as froliñas

color de ouro e de marfil,

a quen cos teus doces labios

tan doces cousas lle dis.

Pasa, pasa, mais non vexan

que te vas ao mar sin fin,

porque estonces, ¡ai, pobriñas,

cánto choraran por ti!

Rosalía de Castro. *Cantares Gallegos*

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Y si la tesis en sí ya resulta extensa... cómo no lo van a ser también los agradecimientos!! 

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Esta tesis tiene sus inicios allá por el verano 2012 cuando, tras haber defendido el trabajo de fin de master, Helena Guasch me ofrece la posibilidad de seguir trabajando en el campo de la Ecotoxicología comenzando una tesis bajo su supervisión. Y así fue como en pleno 2013 me lancé a la *aventura* que supone ser becaria o investigadora en formación... ☺. Sin embargo, en realidad, esta historia se inició unos cuantos años antes, en los últimos de mi licenciatura en Biología en la Universidade da Coruña, cuando un par de becas de colaboración en departamentos me dieron la oportunidad de descubrir y adentrarme por primera vez en el mundo de la investigación bajo la supervisión de mi gran profesor Roberto Bao. En concreto, fue en ese momento que nació mi fascinación por las diatomeas y la Paleoecología que, junto a mi gran colega David Vázquez, disfruté muchísimo! Inolvidable mi primer muestreo en campo con barca hinchable en la Lagoa costeira de Esteiro con David y Manel Leira!! Gracias Roberto, David y Manel!! Vosotros, junto a Álex Blanco, formaréis siempre parte de los gratos recuerdos que tengo sobre mis inicios en investigación.

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LIST OF PUBLICATIONS

List of publication derived from this Doctoral thesis (two of them are published in international journals):

Barral-Fraga L, Morin S, Rovira MD, Urrea G, Magellan K, Guasch H. (2016). Short-term arsenic exposure reduces diatom cell size in biofilm communities. *Environmental Science and Pollution Research*, 23(5): 4257-4270.

doi: http://dx.doi.org/10.1007/s11356-015-4894-8 (see Annex 1)

Magellan K, **Barral-Fraga L**, Rovira M, Srean P, Urrea G, García-Berthou E, Guasch H. (2014). Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae. *Aquatic Toxicology,* 156:116-124.

doi: http://dx.doi.org/10.1016/j.aquatox.2014.08.006 (see Annex 2)

Barral-Fraga L, Martiñá-Prieto D, Barral MT, Morin S, Guasch H. Mutual interaction between arsenic and biofilm in a mining impacted river. *In prep*.

Barral-Fraga L, Barral MT, Prieto D, Rodríguez-Castro MC, Tuulaikhuu B-A, Morin S, Guasch H. Arsenic biogeochemistry in freshwater ecosystems. *In prep*.

LIST OF ACRONYMS

acr3: As^{III} efflux pump-encoding gene
AENOR: Asociación Española de Normalización y Certificación
ANOVA: Analysis of Variance
APHA: American Public Health Association
AQP: aquaglyceroporins
ARMs: arsenate-resistant microorganisms
Arr: arsenate reductase
arrA: arsenate respiratory reductase-encoding gene
Ars: Arsenic resistance system
ArsB: As^{III} efflux pump
ArsB: As^{III} efflux pump
arsB: As^{III} efflux pump-encoding gene
ArsC: arsenate reductase enzyme
arsC: arsenate reductase enzyme-encoding gene
ArsH: arsenate reductase enzyme
arsH: arsenate reductase enzyme-encoding gene
arsM: arsenite methyltransferase enzyme
ArsM: arsenite methyltransferases
arsR: ars operon gen
Arx: anaerobic arsenite oxidation enzyme
As: Arsenic
As-Bet: arsenobetaine
As^{III}: Arsenite
As^{V}: Arsenate
ATP: adenosine triphosphate
CAOs: chemolithoautotrophic arsenite oxidizers
CCC: Criteria Continuous Concentration
Ch: Chapter
Chl-*a*: chlorophyll *a*
CMC: Criteria Maximum Concentration
CTB: chemical time bombs
Cys: cysteine residues
DARPs: Dissimilatory Arsenate-Reducing or Arsenate-Respiring Prokaryotes
df: degrees of freedom
DGT: diffusive gradients in thin films
$DMAA^{III}$: dimethylarsinous acid
$DMAA^{V}$: dimethylarsinic acid
DMA^{III}: dimethylarsenite
DMA^{V}: dimethylarsenate
DNA: Deoxyribonucleic acid
DOC: Dissolved Organic Carbon
DW: Dry Weight
ECOTOX: ECOTOXicology knowledgebase
Eh: Redox potential
EPS: extracellular polymeric substances
F_0: Minimum fluorescence yield
FeAsS: arsenopyrite
Fig.: Figure
GEE: generalized estimating equation
GF/F: glass microfiber filters
GLM: Generalized Linear Model
GSH: glutathione
H: Shannon-Wiener index of diversity
HAOs: heterotrophic arsenite oxidizers
HPLC: High-Performance Liquid Chromatography
iAs: inorganic As
IC20: 20% inhibitory concentration
ICP-MS: Inductively Coupled Plasma Spectrometry
iP: inorganic phosphate
J: Species evenness
L/D: Live/Dead (bacterial viability)
LC50: 50% lethal concentrationLOEL or LOEC: the Lowest Observed Effect Level or the Lowest Observed Effect Concentration
MCL: Maximum Concentration Limit
Met-As: methylarsenicals

µS: microSiemens
$MMAA^{III}$: monomethylarsonous acid
$MMAA^{V}$: monomethylarsonic acid
MMA^{III}: monomethylarsenite
MMA^{V}: monomethylarsenate
MRPP: Multi-Response Permutation Procedures
mV: milliVolts
n: Sample size
NE: North-East
NMDS: Non-Metric Multidimensional Scaling
NOEL or NOEC: No Observable Effect Level or No Observable Effect Concentration
NPL: National Priority List
NW: North-West
OM: organic matter
orgAs: organoarsenicals
P: Phosphorus
p: p-value
PAM: Pulse Amplitude Modulated (fluorimeter)
PAN: Pesticide Action Network
PBET: physiologically based extraction test
PCA: Principal Component Analysis
PCs: phytochelatins
P_i: Proportional abundance of the i^{th} species
p*Ka*: Acid dissociation constant
ppb: parts per billion
PSII: Photosystem II
RDA: Redundancy Rata Analysis
ROS: Reactive Oxygen Species
S: Species richness
SCI: stream and catchment interface
sp.: Species
SPSS: Statistical Package for Social Science (software)
SRP: Soluble reactive phosphorus
SS: Suspended Solids
TC: Total Carbon
TCLP: toxicity characteristic leaching procedure
TMA: trimethylearsine
TMAO: trimethylarsine oxide
TN: Total nitrogen, and Total Kjeldhal nitrogen
TP: Total phosphorus, and Total Phosphate
US EPA: United States Environmental Protection Agency
WHO: World Health Organization
XRF: X-ray fluorescence
Y_{eff}: effective PSII quantum yield or photosynthetic efficiency
Y_{max}: maximum PSII quantum yield or maximal photosynthetic capacity

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4. GENERAL DISCUSSION

CONTENTS

Arsenic (As) contamination of natural waters is a worldwide problem due to its important impacts for human and ecosystem health. Natural (geological processes, mainly) and anthropogenic activities, including mining, are the sources of arsenic pollution in the environment. High concentrations have been reported for water samples in several parts of the world, becoming an environmental concern because of its harmful effects on organisms. Arsenic toxicity depends on numerous interacting factors which makes effects difficult to estimate. In freshwaters, arsenate (As^{V}) can be taken up by microorganisms (especially those forming biofilms) due to its similarity with phosphate (PO_4^{3-}) molecules, resulting its toxicity be dependent on environmental phosphate conditions. Microorganisms play a key role on the arsenic biogeochemistry (speciation, distribution and cycling) in aquatic systems, since they incorporate the dominant iAs (inorganic arsenic) form and may convert it to other arsenic forms. These transformation reactions have a big impact on the environmental behavior of arsenic, since the different chemical forms of this element exhibit different mobility and toxicity. Fish are another key constituent of aquatic ecosystems, and their effects due to arsenic exposure could be influenced by their interaction with microorganisms (i.e biofilms).

Based on the current knowledge about biofilms ecotoxicology and arsenic biogeochemistry in freshwater ecosystems, this thesis is aiming to study, under realistic environmental arsenic concentrations, **i)** the role of benthic biofilms on As-bioavailability and As-detoxification in a freshwater system, **ii)** the toxic effects of arsenic on the structure and function of benthic fluvial biofilms, with especial attention to diatom responses, and **iii)** the interaction between these As-exposed primary producers and As-exposed higher organisms (fish).

In ***Chapter 1***, an experiment combining ecological and ecotoxicological descriptors was conducted to investigate the effects of As^{V} (130 µg L^{-1} over 13 days) on the structure and function of fluvial biofilm under phosphate-limiting conditions. We further incorporated fish (*Gambusia holbrooki*) into our experimental system, expecting fish to provide more available phosphate for algae and, consequently, protecting algae against arsenic toxicity. However, this protective role was not fully achieved. Arsenic inhibited algal growth and productivity but not that of bacteria. The diatom community was clearly affected, showing a strong reduction in cell biovolume; selection for tolerant species, in particular *Achnanthidium minutissimum*, and a reduction in species richness. Our results have important implications for risk assessment, as the experimental arsenic concentration used was lower than the acute toxicity criteria established by the United States Environmental Protection Agency (US EPA), 340 µg As L^{-1}.

In ***Chapter 2***, we examined the effects of arsenic exposure (130 µg L^{-1} over 9 days) in the invasive mosquitofish *G. holbrooki*, in the same laboratory experiment as ***Chapter 1***, incorporating some of the complexity of natural systems by including the interacting effects with the microbial community (the biofilm). Our aims were to quantify the effects of arsenic on some complex behaviors and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas neither food capture efficiency nor consumption

was notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomass. Possibly increased aggression facilitated food resource defense allowing bigger fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation, the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioral and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

In ***Chapter 3***, a biofilm translocation experiment was carried out during 51 days in a mining-impacted river, the Anllóns River (Galicia, NW Spain), where concentrations up to 270 mg As^{V} kg^{-1} are found in sediments. The translocation was performed moving biofilm-colonized substrata from upstream (less As-polluted) to downstream the mine area (more As-polluted site with also more easily extractable As), to explore the effect of arsenic on benthic biofilms and the role of these biofilms on arsenic retention and speciation in the water-sediment interface. Eutrophic conditions (high total dissolved phosphorus and total nitrogen) were detected in water at both sites, while sediments were not considered P-polluted. Translocated biofilms accumulated more arsenic and showed higher potential toxicity (higher As/P ratio). In concordance, their growth was reduced to half that observed in those non-translocated. Moreover, they became less nutritive (less N content) and with higher bacteria and dead diatom densities than the non-translocated biofilms. Besides the higher arsenic exposure, other environmental conditions such as the higher amount of DOC (dissolved organic carbon) and riparian cover in the more As-polluted site could contribute to those effects. Methylated As-species (DMA^{V}) were found in the intracellular biofilm compartment and also in the river water, suggesting a detoxification process by biofilm (methylation) and a contribution to arsenic speciation in the water-benthic biofilm interface. Since most arsenic in sediments and water was arsenate (As^{V}), the high amount of arsenite (As^{III}) detected in the biofilm extracellular compartment may also confirm As^{V} reduction by biofilms. This study provides new arguments to understand microorganism contribution to arsenic biogeochemistry in freshwater environments.

The results obtained in this thesis provide valuable information to understand the contribution of benthic biofilms to the arsenic biogeochemistry in freshwater environments, and specifically in the water-biofilm interface. Also, it was demonstrated once again the importance of using biofilms and a multi-endpoint approach to measure effects of toxicants in freshwater ecosystems, as well as study the toxicity to different trophic organisms, such as biofilm and fish, since aggravated effects resulted in their interaction. Finally, environmental factors such as nutrients or light may influence and modulate arsenic toxicity. Therefore it is crucial to take them into account for the measurement of real toxic effects in the ecosystems.

La contamination par l'arsenic (As) des eaux naturelles est un problème mondial, avec des impacts importants pour la santé humaine et environnementale. Les activités naturelles (processus géologiques, principalement) et anthropiques, notamment minières, sont les sources principales de pollution à l'arsenic dans l'environnement. Des concentrations élevées ont été rapportées pour des échantillons d'eau collectés dans diverses régions du monde, ces niveaux étant préoccupants en raison d'effets néfastes sur les organismes. La toxicité de l'arsenic dépend de nombreux facteurs en interaction, ce qui rend difficile la prédiction des effets. Dans les eaux douces, l'arséniate (As^{V}) peut être accumulé par des micro-organismes (notamment les biofilms) en raison de sa similitude avec la molécule de phosphate (PO_4^{3-}); sa toxicité dépend donc des concentrations environnementales en phosphate. Les micro-organismes jouent un rôle clé sur la biogéochimie de l'arsenic (spéciation, distribution et recyclage) dans les systèmes aquatiques, car ils peuvent convertir la forme dominante iAs (arsenic inorganique) en d'autres formes de l'arsenic. Ces réactions de transformation ont un impact important sur le comportement environnemental de l'arsenic, car les différentes formes chimiques de cet élément présentent une mobilité et une toxicité différentes. Les poissons sont une autre composante clé des écosystèmes aquatiques, et les effets causés par l'exposition d'arsenic pourraient être influencés par la présence et l'action des micro-organismes (i.e. les biofilms).

Sur la base des connaissances actuelles sur l'écotoxicologie des biofilms et de la biogéochimie de l'arsenic dans les écosystèmes d'eau douce, cette thèse vise à étudier, en présence de concentrations environnementalement réalistes **i)** le rôle des biofilms benthiques sur la biodisponibilité de l'arsenic et la détoxification dans un système d'eau douce, **ii)** les effets toxiques de l'arsenic sur la structure et la fonction des biofilms benthiques de rivière, avec une attention particulière portée aux réponses des diatomées, et **iii)** l'interaction entre ces producteurs primaires exposés à l'arsenic et des organismes supérieurs (poissons) également exposés à l'arsenic.

Dans le ***Chapitre 1***, une expérimentation combinant des descripteurs écologiques et écotoxicologiques a été réalisée pour étudier les effets de l'arsenic (130 µg L^{-1} pendant 13 jours) sur la structure et la fonction des *biofilms* fluviaux dans des conditions limitantes en phosphate. Nous avons intégré en plus des poissons (*Gambusia holbrooki*) dans notre système expérimental, en faisant l'hypothèse que la présence des poissons fourniraient plus de phosphate disponible pour les algues et, par conséquent, protégeraient les algues contre la toxicité de l'arsenic. Cependant, ce rôle protecteur n'a pas été pleinement atteint. L'arsenic a inhibé la croissance et la productivité des algues, mais pas celle des bactéries. La communauté de diatomées a été affectée, montrant une forte réduction du biovolume cellulaire, une sélection des espèces tolérantes, en particulier *Achnanthidium minutissimum*, et une réduction de la richesse spécifique. Nos résultats ont des implications importantes pour l'évaluation des risques liés à l'arsenic, car la concentration expérimentale d'arsenic utilisée était plus faible que les critères de toxicité aigue établis par l'US EPA, à savoir 340 µg As L^{-1}.

Dans le ***Chapitre 2***, nous avons examiné les effets de l'exposition à l'arsenic (130 µg L^{-1} pendant 9 jours) sur la gambusie invasive *G. holbrooki*, dans la même expérimentation de laboratoire que le ***Chapitre 1***, intégrant une partie de la complexité des systèmes naturels au travers de l'interaction avec la communauté microbienne (le biofilm). Nos objectifs étaient de quantifier les effets de l'arsenic sur certains comportements complexes de la gambusie et sur ses paramètres physiques, et d'évaluer si les mécanismes de détoxification de l'arsenic par les algues réduisaient les effets de l'exposition à l'arsenic. En présence d'arsenic, le comportement agressif des poissons a augmenté significativement, tandis que ni l'efficacité de capture de nourriture, ni la consommation, n'ont été affectées par la présence d'arsenic. Une augmentation de la bioaccumulation a été observée avec l'exposition à l'arsenic ainsi que, de façon inattendue, de la biomasse de poissons. Il est possible que la stimulation du comportement d'agression ait par ailleurs facilité l'accès aux ressources alimentaires, permettant aux plus gros poissons de prendre du poids. Une aggravation des effets de l'exposition à l'arsenic a été démontrée en présence d'algues. Concernant la biomasse de poissons, la présence d'algues a agi de manière antagoniste avec l'arsenic, entraînant une réduction du poids final. Concernant la bioaccumulation, les effets ont été encore plus marqués, avec des effets additifs de la présence d'algues et de l'arsenic sur l'augmentation de l'absorption et/ou de l'assimilation d'arsenic dans les poissons. Enfin, les comportements d'agression étaient la plus élevés en présence des algues et de l'arsenic. Nous mettons en évidence qu'une recherche multidisciplinaire, utilisant des organismes de différents niveaux trophiques, et considérant les effets comportementaux en plus d'autres effets plus classiquement évalués, est essentielle pour comprendre les impacts de la toxicité de l'arsenic, et donc pour contribuer à la restauration des écosystèmes aquatiques.

Dans le ***Chapitre 3***, une expérimentation de translocation de biofilm a été réalisée pendant 51 jours, dans une rivière impactée par l'exploitation minière: la rivière Anllóns (Galice, nord-ouest de l'Espagne) où les sédiments présentent des concentrations allant jusqu'à 270 mg As^{V} kg^{-1}. Des substrats précolonisés par du biofilm au site amont (moins pollué par l'arsenic) ont été ensuite maintenus sur place ou déplacés dans une zone en aval de la mine (le site le plus pollué par l'arsenic, avec notamment dans le sédiment des formes facilement extractibles de l'arsenic), pour explorer l'effet de l'arsenic sur les biofilms benthiques, et le rôle de ces *biofilms* sur la rétention et la spéciation de l'arsenic à l'interface eau-sédiment. Dans les deux sites, les eaux présentaient des conditions eutrophes (concentrations élevées en phosphore dissous et en azote total), alors que les sédiments n'étaient pas considérés comme pollués au regard de leur teneur en phosphore. Les biofilms transloqués ont accumulé plus d'arsenic et les rapports As/P mesurés, plus élevés, y suggèrent une toxicité potentielle accrue. En concordance, leur croissance a été réduite de moitié, en comparaison avec les biofilms amont. De plus, ils sont devenus moins nutritifs (avec une teneur plus faible en N), avec une augmentation de la densité de bactéries et de diatomées mortes par rapport aux les biofilms non déplacés. En plus des conditions d'exposition à des concentrations élevées en arsenic,

d'autres conditions environnementales, telles que la teneur plus élevée en carbone organique dissous et la couverture végétale riveraine du site, pourraient contribuer à ces effets. L'espèce méthylée de l'arsenic DMA^V a été trouvée dans le compartiment intracellulaire du biofilm ainsi que dans l'eau de la rivière, ce qui suggère un processus de détoxification de l'arsenic par les biofilms (méthylation), et une contribution à la spéciation d'arsenic à l'interface eau-biofilm benthique. L'espèce dominante dans les sédiments et dans l'eau étant l'As^V, les quantités élevées d'arsénite (As^{III}) détectées dans le compartiment extracellulaire du biofilm peuvent également confirmer la réduction en As^V par les biofilms.

Les résultats obtenus dans cette thèse fournissent des informations précieuses pour comprendre la contribution des biofilms benthiques à la biogéochimie de l'arsenic dans les milieux d'eau douce, et plus précisément à l'interface eau-biofilm. En outre, ces travaux confirment l'importance de l'utilisation de biofilms et d'une approche multi-descripteurs pour évaluer les effets des composés toxiques dans les écosystèmes d'eau douce. L'intérêt de considérer dans les études écotoxicologiques les interactions entre différents organismes de l'édifice trophique, tels que les biofilms et les poissons, a été également démontré, car la présence conjointe des algues et de l'arsenic dans les systèmes expérimentaux s'est accompagnée d'une aggravation des effets observés sur le maillon trophique supérieur. Enfin, les facteurs environnementaux tels que la lumière ou les nutriments peuvent influencer et moduler la toxicité, il est donc crucial de les prendre en compte pour une meilleure évaluation des effets réels des toxiques sur les écosystèmes.

A contaminación por arsénico (As) nas augas naturais é un problema global por mor dos seus impactos significativos na saúde humana e nos ecosistemas. Os procesos naturais (procesos xeolóxicos, principalmente) e antropoxénicos, como a minería, son fontes de contaminación por arsénico no medio ambiente. Téñense atopado elevadas concentracións deste metaloide en mostras de auga de varias partes do mundo, tornándose unha preocupación ambiental por mor dos seus efectos nocivos sobre os organismos. A toxicidade do arsénico depende de moitos factores que interactúan entre sí, o que fai que os seus efectos sexan difíciles de estimar. Nas augas doces, o arsenato (As^{V}) pode ser absorbido por microorganismos (*biofilm*s ou biofilmes), debido á súa semellanza coa molécula dun nutrinte, o fosfato (PO_4^{3-}), dependendo así a súa toxicidade das concentracións ambientais de fosfato. Os microorganismos xogan un papel fundamental na bioxeoquímica do arsénico bioquímico (é dicir, na súa especiación, distribución e no seu ciclo) en sistemas acuáticos, xa que incorporan a forma dominante de iAs (arsénico inorgánico), que soe ser o arsenato, podendo despois convertelo noutras formas de arsénico. Estas reaccións de transformación teñen un impacto importante sobre o comportamento ambiental do arsénico, porque diferentes formas químicas deste metaloide teñen tamén diferente mobilidade e toxicidade. Os peixes son outro compoñente clave dos ecosistemas acuáticos, e os efectos debidos á súa exposición ao arsénico poderían verse influenciados ao interactuaren cos *biofilms*).

Baseándose no coñecemento actual sobre a ecotoxicoloxía dos *biofilms* e a bioxeoquímica do arsénico nos ecosistemas de augas doces, esta tese pretende estudar, empregando concentracións ambientais realistas, **i)** o papel dos *biofilm*s bentónicos na biodispoñibilidade e desintoxicación do arsénico nun sistema de auga doce, **ii)** os efectos tóxicos do arsénico sobre a estrutura e a función dos *biofilm*s fluviais, con especial atención ás diatomeas (microalgas marróns), e **iii)** a interacción entre estes produtores primarios e organismos superiores coma os peixes cando se ven todos eles expostos a este metaloide.

Así pois, no ***capítulo 1*** levouse a cabo un experimento combinando descritores ecolóxicos e ecotoxicolóxicos, para investigar os efectos do As^{V} (130 µg L^{-1} durante 13 días) sobre a estrutura e a función do *biofilm* fluvial en condicións de limitación de fosfato. Ademáis, incorporáronse peixes (o mosquitofish oriental *Gambusia holbrooki*) no sistema experimental, esperando que puidesen proporcionar máis fosfato ás algas a través das súas excrecións e, polo tanto, protexelas contra a toxicidade do arsénico. Con todo, este papel protector non foi alcanzado por completo, pois o arsénico inhibiu o crecemento e a produtividade algal, anque non o crecemento das bacterias. A comunidade de diatomeas viuse claramente afectada, mostrando unha forte redución no seu biovolume celular e unha especial selección cara especies tolerantes -particularmente *Achnanthidium minutissimum*- causando, polo tanto, unha redución no número de especies (menor riqueza específica). Os nosos resultados teñen implicacións importantes para a avaliación dos riscos ambientais do arsénico, xa que a concentración utilizada neste experimento (130 µg L^{-1}) foi inferior aos criterios de toxicidade

aguda establecidos pola Axencia de Protección Ambiental dos Estados Unidos (US EPA), 340 µg As L^{-1}.

No ***capítulo 2***, preséntanse os resultados dun experimento de laboratorio (o mesmo que no ***capítulo 1***) no que se examinaron os efectos do arsénico (130 µg L^{-1} durante 9 días) sobre o peixe *G. holbrooki*, unha especie invasora. O experimento incorporou parte da complexidade dos sistemas naturais incluíndo a interacción dos peixes coa comunidade microbiana (*biofilm*). O noso obxectivo foi cuantificar os efectos do arsénico no peixe analizando algúns comportamentos complexos e parámetros físicos, e avaliar o papel detoxificador do biofilm. A agresividade dos peixes aumentou significativamente en presenza de arsénico, mentres que nin a eficiencia de capturas dos alimentos nin o consumo dos mesmos se viron afectados polo dito tóxico. Observouse unha maior acumulación de arsénico nos peixes e, de forma inesperada, un aumento do seu peso (biomasa) no tratamento con arsénico. Probablemente, o aumento da agresividade facilitou o acceso aos recursos alimenticios, permitindo que os peixes máis grandes gañasen máis peso. O máis salientable é que a presenza de *biofilm* agravou os efectos da exposición ao arsénico en peixes. En canto á biomasa dos peixes, o *biofilm* actuou de forma antagónica co arsénico, resultando na redución desvantaxosa de peso nos peixes. En canto á bioacumulación, os efectos foron aínda máis graves, xa que na presenza de *biofilm* a captación e/ou asimilación do arsénico nos peixes aumentou. A agresividade nestes animais resultou tamén ser máis forte na presenza de arsénico e *biofilm*. Queremos salientar a importancia da investigación de tipo multidisciplinaria, na que se teña en conta a interacción entre distintos organismos da rede trófica, e integrando o estudo de diferentes efectos sobre os organismos (coma os cambios no comportamento, por exemplo, entre outros), sendo crucial para entender mellor os impactos reais do arsénico nos ecosistemas acuáticos.

O ***capítulo 3*** baséase nun experimento de translocación de *biofilm* levado a cabo durante 51 días no río Anllóns (Galicia), o cal se acha afectado pola actividade mineira, podéndose atopar ata 270 mg kg^{-1} de As^{V} nos seus sedimentos. A translocación realizouse movendo substratos colonizados con *biofilm* dende un tramo do río situado augas arriba da zona mineira (menos contaminada) a un tramo augas abaixo da mesma (máis contaminado e cunha maior proporción da fracción máis móbil de arsénico). O experimento tiña un doble obxetivo: i) examinar o efecto do arsénico sobre o *biofilm* bentónico, e ii) o papel deste *biofilm* na retención e especiación do arsénico na interface auga-sedimento. Detectáronse condicións eutróficas na auga de ámbolos dous tramos (concentracións elevadas de fósforo total disolto e de nitróxeno total), mentres que os sedimentos non se atoparon contaminados por fósforo. Os *biofilms* do tramo máis contaminado acumularon máis arsénico e mostraron unha maior toxicidade potencial (maior relación As/P). Por conseguinte, o seu crecemento viuse reducido á metade do observado nos *biofilms* do tramo menos contaminado.Ditos *biofilms* perderon calidade nutricional (menor contido de N) e mostraron unha maior densidade de bacterias e diatomeas mortas ca nos *biofilm*s non translocados. Ademáis da exposición ao arsénico, outras

condicións ambientais coma o carbono orgánico disolto ou a cuberta do bosque de ribeira (superiores no tramo situado augas abaixo) poderían explicar os efectos observados. En canto ao efecto do *biofilm* sobre o arsénico, a presenza de especies químicas metiladas e menos tóxicas (DMA^V) tanto na auga coma no interior das células do *biofilm*, indican que o biofilm contribuiu á especiación do arsénico na interface auga-*biofilm* bentónico, reducindo a súa toxicidade. Por outra banda, xa que a maior parte do arsénico en sedimentos e auga é arsenato (As^V), a gran cantidade de arsenito (As^{III}) detectado no compartimento extracelular confirmaría a redución de As^V por parte deste *biofilm*.

Os resultados obtidos nesta tese proporcionan información valiosa para comprender a contribución dos *biofilm*s á bioxeoquímica do arsénico en ambientes de auga doce e, especialmente, na interface auga-*biofilm*. Unha vez máis, vólvese a demostrar a importancia do uso dos *biofilm*s e cun enfoque multi-resposta para avalia-la magnitude dos efectos dos contaminantes (substancias tóxicas) sobre os ecosistemas de auga doce. Queremos salientar tamén o valor dos estudos de toxicidade nas interaccións entre diferentes organismos tróficos, coma os *biofilm*s o os peixes, xa que os efectos máis graves observados nestes organismos superiores resultaron desta interacción. Finalmente, os estudos de campo mostran que a resposta dos organismos aos factores ambientais (coma a luz ou a concentración de nutrintes) pode enmascarar o efecto dos contaminantes, polo que é fundamental tomalos en consideración.

La contaminació per arsènic (As) en el medi aquàtic és considerada un problema a nivell mundial, degut als seus efectes sobre la salut humana i la dels ecosistemes. Aquesta contaminació prové de processos naturals (principalment geològics) i d'activitats antropogèniques, com la mineria. En diverses parts del món, se n'han trobat concentracions elevades, esdevenint un problema ambiental. Si més no, la toxicitat de l'arsènic és difícil d'estimar ja que depèn de la interacció entre nombrosos factors. En aigües dolces, l'arseniat (As^V) pot ser absorbit pels microorganismes (especialment pels biofilms) a causa de la seva similitud amb el fosfat (PO_4^{3-}), sent la seva toxicitat depenent de la concentració de fosfat. Els microorganismes tenen un paper clau en la biogeoquímica de l'arsènic (especiació, distribució i cicle) en els sistemes aquàtics, ja que n'incorporen la forma dominant, que és el iAs (arsènic inorgànic) i poden convertir-lo en altres formes químiques. Aquestes reaccions de transformació tenen un gran impacte en el seu comportament ambiental, ja que les diferents formes químiques d'aquest element difereixen en quan a la seva mobilitat i toxicitat. Els peixos són un altre element clau dels ecosistemes aquàtics, sensibles a la presència d'arsènic, la toxicitat del qual pot estar influenciada per la seva interacció amb els microorganismes (és a dir, els biofilms).

Basant-nos en els coneixements actuals sobre ecotoxicologia dels biofilms i biogeoquímica de l'arsènic en ecosistemes d'aigua dolça, aquesta tesi té com a objectius estudiar, en concentracions ambientals i realistes d'arsènic, ***i)*** el paper dels biofilms bentònics en la biodisponibilitat i de-toxicació de l'arsènic en un sistema d'aigua dolça, ***ii)*** els efectes tòxics de l'arsènic en l'estructura i funció dels biofilms fluvials bentònics, fent especial atenció a la resposta de les diatomees, i ***iii)*** la interacció entre els productors primaris i altres organismes superiors (peixos) quan es troben sota l'efecte de l'arsènic.

Al ***capítol 1***, s'exposen els resultats d'un experiment en el que es van investigar els efectes del As^V (130 µg L^{-1} durant 13 dies) en l'estructura i funció del biofilm fluvial en condicions de limitació de fosfat. A més, es van incorporar peixos (*Gambusia holbrooki*) a l'experiment, esperant que aquests proporcionessin més fosfat a les algues i, en conseqüència, les protegissin de la toxicitat de l'arsènic. No obstant això, no es va aconseguir plenament aquesta funció protectora. L'arsènic va inhibir el creixement algal i la seva productivitat. Per altra banda, els bacteris no es van veure afectats. L'arsènic va afectar de manera clara a la comunitat de diatomees, mostrant una forta reducció del biovolum cel·lular; una selecció d'espècies tolerants, en particular *Achnanthidium minutissimum*, i una reducció en la riquesa d'espècies. Els nostres resultats tenen implicacions importants per a l'avaluació dels riscos ambientals de l'arsènic, ja que la concentració utilitzada en aquest experiment (130 µg As L^{-1}) és inferior als criteris de toxicitat aguda establerts per l'Agència de Protecció Ambiental dels Estats Units (US EPA), 340 µg As L^{-1}.

Al ***capítol 2***, es presenten els resultats d'un experiment de laboratori (el mateix que al ***capítol 1***) en el que es van examinar els efectes de l'arsènic (130 µg L^{-1} durant 9 dies) sobre el peix *G. holbrooki,* una espècie invasora. L'experiment incorpora part de la complexitat dels

sistemes naturals mitjançant la inclusió dels efectes interactius del tòxic amb la comunitat microbiana (el biofilm). El nostre objectiu va ser quantificar els efectes de l'arsènic en alguns comportaments complexos i paràmetres físics dels peixos, i avaluar el paper de-toxificador del biofilm. L'agressivitat dels individus de gambúsia va augmentar de manera significativament amb l'arsènic mentre que el moviment de l'opercle disminuí lleugerament (de manera no significativa). A més, ni l'eficiència de captura dels aliments ni el consum es van veure afectats pel tractament amb arsènic. L'arsènic es va bioacumular de manera significativa. Per altra banda, el pes (biomassa) dels peixos va augmentar en el tractament amb arsènic, resultat que no havíem anticipat. Possiblement, l'augment de l'agressió va facilitar l'accés a l'aliment, fent que els peixos guanyessin més pes. La presència de biofilm va alterar la resposta dels peixos a l'arsènic, actuant antagònicament. Pel que fa a la bioacumulació, els efectes van ser encara més greus, ja que en presència de biofilm la captació i/o assimilació d'arsènic va incrementar. L'agressivitat en aquests animals va ser també més important en la presència de biofilm i arsènic. Volem destacar la importància d'una investigació de tipus multidisciplinària, en la qual es tingui en compte la interacció entre diferents organismes de la xarxa tròfica, i integrant l'estudi de diferents efectes sobre els organismes (com els canvis en el comportament, per exemple, entre d'altres), sent crucial per entendre millor els impactes reals de l'arsènic en els ecosistemes aquàtics.

El ***capítol 3*** es basa en un experiment de translocació de biofilm dut a terme durant 51 dies en un riu gallec afectat de l'activitat minera, el riu Anllóns, que conté concentracions de fins a 270 mg As^{V} kg^{-1} en el sediment. La translocació va consistir en transportar substrats colonitzats amb biofilm des d'un tram de riu situat aigües amunt de la zona minera (amb menor contaminació d'arsènic) a un altre tram situat aigües avall (més contaminat i amb una proporció més gran de la fracció més mòbil d'arsènic). L'experiment tenia un doble objectiu *i)* examinar l'efecte de l'arsènic sobre el biofilm bentònic, i *ii)* analitzar el paper d'aquest biofilm sobre la retenció i especiació de l'arsènic en la interfície aigua-sediment. Es van detectar condicions eutròfiques (concentracions elevades de fòsfor dissolt total i nitrogen total a l'aigua) a ambdós trams, si bé els sediments no estaven contaminats amb fòsfor. Els *biofilms* translocats van acumular més arsènic i van mostrar una major toxicitat potencial (major relació de As/P). En concordança, el seu creixement es va reduir a la meitat de l'observat en els biofilms del tram menys contaminat. A més, el biofilm es va fer menys nutritiu (menor contingut de N) i va augmentar el nombre de bacteris i la densitat de diatomees mortes en relació amb el biofilm no translocat. A més de l' exposició a l'arsènic, altres condicions ambientals, com ara el carboni orgànic dissolt (DOC) i la cobertura del bosc de ribera (superiors al tram situat aigües avall) podrien explicar els efectes observats. En relació amb l'efecte del biofilm sobre l'arsènic, la presència d'espècies químiques metilades d'arsènic (DMA^{V}) tant a l'aigua del riu com a l'interior de les cèl·lules del biofilm, indiquen que el biofilm contribueix a l'especiació de l'arsènic en la interfície aigua-biofilm bentònic reduint-ne la seva toxicitat (ja que la forma metilada té menor toxicitat). Per altra banda, atès que la major part de l'arsènic en aigua i en els sediments és

arseniat (As^{V}), l'elevada quantitat de arsenit (As^{III}) detectat en el compartiment extracel·lular del biofilm confirmaria la reducció d'As^{V} per part del biofilm.

Els resultats obtinguts en aquesta tesi proporcionen informació valuosa per comprendre la contribució del biofilm a la biogeoquímica de l'arsènic en ambients d'aigua dolça, i especialment a la interfície biofilm-aigua. A més, es va demostrar una vegada més la importància de l'ús de biofilms i amb un enfoc multi-resposta per avaluar la magnitud dels efectes dels contaminants (substàncies tòxiques) sobre els ecosistemes d'aigua dolça. També volem remarcar en el valor dels estudis de toxicitat en les interaccions entre els diferents organismes tròfics, com ara els biofilms o els peixos, ja que els efectes més greus observats en aquests organismes superiors van ser resultat d'aquesta interacció. Finalment, els estudis de camp ens mostren que la resposta dels organismes als factors ambientals (la llum o la concentració de nutrients) pot emmascarar l'efecte dels contaminants, pel que cal tenir-los en compte.

La contaminación por arsénico (As) de las aguas naturales es un problema mundial debido a sus importantes impactos en la salud humana y en los ecosistemas. Los procesos naturales (procesos geológicos, principalmente) y antropogénicos, como la minería, son las fuentes de contaminación por arsénico en el medio ambiente. Se han publicado altas concentraciones de arsénico en muestras de agua de varias partes del mundo, convirtiéndose en una preocupación ambiental debido a sus efectos nocivos sobre los organismos. La toxicidad del arsénico depende de numerosos factores que interactúan entre sí, lo que hace que los efectos sean difíciles de estimar. En aguas dulces, el arseniato (As^{V}) puede ser absorbido por microorganismos (especialmente como biofilms) debido a su similitud con la molécula de fosfato (PO_4^{3-}), siendo su toxicidad dependiente de las condiciones ambientales de este nutriente. Los microorganismos desempeñan un papel clave en la biogeoquímica del arsénico (en su especiación, distribución y ciclo) en los sistemas acuáticos, ya que incorporan la forma dominante de iAs (arsénico inorgánico), que suele ser el arseniato, y pueden convertirla en otras formas de arsénico. Estas reacciones de transformación tienen un gran impacto en el comportamiento ambiental del arsénico, ya que las diferentes formas químicas de este elemento presentan diferente movilidad y toxicidad. Los peces son otro componente clave de los ecosistemas acuáticos, y sus efectos debidos a la exposición al arsénico podrían verse influidos por su interacción con los microorganismos (es decir, con biofilms).

Basándonos en los conocimientos actuales sobre la ecotoxicología del biofilm y la biogeoquímica del arsénico en los ecosistemas de agua dulce, esta tesis pretende estudiar, bajo concentraciones ambientales realistas, **i)** el papel de los biofilms bentónicos en la biodisponibilidad y destoxificación del arsénico en un sistema de agua dulce, **ii)** los efectos tóxicos del arsénico sobre la estructura y función de los biofilms bentónicos fluviales, prestando especial atención a las respuestas de las diatomeas, y **iii)** la interacción entre estos productores primarios y organismos superiores como los peces cuando se encuentran bajo el efecto del arsénico.

Así, en el ***capítulo 1*** se realizó un experimento que combinaba descriptores ecológicos y ecotoxicológicos para investigar los efectos del As^{V} (130 µg L^{-1} durante 13 días) sobre la estructura y función del biofilm fluvial y bajo condiciones de limitación de fosfato. Además incorporamos peces (*Gambusia holbrooki*) en nuestro sistema experimental, esperando que pudiesen proporcionar más fosfato a las algas y, en consecuencia, protegerlas contra la toxicidad de arsénico. Sin embargo, este papel protector no se logró por completo. El arsénico inhibió el crecimiento y la productividad de las algas, pero no el de las bacterias. La comunidad de diatomeas fue claramente afectada mostrando una fuerte reducción en el biovolumen celular y una selección de especies tolerantes -en particular *Achnanthidium minutissimum*- causando, por tanto, una reducción en la riqueza de especies. Nuestros resultados tienen implicaciones importantes para la evaluación de los riesgos ambientales del arsénico, ya que la concentración experimental utilizada de este elemento fue menor que la concentración límite establecida por la Agencia de Protección Ambiental de los Estados Unidos (US EPA) para la toxicidad aguda, 340 µg As L^{-1}.

En el ***capítulo 2***, se examinaron los efectos de la exposición al arsénico en el pez mosquito *G. holbrooki* (130 µg L^{-1} a lo largo de 9 días) en un experimento de laboratorio (el mismo que en el ***capítulo 1***) que incorporaba parte de la complejidad de los sistemas naturales al incluir los efectos interactivos del tóxico con la comunidad microbiana (el biofilm). Nuestro objetivo era cuantificar los efectos del arsénico sobre algunos comportamientos complejos y sobre parámetros físicos en los peces y evaluar el papel detoxificador del biofilm. La agresividad aumentó significativamente en presencia de arsénico, mientras que el movimiento opercular disminuyó de forma no significativa, y ni la eficiencia ni el consumo de la captura de alimentos se vieron notablemente afectados. La bioacumulación aumentó con el arsénico y, de forma inesperada, también lo hizo la biomasa de los peces. Posiblemente el aumento de la agresividad facilitó la defensa por los recursos alimentarios permitiendo que los peces más grandes aumentaran de peso. Lo más destacable es que la presencia de biofilm agravó los efectos de la exposición al arsénico en los peces. En cuanto al aumento de la biomasa de peces, el biofilm actuó de forma antagónica con el arsénico, dando como resultado una reducción desventajosa del peso ganado en los peces. En cuanto a la bioacumulación, los efectos fueron aún más graves, ya que las algas también contenían arsénico y, por tanto, proporcionaban un aumento de absorción y/o asimilación de arsénico en los peces. La agresividad en estos animales resultó también más importante en presencia de algas y arsénico. Queremos destacar la importancia de una investigación de tipo multidisciplinaria, en la que se tenga en cuenta la interacción entre distintos organismos de la red trófica, e integrando el estudio de distintos efectos posibles en los organismos (como los cambios en el comportamiento, por ejemplo, entre otros), siendo fundamental para entender mejor los impactos reales del arsénico en los ecosistemas acuáticos.

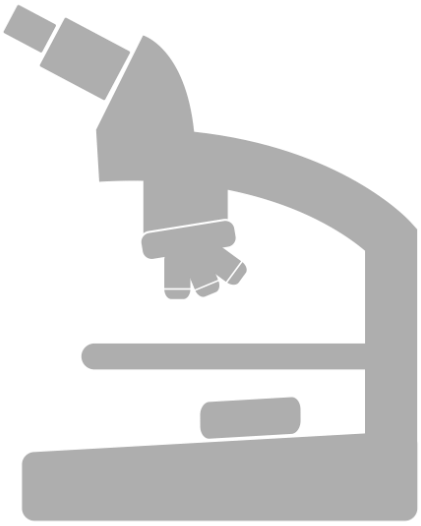
En el ***capítulo 3***, se realizó un experimento de translocación de biofilm durante 51 días en un río impactado por la minería, el río Anllóns (Galicia, noroeste de España), donde se suelen encontrar concentraciones de hasta 270 mg As^{V} kg^{-1} en sus sedimentos. La translocación se realizó moviendo los sustratos colonizados por biofilm desde aguas arriba (menos contaminado) hasta aguas abajo del área de la mina (en el punto más contaminado por arsénico y, además, más fácilmente extraíble del sedimento al agua), para explorar el efecto del arsénico en biofilms bentónicos y el papel de estos biofilms sobre la retención y especiación de arsénico en la interfaz agua-sedimento. Se detectaron condiciones eutróficas (altos niveles de fósforo total disuelto y nitrógeno total) en el agua en ambos puntos de muestreo, mientras que los sedimentos no se consideraron contaminados por fósforo. Los biofilms translocados acumularon más arsénico y presentaron mayor toxicidad potencial (mayor relación As/P). En concordancia, su crecimiento se redujo a la mitad de lo observado en aquellos no translocados. Además, perdieron calidad nutricional (menor contenido de N) y mostraron mayor densidad de bacterias y de diatomeas muertas que los biofilms no translocados. A mayores de la alta concentración de arsénico a la que estaban expuestos, otras condiciones ambientales tales como una mayor cantidad de carbono orgánico disuelto y de cubierta ribereña en el sitio de muestreo situado aguas abajo de la mina podrían contribuir a dichos efectos. En el

compartimiento intracelular de los biofilms, así como en el agua del río, se encontraron especies de arsénico metiladas menos tóxicas (principalmente, DMA^{V}), indicando que el *biofilm* contribuyó a la especiación del arsénico en la interfaz agua-biofilm bentónico, reduciendo así su toxicidad. Dado que la mayoría de arsénico en sedimentos y agua era As^{V}, la gran cantidad de arsenito (As^{III}) detectada en el compartimiento extracelular de estos biofilms puede también confirmar la existencia de un proceso de reducción de As^{V} por biofilms.

Los resultados obtenidos en esta tesis proporcionan información valiosa para comprender la contribución de los biofilms a la biogeoquímica del arsénico en ambientes de agua dulce y, específicamente, en la interfaz agua-biofilm. Una vez más, se volvió a demostrar la importancia del uso del biofilm y con un enfoque multi-respuesta para evaluar la magnitud de los efectos de los contaminantes (substancias tóxicas) sobre los ecosistemas de agua dulce. También queremos destacar el valor del estudio de la toxicidad en las interacciones entre diferentes organismos tróficos, como los biofilms y los peces, ya que los efectos más graves observados en los peces resultaron de esta interacción. Finalmente, los estudios de campo muestran que la respuesta de los organismos a los factores ambientales (como la luz o la concentración de nutrientes) puede enmascarar el efecto de los contaminantes, por lo que es fundamental tomarlos en consideración.

1. General Introduction

2. MATERIALS AND METHODS



In this general section of Materials and Methods, the experimental designs and the main techniques used in the thesis are indicated. Different analyses were done in the three studies of this thesis. Laboratory experiments **(*Chapter 1*** and ***2*)** were performed using 12 artificial stream channels, while the field experiment **(*Chapter 3*)** was conducted in an Atlantic river: the Anllóns River (Galicia, NW Spain). In all these studies, biofilms were developed on artificial hard substrates (glass tiles), which are typically used in biofilm investigations as substitutes for natural rocky substrates (Mora-Gómez *et al.* 2016). The methodology followed in this thesis is summarized in this section but described in more detail within each chapter.

1. EXPERIMENTAL DESIGNS

Laboratory experiments **(*Chapter 1*** and ***2*)**

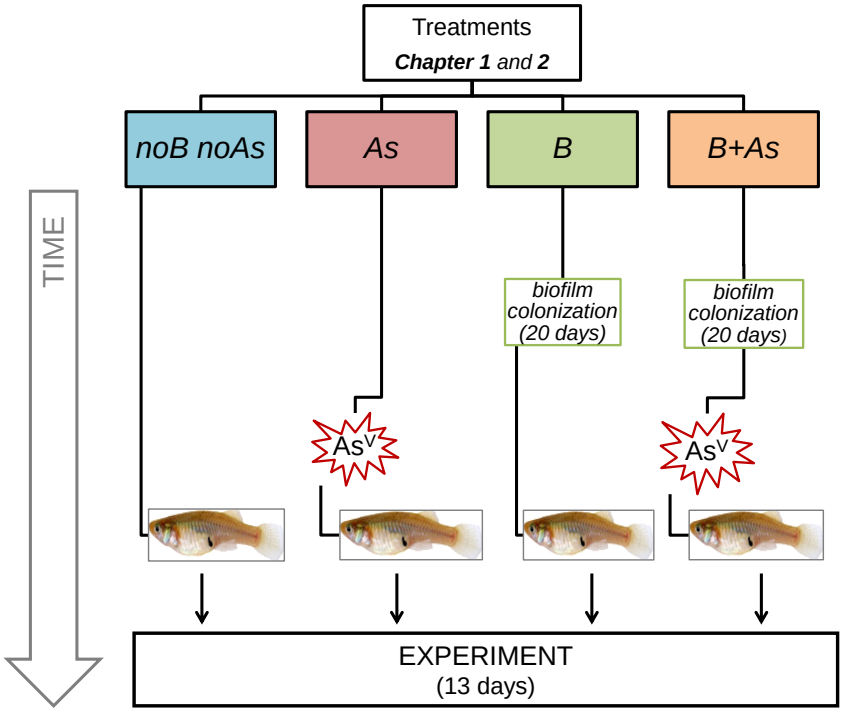
For the laboratory experiments, experimental units consisting in artificial channels simulating streams were used, some of them with colonized natural-biofilm on the bottom, and all of them with fish (placed separately). Different treatments were constituted with biofilms, fish and arsenic (Fig. 1.a):

- ✓ *noB noAs* (without biofilm or arsenic) in ***Chapter 1***, named "*control*" in ***Chapter 2***
- ✓ *As* (with arsenic only) in ***Chapter 1***, named *A* in ***Chapter 2***
- ✓ *B* (with biofilm only) in ***Chapter 1*** and ***2***
- ✓ *B+As* (with both biofilm and arsenic) in ***Chapter 1***, named *B+A* in ***Chapter 2***.

Field experiment **(*Chapter 3*)**

For the field experiment, the experimental units consisted on cement cobbles with fixed glass tiles colonized by natural biofilm, placed horizontally upstream and downstream a mine area in the Anllóns River riverbed and, therefore, exposed to different arsenic concentrations. The two sites constituted the different treatments of this study (Fig. 1.b).

a)



b)

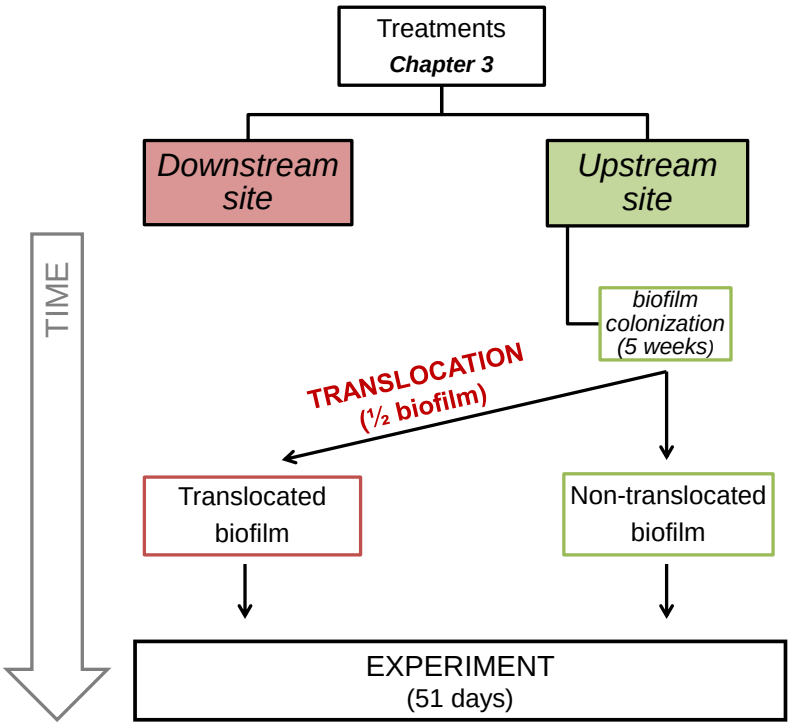


Figure 1 Diagrams of the experimental designs peformed in the different studies of this thesis. Specifically, it is shown how it was performed or considered the different treatments in a) ***Chapter 1*** and ***2***; and b) in ***Chapter 3***. See main text on the respective chapters for more information.

2. MAIN ANALYTICAL METHODS

Environmental light intensity (µmol photons m^{-2} s^{-1}), using a light sensor (LI-COR Inc., Lincoln, Nebraska, USA), in ***Chapter 3***:

- ✓ *Riparian cover*
- ✓ *Light reaching benthic biofilm*

Water chemical analyses

Chapter 1 and ***2***:

- ✓ *Physical* and *chemical parameters* (water temperature, dissolved oxygen, electrical conductivity and pH) using HQ Portable Meters, HQ40d18, HACH Company.
- ✓ *Inorganic phosphate (iP)* concentration, by a modified molybdenum blue method (Carvalho *et al.* 1998) to avoid arsenate interference.
- ✓ *Total dissolved arsenic concentration*, using Inductively Coupled Plasma Spectrometry (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, Denmark)

Chapter 3:

- ✓ *Suspended solids* (SS; according to APHA, 1995)
- ✓ *Total dissolved nitrogen* (TN), using the Kjeldhal method (following UNE-EN 25663:1994)
- ✓ *Total dissolved phosphorus* (TP; following APHA, 2005)
- ✓ *Soluble reactive phosphorus* (SRP; according to Murphy and Riley 1962)
- ✓ *Dissolved organic carbon* (DOC), using a Total Organic Carbon Analyser Model TOC-5000 (Shimadzu, Kyoto, Japan
- ✓ *Total dissolved As*, using ICP-MS (Varian 820MS)
- ✓ *Arsenic speciation*, using High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Spectrometry, HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS).

Sediment analyses (*Chapter 3)*:

- ✓ *pH* and *Eh* determination (in the field), using a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, Eibar, Spain)
- ✓ *Bioavailable arsenic* measurement *in situ* using diffusive gradients in thin films (DGT) (DGT Research Ltd., Lancaster, UK).

In the <2mm fraction:

- ✓ Determination of the *particle size distribution* (2, 1, 0.5, 0.25, 0.1 and 0.05 mm) by dry sieving.
- ✓ Extraction of the arsenic content from this sediment fraction (extracted with phosphate buffer, following Gleyzes *et al.* 2002), named *easily-extractable arsenic concentrations* in the text, and further measure of total arsenic concentration by ICP-MS.
- ✓ Determination of arsenic *speciation* in the previous extracts, measured by HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS).

In the <2mm fraction, after milled and sieved (<50 µm):

- ✓ *Total phosphate* (TP; following Murphy and Riley 1962),
- ✓ *Total Kjeldhal nitrogen* (TN, following Guitián and Carballas 1976)
- ✓ *Total organic matter* (OM), through calcinations at 450 °C during 2h following the UNE-EN 13039 standard (AENOR 2012).
- ✓ Determination of *total arsenic concentration*, (following Devesa-Rey *et al.* 2008), using a X-ray fluorescence spectrometry (custom built, equipped with a Philips high-voltage generator and a Mo anode of 2.2 Kw as X-ray source).

Biofilm measurements

Chapter 1:

- ✓ *Chlorophyll-a fluorescence-related endpoints,* using PhytoPAM (Pulse Amplitude Modulated) fluorimeter (HEINZ WALZ, Effeltrich, Germany)
- ✓ *Benthic chlorophyll-a,* extracted with 90% acetone (following the method described in Jeffrey and Humphrey 1975)
- ✓ *Bacterial abundance (life-dead method)*, using the double staining Live/Dead BacLight Bacterial Viability Kit (Molecular Probes), and subsequent cells counting using epifluorescence microscopy at a magnification of 1000x in immersion oil (Nikon E600, Tokyo, Japan).
- ✓ *Diatom community identification* (following Leira and Sabater, 2005, for samples preparation; Krammer and Lange-Bertalot 1986–1991 for species identification), and *diversity indices* (Shannon and Weaver 1949; Pielou 1975) using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of 1000x for species identification.
- ✓ *Diatom biovolume or cell size determination* (following a set of geometrical shapes proposed by Hillebrand *et al.* 1999; see Fig. 2), using a light microscope with Nomarski differential interference contrast optics at a magnification of 1000x.

Arsenic content in biofilm (using ICP-MS):

- ✓ *Total arsenic accumulated in biofilm,* previously freeze-dried and digested with HNO_3 (65%) using a high performance microwave digestion unit (Milestone, Ethos Sel, Sorisole (BG), Italy)

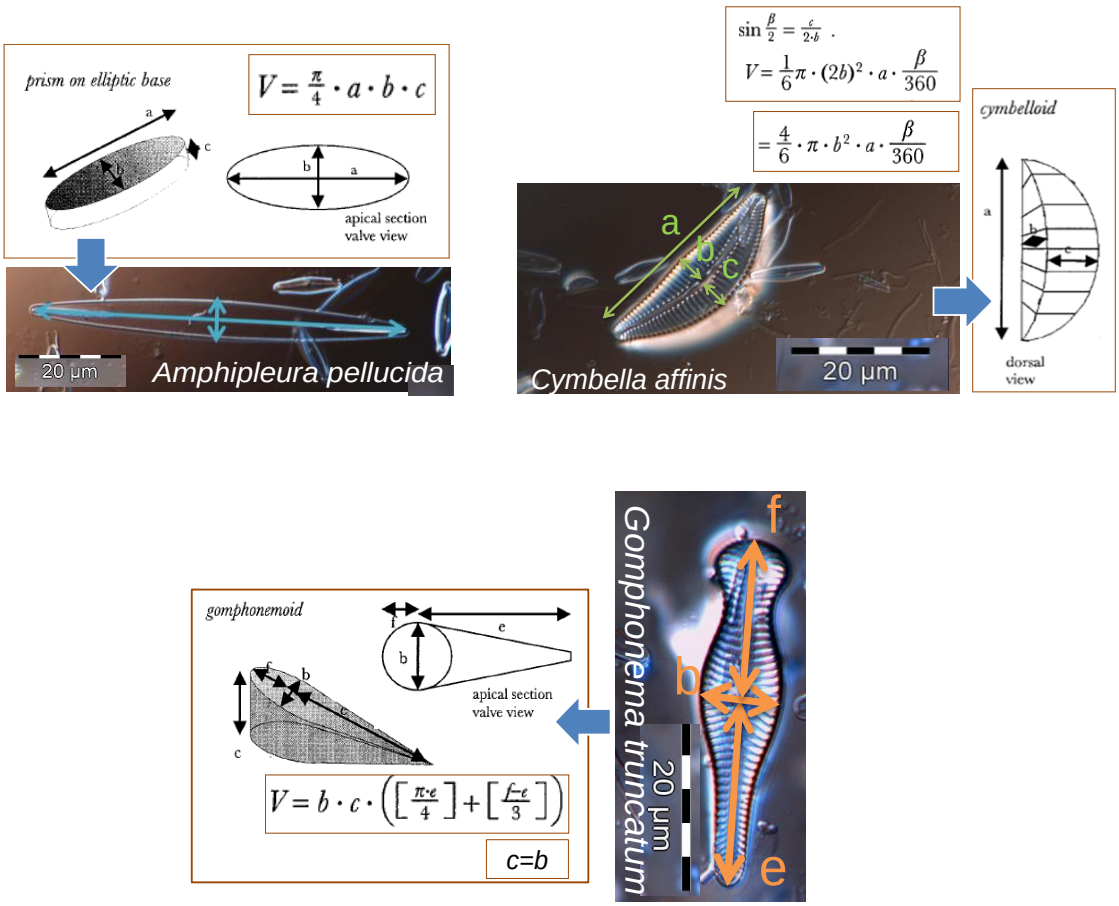


Figure 2 Examples of real measures done to diatom cells, following the set of geometrical shapes proposed by Hillebrand *et al.* (1999).

Chapter 3:

- ✓ *In vivo fluorescence measurements* (*Fo, Ymax, Yeff* parameters), using MINI-PAM fluorimeter**.**
- ✓ *Total dry weigh biomass* (DW).
- ✓ *Elemental composition* (C:N:P), using an elemental analyser (PerkinElmer 2400) for C and N; and ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE) for P determination (Sterner and Elser 2002; Muñoz *et al.* 2009; Scharler *et al.* 2015).
- ✓ *Bacterial density* (adapted from Amalfitano *et al.* 2009 and Perujo *et al.* 2015), determined by flow cytometry (FACSCalibur, Becton–Dickinson)**.**
- ✓ *Quantitative estimates of live diatom community* (following Morin *et al.* 2010), using a Nageotte counting chamber and a light microscope (Nikon E600, Tokyo, Japan).
- ✓ *Relative abundances of the diatom species* (Krammer and Lange-Bertalot 1986-1991; and Coste and Rosebery 2011) using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of 1000x, and *diatom diversity indices* (Shannon and Weaver 1949; Pielou 1975).

Arsenic content in biofilm*:*

- ✓ *Total arsenic accumulated* (measured by ICP-MS) in biofilm samples previously freeze-dried and digested with HNO_3 (65%) and H_2O_2 (31%) in a high performance microwave digestion unit.
- ✓ *Extracellular and intracellular arsenic content* (following Levy *et al.* 2005 for the measures in the extracellular compartment, and Myashita *et al.* 2009 for the intracellular compartment): determination of total arsenic (ICP-MS) and arsenic speciation (HPLC-ICP-MS).

Fish measurements (***Chapter 2***):

- ✓ *Direct behavior:* frequencies of operculum movements were recorded during 1 minute.
- ✓ *Complex behaviors:* the frequencies of aggressive interactions initiated for each fish (mostly females) as lunges, chases and bites were also recorded.
- ✓ *Physiological parameters:*
  - o Change in biomass, by weighting fish at the beginning and at the end of the experiment.
- ✓ *Total arsenic accumulation in female fish tissue (liver and gills)* in previously frozen, freeze dried and finally digested samples with HNO_3 (65%) and H_2O_2 (31%).

Table 1 Summary of the different analytical methods used in a) environmental samples (light, river water and sediments) and b) biological samples (biofilm and fish), in each chapter (***Ch***) of this thesis.

a)

		Ch 1	***Ch 2***	***Ch 3***
LIGHT	Riparian cover			
	Light arriving benthic biofilms			
WATER	Physical and chemical parameters			
	SS			
	TN			
	TP			
	iP modified molybdenum blue method			
	SRP			
	DOC			
	Total As			
	As speciation			
SEDIMENT	pH and Eh			
<2mm fraction	Particle size distribution			
	Easily-extractable As concentrations			
	Extracts As speciation			
<2mm fraction, after milled and sieved (<50 µm)	TP			
	TN			
	OM			
	Total As concentration			
DGTs	Total As concentration			

b)

		Ch 1	*Ch 2*	*Ch 3*
BIOFILMS				
	In vivo fluorescence measurements			
	Benthic chl-a			
	DW			
	C:N:P			
	Bacterial density			
	Bacterial viability (L/D)			
	Live diatom			
	Diatom specific relative abundances			
	Diatom biovolume or cell size determination			
	Diatom specific diversity indices			
Arsenic content	Total bioaccumulated-As			
	Extracellular and intracellular As			
	As speciation			
FISH				
Direct behavior	Frequencies of operculum movements			
Complex behaviors	Frequencies of aggressive interactions (lunges, chases and bites)			
Physiological parameters	Change in biomass			
Arsenic content	Total bioaccumulated-As in tissue			

Table 2 Summary of the different statistical analysis used in each chapter (***Ch***) of this thesis

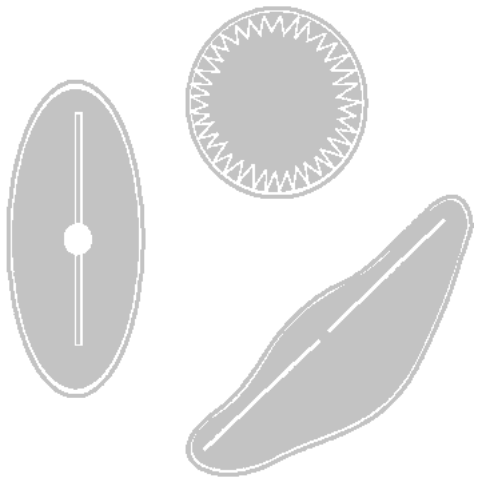
STATISTICAL ANALYSIS	Purpose of the analysis	*Ch 1*	*Ch 2*	*Ch 3*
Student's t-tests	To assess differences in specific diatom cell biovolume between treatments (*B* and *B+As*)			
One-Way ANOVAs	To assess differences in parameters measured only at the end of the experiment (chlorophyll-*a* content, arsenic bioaccumulated in biofilm and fish) and other diatom metrics, between treatments (*B* and *B+As*) only during the *As+Fish* period			
	To assess differences in diatom diversity indices (*S, H, J*) between sites (*Downstream* and *Upstream*)			
Two-Way ANOVAs	To assess differences in live bacteria between treatments (*B*, *B+As*) and in physical and chemical parameters between treatments (*noB noAs*, *As*, *B*, *B+As*), across periods (*Biofilm colonization, Arsenic* and *As+Fish)*			
	To assess differences in bacterial density, and in the arsenic accumulation (total arsenic and species) in different biofilm compartments (*rinse solution, extracellular, intracellular*) between sites (*Downstream* and *Upstream*), across time.			
Two-Way Repeated Measures ANOVA	To assess differences in biofilm photosynthetic parameters between treatments (*B*, *B+As*) and time (biofilm colonization days)			
	To assess differences in biofilm metrics and light measurements between sites (*Downstream* and *Upstream*) and time (*translocation days)*			

STATISTICAL ANALYSIS	Purpose of the analysis	*Ch 1*	*Ch 2*	*Ch 3*
Generalized Estimating Equation (GEE)	To assess differences in fish aggression (*Direct behavior*) between treatments (*C, B, A, B+A*), controlling for time (covariate)			
	To assess differences in capture efficiency and consumption (*Complex behaviors)* by fish between treatments (*C, B, A, B+A*)			
	To assess differences in the change in fish biomass (*Physical parameter)* between treatments (*C, B, A, B+A*), controlling for the total length of each fish (covariate)			
Factorial Generalized Linear model (GLM)	To assess differences in arsenic bioaccumulation in fish tissue (*Physical parameter)*			
Fitting to a 3-parameter log-normal curve	To assess changes in biofilm biomass during time (*colonization* and *experiment* period)			
Non-Metric Multidimensional Scaling plot (NMDS)	To detect possible variations of diatom community composition between sites (*Downstream* and *Upstream*), based on Bray Curtis distance			
Multi-Response Permutation Procedures (MRPP)	To test for inter-site (*Downstream* and *Upstream*) *versus* intra-site heterogeneity in diatom community structure (Zimmerman *et al.* 1985), based on Bray Curtis distance			
Redundancy Data Analysis (RDA)	To assess the effect of the environmental factors on the biological responses, using variables taken at both sites (*Downstream* and *Upstream*) and every sampling day			

3. RESULTS

CHAPTER 1

SHORT-TERM ARSENIC EXPOSURE REDUCES DIATOM CELL SIZE IN BIOFILM COMMUNITIES



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ABSTRACT

Arsenic (As) pollution in water has important impacts for human and ecosystem health. In freshwaters, arsenate (As^V) can be taken up by microalgae due to its similarity with phosphate molecules, being its toxicity aggravated under phosphate depletion. An experiment combining ecological and ecotoxicological descriptors was conducted to investigate the effects of As^V (130 µg L^{-1} over 13 days) on the structure and function of fluvial biofilm under phosphate-limiting conditions. We further incorporated fish (*Gambusia holbrooki*) into our experimental system, expecting fish to provide more available phosphate for algae and, consequently, protecting algae against arsenic toxicity. However, this protective role was not fully achieved. Arsenic inhibited algal growth and productivity but not that of bacteria. The diatom community was clearly affected showing a strong reduction in cell biovolume; selection for tolerant species, in particular *Achnanthidium minutissimum*; and a reduction in species richness. Our results have important implications for risk assessment, as the experimental arsenic concentration used was lower than acute toxicity criteria established by the US EPA.

1. BACKGROUND

Arsenic (As) is a widely distributed metalloid in natural ecosystems and it is considered a Priority Pollutant, being the second most common inorganic contaminant in the original National Priority List (NPL), created by the US EPA (Davis *et al.* 2001). The Aquatic Life Criteria (US EPA 2014) establishes at 340 µg L^{-1} the limit of arsenic concentration during an acute arsenic exposure in freshwaters (Criteria Maximum Concentration, CMC).

In rivers, water contaminated with arsenic have baseline concentrations ranging between 0.1 – 2.1 µg L^{-1}, with an average of 0.8 µg L^{-1} (Smedley and Kinniburgh 2002; Rahman *et al.* 2012).

A key factor in arsenic toxicity is its chemical speciation, and biological activity plays a major role in arsenic biogeochemistry (speciation, distribution and cycling) in freshwaters (Smedley and Kinniburgh 2005; Rahman *et al.* 2012). The pentavalent arsenate oxyanion (As^V) is the stable and predominant arsenic species in well oxygenated aquatic environments such as river and lake waters and oxic seawater (Smedley and Kinniburgh 2005). Little is known about As^V toxicity in algae, especially in rivers, although some studies have found that arsenic is toxic to freshwater microalgae at high concentrations, particularly at low ambient concentrations of phosphate (referred in this chapter as PO_4^{3-} or P) (e.g. Levy *et al.* 2005; Wang *et al.* 2013). Arsenate is an analog of phosphate and algae may uptake both molecules through phosphate transporters, because they share the same internalization mechanisms (Guo *et al.* 2011; Wang *et al.* 2013). It could thus be anticipated that As^V modes of toxic action might depend on phosphate availability in the environment and subsequent synthesis of phosphate transporters (Miot *et al.* 2009). In fact, aggravated arsenic toxicity has been found under phosphate depletion

in several freshwater experiments (e.g. Levy *et al.* 2005; Wang *et al.* 2013; 2014; Rodriguez Castro *et al.* 2015). In literature, laboratory experiments generally use high arsenic concentrations, and field studies are more focused on lakes. Therefore, more research on As^V toxicity and its relationship with phosphate in environmental systems is necessary, especially in rivers.

Biofilms are crucial in ecosystem functioning and have an excellent ability to degrade and transform pollutants (Mora-Gómez *et al.* 2016). In rivers, evidence of the link between metal exposure (water concentration) and metal content in biofilms has already been demonstrated, highlighting their likely effects through the trophic chain (Guasch *et al.* 2012). Biofilm complexity produces a large panel of functional and structural endpoints in both autotrophs and heterotrophs, which are often used to assess the effects of chemicals on biofilm communities (Sabater *et al.* 2007). For instance, photosynthetic parameters (Corcoll *et al.* 2012a) are early warning functional endpoints, which are usefully complemented by more structural information.

The diatom component of fluvial biofilms is among the most studied of algal organisms, due to their cosmopolitanism and predominance. Their sensitivity to many environmental factors has resulted in their wide use in water quality assessment (e.g. Coste *et al.* 2009). They respond quickly to environmental changes such as water metal contamination, as extensively documented in field and laboratory experiments. Responses of diatoms to metal pollution have generally been detected at the individual level (e.g. size, growth form, and morphological abnormalities) and/or through changes to community structure (replacement of sensitive species by tolerant ones, or decrease in species diversity) (Morin *et al.* 2012). Concerning the whole algal component, alterations of algal succession (i.e., the temporal variation in community composition during colonization, from diatoms at the beginning to cyanobacteria and filamentous green algae at the end) in biofilms exposed to metals, such as copper and zinc, have already been documented (Serra 2009; Bonet 2013).

The use of different trophic levels, e.g. fish and biofilm together, give complementary results (e.g. Griffith *et al.* 2005; Passy 2012) and may interact to modify expected toxicity (***Chapter 2***). Fish are highly sensitive to small environmental changes and arsenic is considered to be one of the most toxic elements to them (Bhattacharya *et al.* 2007). One fish species for which arsenic impacts have been demonstrated is the mosquitofish *Gambusia holbrooki* (Newman *et al.* 1989; Moeller *et al.* 2003).

In this study, we investigated the effects of short term arsenate (As^V) exposure on fluvial biofilm under the influence of fish (*Gambusia holbrooki*). Therefore, by adding fish we implemented a complex scenario in a laboratory experiment that was consequently much closer to reality than those used in classic toxicity tests. We conducted an experiment simulating a well oxygenated environment, to ensure that As^V was the dominant arsenic species, and biofilm was grown under conditions of phosphorus limitation, which is likely to lead to high arsenic toxicity.

We expected to see arsenic effects on biofilm at different scales, from diatom community structure to general algal and bacterial behavior. Effects on biofilm function and structure were anticipated, but we had no *a priori* assumptions about the intensity of effects, as both arsenate concentration and time of exposure were relatively low. Particular attention was given to diatoms, with the expectations that arsenic would cause a change in species composition and in their biovolume or cell size.

2. MATERIALS AND METHODS

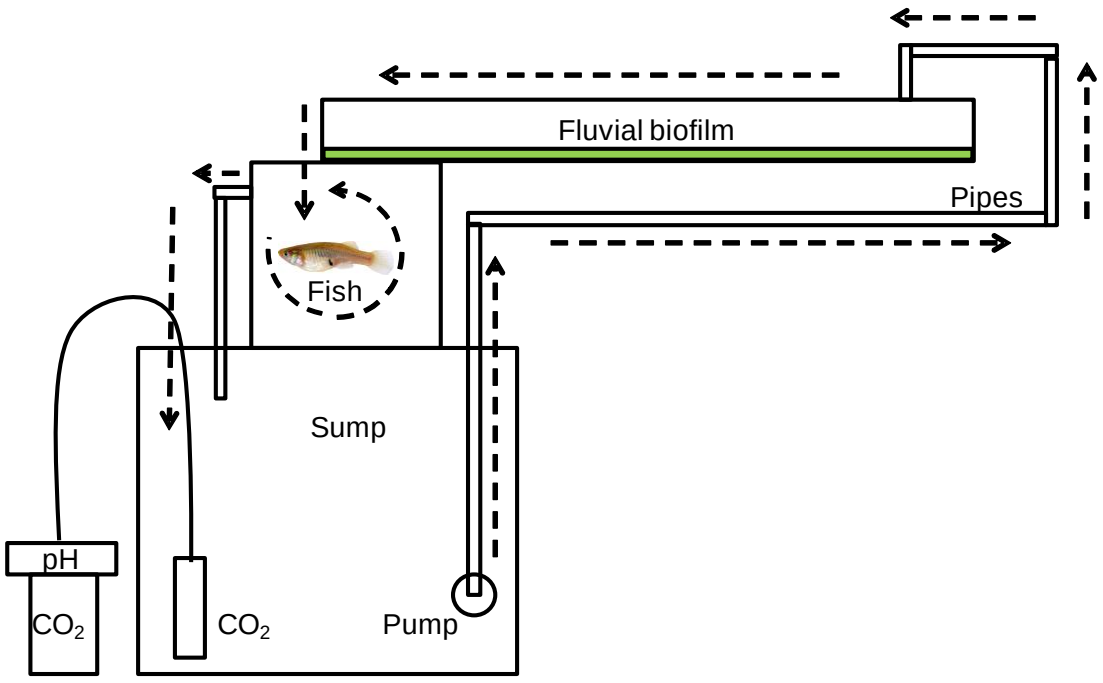
2.1. Experimental units

We constructed twelve experimental units, each consisting of a long channel (90 x 8.5 x 7.5 cm^3), as a laboratory stream, containing small (1.2 x 1.2 cm^2) and larger (7 x 7 cm^2) sandblasted glass tiles placed on the floor for biofilm colonization; a four-liter aquarium (31.5 x 11 x 31.5 cm^3) to house the fish and a sump tank (60 x 25 x 75 cm^3) filled with approximately 90 liters of water. This large volume of water ensured that changes in water chemistry were minimized. Each experimental unit was an independent system recirculating dechlorinated tap water in a constant and controlled flow rate using a hose and a submersible pump (EHEIM Universal Pumps, Germany) placed in the sump tank. Water was thus pumped from the sump tank to the head of the algal biofilm channel, passed through this channel into the 4 liter fish aquaria, where it circulated and finally returned to the sump tank (Fig. 1). The physicochemical composition of the dechlorinated tap water was characterized (see methodology in the "Water chemical sampling and analyses" section later): it is neutral water (pH 7.55±0.09), with conductivity 446.83±8.57 µS cm^{-1}, O_2 concentration 8.66±0.03 mg L^{-1} and P- PO_4^{3-} 3.70±2.93 µg L^{-1} (determined by a modified molybdenum blue method of Carvalho *et al.* 1998). Concentrations of major cations and anions dissolved in water were previously analyzed using ion-chromatography (Metrohm Ltd., Herisau Switzerland). Anions were measured using a METROSEP A SUPP 5 column and $NaHCO_3$ (84 mg L^{-1}) and Na_2CO_3 (229 mg L^{-1}) as eluents. Cations were measured using a METROSEP C 2 column and tartaric acid (2,3-dihydroxybutanedioic acid; 4 mM) and dipicolinic acid (pyridine-2,6-dicarboxylic acid; 0.75 mM) as eluents. The water contains: NO_3^- 12.73±3.58 mg L^{-1}, NO_2^- <0.01 mg L^{-1}, NH_4^+ <0.1 mg L^{-1}, SO_4^{2-} 43.74±1.03 mg L^{-1}, Ca^{2+} 33.38±1.27 mg L^{-1}, Mg^{2+} 8.43±0.35 mg L^{-1}, Na^+ 27.12±1.70 mg L^{-1} and Cl^- 46.64±0.73 mg L^{-1}.

All experimental units were housed in a room under controlled environmental conditions. Temperature was maintained at 19.5 ± 0.5 °C. Water pH was automatically controlled with a system based on CO_2 addition (JBL Proflora m630: JBL, Ludwigshafen, Germany), from 7.5 to 7.9, to provide enough inorganic carbon for algal growth. Light irradiance without heat (120W LEDs Grow Light, Lightech, Girona, Spain) was also automatically controlled, with a 12h:12h light:dark cycle.

Mosquitofish (*Gambusia holbrooki*) were collected from the Ter, Fluvià and Muga rivers (NE Spain) and transported to the laboratory where they were placed in 60 L stock aquaria (60 cm × 30 cm × 32 cm) each containing conditioned water and a filtered air supply. *Gambusia holbrooki* from all three rivers were housed together. Fish were fed to satiation once per day with commercial food flakes or defrosted frozen bloodworms (*Chironomus* spp.) and were able to acclimate to captivity conditions for at least 6 months, with a further month to acclimate to experiment-specific environmental parameters (e.g. temperature). During the experiments, fish and biofilms were not together but separated into different compartments of the experimental units: fish were placed in the four-liter aquarium, while biofilms were grown in the channels. This ensured that fish could not graze biofilms. Fish were also fed to satiation during the experiment with the commercial frozen bloodworms (*Chironomus* spp.).

a)



b)

c)

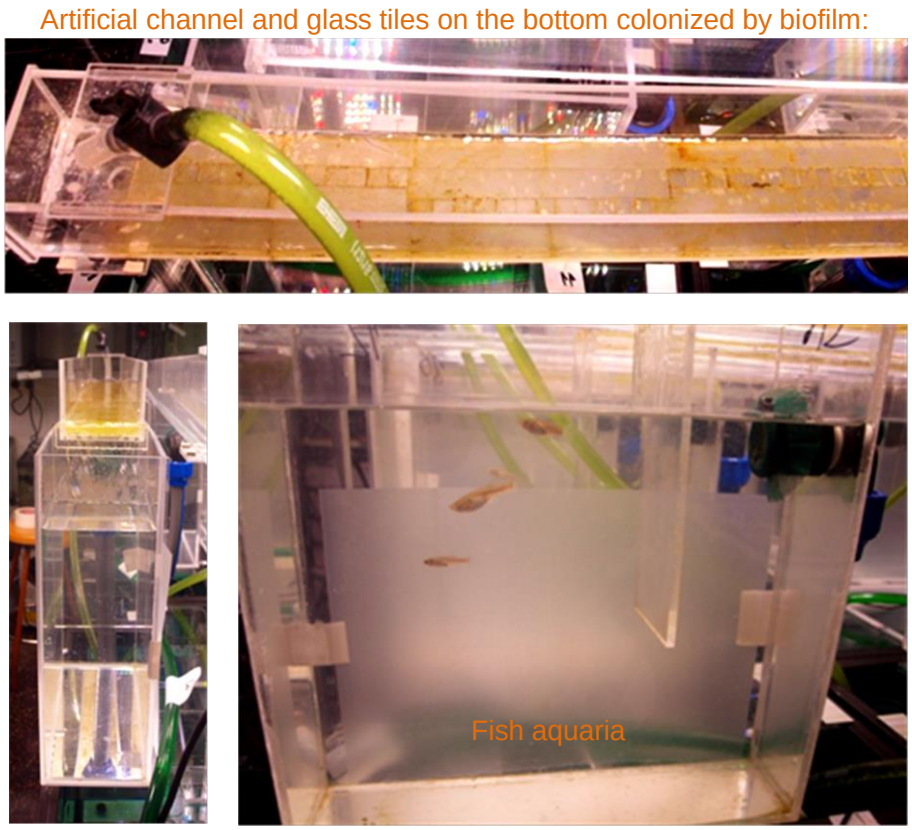


Figure 1 Experimental unit: a) schematic diagram (the dashed arrows show the direction of water flow); b) and c) are pictures of the experimental units and the detail of the different parts (see main text for details).

2.2. Experimental design

Our experimental design consisted of three replicates of each of four different treatments. Treatments were: *noB noAs*, arsenic (with arsenic only), *B* (with biofilm only) and *B+As* (with both biofilm and arsenic) (Fig. 2). First, natural biofilm inoculum was added to six of the experimental units and allowed to grow and colonize the sandblasted glass tiles (*Biofilm colonization period*). After colonization (20 days), As^V (130 μg L^{-1}) was added to six of the experimental units (*As period*). This time lag was expected to influence dissolved arsenic concentration in the *B+As* treatment due to uptake and/or adsorption. Four days later, four fish (1 male, 3 females) were added to each experimental unit, such that each contained the same fish biomass (*As+Fish period*). The experiment ended after 33 days of biofilm colonization. Thus, biofilms were exposed to As^V for 13 days, and fish exposure lasted for 9 days (Fig. 2).

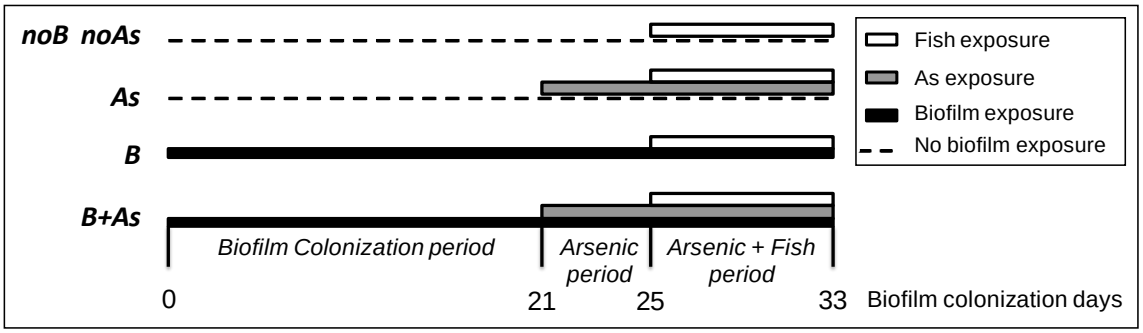


Figure 2 Timeline (biofilm colonization days) of this experimental study. White, gray and black rectangles represent the exposure time of fish, arsenic and fluvial biofilm respectively, in the experimental units. Black dotted lines represent absence of biofilm in the experimental units. Time was divided into three parts: *Biofilm Colonization period, Arsenic period, Arsenic+Fish period* (see main text for details).

Biofilm colonization period

Biofilm was colonized on sandblasted glass tiles (1.44 cm^2 and 49 cm^2), placed at the bottom of each channel. Several rocks were chosen at random from the upstream zone of the Llémena Stream (NE Spain), a small calcareous tributary of the Ter River that had minimal human impact. Rocks were transported to the laboratory in boxes filled with river water that were placed inside a portable fridge to ensure biofilms were always wet and fresh. Once in the laboratory, all rock surfaces were scraped and, then scraped biofilm was added as an inoculum to the channel (artificial stream) of each experimental unit (the same volume in each one) twice per week during the three-week colonization period (from biofilm colonization day 1 to 20). Once per week, water levels were adjusted and 10 μg L^{-1} of phosphate (KH_2PO_4, Merk, Darmstadt, Germany) were added to reproduce phosphate limiting conditions for algal growth (Dodds *et al.* 1998). The use of clean artificial substrates, instead of already colonized rocks, allowed monitoring of biofilm colonization and algal succession in experimental conditions.

Biofilm development was controlled regularly, measuring the *Fo* parameter (the minimal fluorescence yield of a dark adapted cell) that gives a fluorescence proportional to the biofilm chlorophyll-a concentration. This parameter was obtained by using the PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany), as detailed in the "Biofilm measurements" section.

Arsenic period

After 20 days of colonization, young biofilms, but close to maturity, had developed indicating the best time to begin the arsenic exposure while avoiding senescence at the end of the experiment. Thus, on biofilm colonization day 21, As^V solution as $NaH_2AsO_4.7H_2O$ (Merk, Darmstadt, Germany) was added to six of the experimental units without (*As* treatment) and with (*B+As* treatment) biofilm, to reach the nominal concentration of 130 µg As^V L^{-1}. After arsenic addition the only addition of water was to replace water lost through evaporation. Therefore, the *Arsenic period* began on biofilm colonization day 21 and ended on day 24.

As it was expected that biofilm would retain arsenic, the arsenic was added before adding fish in order to check the influence of this retention on the reduction of exposure to fish.

As+Fish period

On day 25, all fish were weighed, total length was measured, and four fish were added to each experimental unit. Different sized females were used primarily to allow identification of individuals within an aquarium so any overlap in sizes between aquaria was tolerated.

2.3. Water chemical sampling and analyses

Physical and chemical parameters (water temperature, dissolved oxygen, conductivity and pH) were measured with appropriate probes during the whole experimental period (33 days). Dissolved oxygen and conductivity were measured 6-10 times (HQ Portable Meters, HQ40d18, HACH Company), whereas phosphate and total dissolved arsenic were measured 10 and 7 times respectively for each experimental unit.

Triplicate water samples (10 mL) were taken for chemical analyses from each experimental unit 10 times during the experiment. Water was filtered with GF/F Glass Microfiber Filters (Whatman, 0.7 µm of pore size) for phosphorus determination, but for total dissolved arsenic water samples were filtered with 0.2 µm nylon membrane filters (Whatman) and immediately acidified with 1% of HNO_3 (65% suprapure, Merck). All water samples were frozen (at -20 °C) until analysis.

Inorganic phosphate (iP) concentration was determined by a modified molybdenum blue method (Carvalho *et al.* 1998) to avoid arsenate interference. Briefly, 10 mL of the sample were pipetted into a digestion tube and 2 mL of L-cysteine (5% w/v in 0.6 M HCl) were added. The

tube was tightly capped and incubated for 5 min at 80ºC to allow complete reduction of arsenate into arsenite. The solution was cooled to ambient temperature (25 °C) and then inorganic phosphate was determined with 0.5 mL of ascorbic acid (5% w/v in deionized water), 1 mL of acetone and 2 mL of mixed reagent (50 mL of sulfuric acid 20%, 5 mL of antimony potassium tartrate, 15 mL of ammonium molybdate and made up to 100 mL with Milli-Q water). Absorbance was quantified at 875 nm.

2.4. Biofilm measurements

Chlorophyll-a fluorescence-related endpoints

Photosynthetic activity and algal biomass of the biofilm were measured on days 7, 10, 14, 17, 21, 25, 26, 28, 31 and 33 using the PhytoPAM (Pulse Amplitude Modulated) fluorometer (HEINZ WALZ, Effeltrich, Germany) connected to an Emitter Detector Fiberoptics Unit (PHYTO-EDF) and "PhytoWin" software. PAM fluorometry is a rapid, non-invasive and reliable method to assess photosynthesis performance, and has been found to be the most sensitive tool for the rapid detection of harmful compounds (Corcoll *et al.* 2012a). Five replicates (small colonized sandblasted glass tiles) were used from each experimental unit (*B* and *B+As* treatments) each time. Temperature (19 ºC) and distance between light emitting diode and samples (8mm) were kept constant for all the measurements. First, measurements of dark adapted samples were done at the end of the darkness cycle. A saturation pulse was applied and the minimum fluorescence yield was obtained. According to Corcoll *et al.* (2012a), the minimal fluorescence yield of a dark adapted cell (F_0) is proportional to its chlorophyll-a concentration. Thus, it can be used as an estimation of algal biomass. The maximum PSII quantum yield (*Ymax)* was also obtained during the saturation pulse performed under dark conditions. This parameter is defined as a measure of the photosynthetic capacity of the community (Corcoll *et al.* 2012a). Thereafter, light adaptation of the samples was carried out for 15 minutes for light measurements. Actinic light provided by the instrument was used. One saturation pulse was applied and the effective PSII quantum yield (Photosynthetic efficiency, *Yeff*) was obtained. Effective PSII quantum yield is defined as a measure of the photosynthetic efficiency of the community (Corcoll *et al.* 2012a). After all measures, colonized sandblasted glass substrata were returned into the experimental units channels.

Bacterial abundance

The double staining Live/Dead BacLight Bacterial Viability Kit (Molecular Probes) was used to measure the abundance of live and dead bacteria in the biofilm samples. Four times during the experiment, small colonized sandblasted glass tiles were collected into autoclaved glass vials, resuspended and then diluted in autoclaved Milli-Q water. All cells were firstly individualized by sonication (less than one minute to avoid damaging cell membranes) and stained using a mixture of 3.34mM SYTO® 9. Then, only dead cells (those with cell membranes

damaged during the experiment) were stained by 20mM propidium iodide (Freese *et al.* 2006). After 30 minutes in dark conditions, each sample was filtered through a 0.2 µm black polycarbonate filter (Nuclepore, Whatman). Twenty random microscopy fields were counted for each sample (filter) using epifluorescence microscopy at a magnification of 1000x in immersion oil (Nikon E600, Tokyo, Japan). Data are expressed as live bacteria (cell cm^{-2}).

Benthic chlorophyll-a

On the last day of the experiment (after 13 days of biofilm arsenic exposure), small and colonized sandblasted glass tiles were collected from each channel into falcon tubes, immediately frozen in liquid nitrogen and stored at -80ºC until chlorophyll-a extraction. The chlorophyll-a content was extracted with 90% acetone for 12 h. Sonication (Ultrasonic bath, J.P Selecta) for 2 minutes improved the pigment extraction and chlorophyll-a concentration was subsequently estimated from spectrophotometric measurements (spectrophotometer UV-1800, Shimadzu), following the method described in Jeffrey and Humphrey (1975). Since the biofilm was colonized on the surface of the tile, when the tile was submerged in 90% acetone for chlorophyll-a extraction and then sonicated, chlorophyll-a from the whole biofilm colonized on the tile was obtained.

Diatom community identification and metrics

Diatoms were collected from 1 small colonized sand blasted glass substratum from each channel at the end of the experiment. Biofilm was immediately resuspended and conserved in a glass vial with 4.5 mL of Milli-Q water and 0.5 mL of 40% formaldehyde. Then, samples were digested with 10 mL of hydrogen peroxide (30% H_2O_2) to eliminate organic matter and obtain clean frustules according to Leira and Sabater (2005). Frustules were then washed with distilled water, dehydrated on cover glasses and finally mounted on permanent slides using Naphrax (Refractive index 1.74; Brunel LTD, UK). All these steps were carefully performed with controlled volumes, to allow a final quantitative assessment of diatom densities. Up to 600 diatom valves per slide were counted and identified to assess species richness and diversity in our samples. Random transects were scanned under a light microscope (Nikon E600, Tokyo, Japan) using Nomarski differential interference contrast optics at a magnification of 1000x. Identification mainly followed Krammer and Lange-Bertalot (1986–1991), and recent nomenclatural updates. Diatom species relative abundance and density were calculated, as well as the species richness (*S*), Shannon-Wiener index of diversity (*H*) and species evenness (*J*). Calculations for *H* and *J* were performed using the following equations:

$$H = -\sum_{i=1}^{S} P_i \ln P_i \quad ; \quad J = \frac{H}{H_{max}} = \frac{-\sum_{i=1}^{S} P_i \ln P_i}{\ln S},$$

where P_i is the proportional abundance of the *i*th species and S is the total number of species present in the community (species richness).

Diatom biovolume determination

Diatom specific biovolume was determined using light microscopy with Nomarski differential interference contrast optics at a magnification of 1000x and following a set of geometrical shapes proposed by Hillebrand *et al.* (1999). Cell size (or cell biovolume) was calculated by measuring different dimensions (length, width, diameter and some heights) of 25 randomly selected valves per species, as far as possible, and using equations from a set of geometrical shapes proposed by Hillebrand *et al.* (1999). Total species biovolume was then calculated.

In addition, since theoretical cell biovolume data has been used in several studies, our measured cell biovolumes were compared with the theoretical ones (http://hydrobio-dce.irstea.fr/cours-deau/diatomees/) corresponding to each species.

2.5. Arsenic measurements

The level of arsenic in the circulating system was measured 7 times during the whole exposure period: 4 times before adding fish and 3 times after adding fish. For biofilm samples, total arsenic accumulation was measured at the end of the exposure (6 samples/channel). For all analyses, the detection limit was 0.08 µg L^{-1}; Rhodium (Rh) was used as the internal standard and the accuracy of the analytical method was checked periodically using a certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

Total dissolved arsenic concentration

Total dissolved arsenic concentration (µg L^{-1}) was analysed by inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies, Inc. Wilmington, DE).

Total arsenic accumulation in biofilm

Total arsenic accumulation in biofilm was analyzed in triplicate for treatments *B* and *B+As* (using large sand blasted glass substrata). Colonized glass substrates were collected at the end of the experiment, placed on filter paper to remove excess water, and immediately frozen before analysis. Then, biofilm was freeze-dried, weighed and digested using 4 ml of concentrated HNO_3 (65% suprapure, Merck, Germany) in a high performance microwave digestion unit (Milestone, Ethos Sel). They were then diluted to 15 mL with milli-Q water and the subsequent liquid samples were treated as dissolved metal water samples. Total dissolved arsenic concentration was measured by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE).

2.6. Data analysis

Prior to statistical analyses, some variables had to be log-transformed (from water physical and chemical data, only phosphate concentration and total dissolved arsenic were log-transformed; from biological data, live bacteria and bioaccumulated arsenic on biofilm; and photosynthetic parameters were also log-transformed), or log(x+1) transformed (diatom relative frequencies) to reduce skewed distributions and fix heteroscedasticity. For chemical measurements, half of the detection limit was used for data treatment when the value obtained was below the detection limit (Helsel 1990).

Most data were taken several times during the experiment. Significant differences between treatments and time together were analyzed. Two-Way ANOVAs were applied to physical and chemical data, where the *Time* variable was categorized in three periods: *Biofilm colonization, arsenic* and *As+Fish*. Biofilm photosynthetic parameters were analyzed by Two-Way Repeated Measures ANOVA, where the *Time* variable (expressed in biofilm colonization days) was the within-subject continuous variable, and *Treatment* (biofilm treatment, *B*, *versus* biofilm with arsenic exposure, *B+As*) was the between-subject variable. Finally, post-hoc Bonferroni's tests were applied to check exactly where significant differences were found.

For data taken only at the end of the experiment (chlorophyll-*a* content, arsenic bioaccumulated in biofilm and fish) and diatom metrics, One-Way ANOVAs were performed to analyze significant differences between treatments. For diatom species relative abundance, only the species that represented more than 0.5% of the total relative abundance were considered in the ANOVA analysis. For total diatom cell biovolume, One-Way ANOVA was also performed. However, specific diatom cell biovolume were analyzed with Student's t-tests, since heteroscedasticity was not reduced with the log-transformation. Student's t-test is analogous to the One-Way ANOVA with two treatments, but it allows to obtain results even in case of heteroscedasticity. Statistical significance for all the ANOVA's and Student's t-tests was set at $p \leq 0.05$; while marginal significance was set at $0.05 < p \leq 0.1$. Correlation analysis was done to compare measured and theoretical diatom cell biovolume data.

SPSS software (version 15.0) was used for statistical analyses. Boxplots for the description of the diatom cells biovolume, as well as the correlation analysis between measured and theoretical data, were done with Microsoft Excel 2010 software. The graphics for the photosynthetic parameters and physicochemical variables were developed using Sigmaplot software (version 11.0).

3. RESULTS

3.1. Physico-chemical and bioaccumulation data

A time effect was observed with water chemistry and arsenic also had a significant effect, especially after fish addition. Physico-chemical data, as well as the ANOVAs' results and comparison per pairs, are summarized in Table 1. Water conductivity slightly decreased over the whole experiment (time effect), and was lower in the experimental units with biofilms (*B* and *B+As*; mean values of 427.19 ± 6.39 µS cm^{-1} over the experiment) than in those without biofilm (*noB noAs* and *As*; 441.75 ± 7.48 µS cm^{-1}). In general, lower values were found in the *B* treatment than in the *B+As* treatment. For dissolved oxygen, a general decrease was observed during the *As+Fish period*, being significantly lower (p<0.001) in biofilm exposed to arsenic than in biofilm without arsenic. On the other hand, a significant increase in phosphate concentration in water was observed except in the arsenic treatment at the end of the experiment (p<0.001), during the *As+Fish period*. Also in that period, arsenic accumulation in the biota reflected exposure (Table 2), with higher arsenic content in biofilm (p<0.001) and fish (p=0.012).

3.2. Biofilm measurements

Bacteria

Live bacteria (cell cm^{-2}) increased in both biofilm treatments over the experiment (p=0.015) from a mean of $4.09x10^6$ ± $1.25x10^6$ cell cm^{-2} during the *Biofilm colonization period* to a mean of $13.17x10^6$ ± $8.23x10^6$ cell cm^{-2} in the *As+Fish period* (Table 2). No significant difference was observed between treatments *B vs*. *B+As*.

Table 1 Water physical and chemical data, with statistical results. Water physical and chemical data are represented by the mean ± standard deviation, and sample size (n). Statistical results (F and *p*) for effects on Time (degrees of freedom, d*f*=2) and Treatment (d*f*=3) were achieved by Two-Way ANOVA and Bonferroni's test (different letters indicate significant differences between sampling time or treatments at $p \leq 0.05$). bdl: below detection limit. *Stars indicate marginal significance ($0.05 < p \leq 0.1$).

Time period	Treatment	Conductivity (µS cm^{-1})	O_2 (mg L^{-1})	PO_4^{3-} (µg L^{-1} - P)	Total As (µg L^{-1})
Biofilm colonization	***B***	444.00 ± 3.10 (n=6)	8.66 ± 0.04 (n=6)	5.83 ± 5.46 (n=6)	
	B+As	448.50 ± 7.34 (n=6)	8.62 ± 0.02 (n=6)	bdl (n=6)	
As	***noB noAs***	439.00 ± 2.92 (n=9)	8.80 ± 0.09 (n=9)	bdl (n=3)	1.98 ± 0.12 (n=12)
	As	435.78 ± 4.71 (n=9)	8.77 ± 0.03 (n=9)	bdl (n=3)	124.89 ± 2.43 (n=12)
	B	424.50 ± 2.46 (n=9)	8.82 ± 0.23 (n=9)	2.51 ± 0.02 (n=3)	2.01 ± 0.15 (n=12)
	B+As	429.40 ± 1.51 (n=9)	8.56 ± 0.08 (n=9)	3.07 ± 0.99 (n=3)	121.00 ± 4.06 (n=12)
As + fish	***noB noAs***	446.44 ± 9.68 (n=9)	8.58 ± 0.10 (n=9)	12.11 ± 4.10 (n=3)	1.92 ± 0.09 (n=9)
	As	445.78 ± 5.33 (n=9)	8.50 ± 0.06 (n=9)	3.18 ± 1.17 (n=3)	127.96 ± 5.55 (n=9)
	B	419.40 ± 1.94 (n=9)	8.69 ± 0.27 (n=9)	12.28 ± 3.34 (n=3)	1.89 ± 0.11 (n=9)
	B+As	435.30 ± 3.28 (n=9)	8.34 ± 0.12 (n=9)	15.96 ± 4.14 (n=3)	124.20 ± 2.64 (n=9)
Time effects	ANOVA	F=78.177, $p<0.001$	F=21.076, $p<0.001$	F=34.690, $p<0.001$	F=0.801, $p=0.374$
	Biofilm colonization	a	a	a	
	As	b	a	a	a
	As + fish	c	b	b	a
Treatment effects	ANOVA	F=66.824, $p<0.001$	F=11.293, $p<0.001$	F=5.226, $p=0.006$	F=48006.691, $p<0.001$
	noB noAs	a	a	a	a
	As	a,c	a	b*	b
	B	b	a	a	a
	B+As	c	b	a*	b

Table 2 Biological data with statistical results. Biological data are represented by the mean ± standard deviation, and sample size (n). Statistical results (F and *p*) for effects on Time (degrees of freedom, d*f*=2) and Treatment (d*f*=1) were achieved by Two-Way ANOVA (for Live bacteria data) and One-Way ANOVA (for Chl-a, arsenic biofilm and arsenic fish data). Bonferroni's tests were also carried out (different letters indicate significant differences between sampling time or treatments at $p \leq 0.05$).

Time period	Treatment	Live bacteria (x10^6 cell cm^{-2})	Chl-a (µg cm^{-2})	As biofilm (µg g^{-1})	As fish (µg g^{-1})
Biofilm colonization	***B***	5.12± 0.50 (n=3)			
	B+As	3.05 ± 0.69 (n=3)			
As	***B***	9.81 ± 7.52 (n=3)			
	B+As	5.83 ± 2.04 (n=3)			
As + fish	***B***	12.23 ± 8.47 (n=6)	40.61 ± 7.56 (n=3)	3.251 ± 0.21 (n=6)	470.95 ± 61.38 (n=3)
	B + As	14.11 ± 8.67 (n=6)	22.72 ± 8.64 (n=3)	79.59 ± 9.39 (n=6)	758.09 ± 95.32 (n=3)
Time effects	ANOVA	F=4.980, *p*=0.019			
	Biofilm colonization	a			
	As	a,b			
	As + fish	b			
Treatment effects (*B vs. B+As*)	ANOVA	F=0.623, *p*=0.440	F=7.282, *p*=0.054	F=3297.04, *p*<0.001	F=19.243, *p*=0.012

Chlorophyll-a fluorescence measurements and Chlorophyll-a content

Minimum fluorescence yield (F_0) increased over time and showed significant differences between treatments *B* and *B+As* (Fig. 3, Table 3) during the *As+Fish period*, revealing a significant inhibition of algal biofilm growth from day 25 to day 33 (Fig. 3). Chlorophyll-a concentration showed a similar result (Table 2).

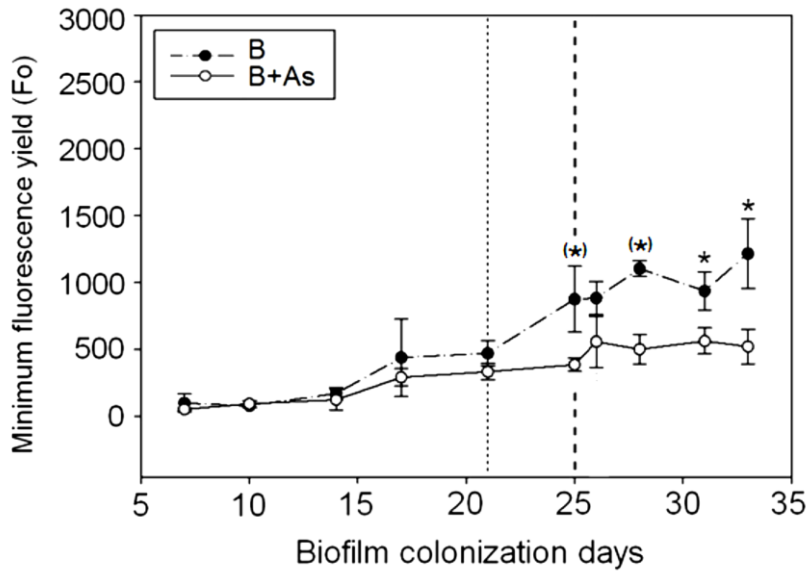


Figure 3 Biofilm growth: Evolution of Minimum fluorescence yield (F_0) during the biofilm colonization days until the end of the experiment in the different treatments (*B*, biofilm without arsenic exposure; *B+As*, biofilm with arsenic exposure). Vertical lines indicate arsenic addition (on biofilm colonization day 21) and fish addition (on biofilm colonization day 25). Stars indicate significant differences (at $p \leq 0.05$) between treatments for each day. Stars in brackets indicate marginal significance ($0.05 < p \leq 0.1$).

Arsenic also affected algal succession and photosynthetic parameters of the different groups of algae and cyanobacteria. In the *B* treatment, diatoms and cyanobacteria increased in biomass during the 4 first weeks, then decreased, and were followed by a progressive growth of filamentous green algae. In contrast, green algae did not grow with arsenic (Fig. 4). Significant differences in the maximum PSII quantum yield (*Ymax*) between treatments were found. *Ymax (diatoms)* was lower during the whole period of arsenic exposure, in contrast to *Ymax (cyanobacteria)* and *Ymax (general)* that showed more scattered results (Fig. 5). The effective PSII quantum yield (*Yeff*) also showed significant differences, except in diatoms (*Yeff diatoms*), at the end of the experiment (Fig. 5, Table 3).

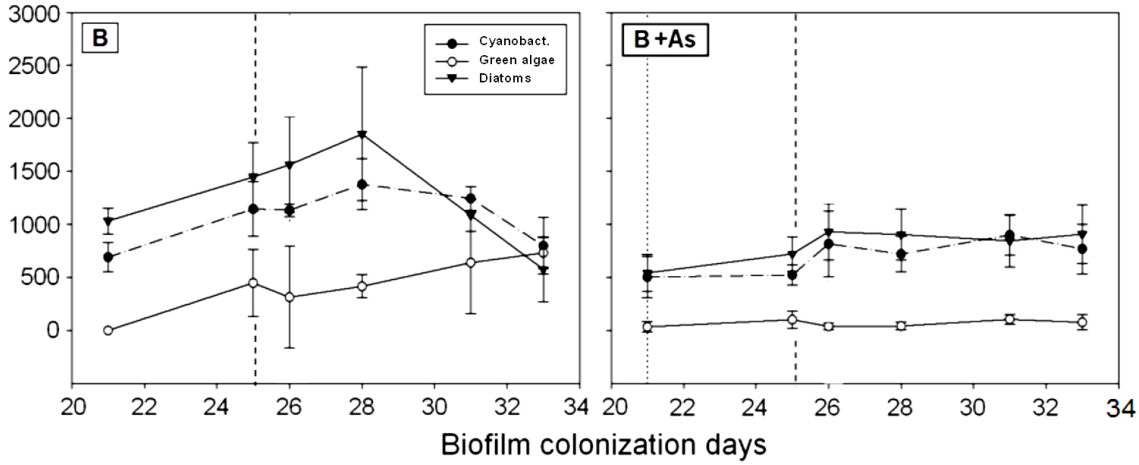


Figure 4 Algal succession: Evolution of Minimum fluorescence yield (F_0) of each algal group (Cyanobacteria, Green algae and Diatoms) during the arsenic exposure and until the end of the experiment, compared between treatments (*B vs. B+As*). Vertical lines indicate arsenic addition (biofilm colonization day 21) and fish addition (biofilm colonization day 25).

Table 3 Statistical results of biofilm photosynthetic parameters. Two-Way Repeated Measures ANOVA (F and *p*) of photosynthetic parameters was performed for all algae (general), cyanobacteria, filamentous green algae and diatoms to analyze statistical differences in time (sample size, n=6; degrees of freedom, d*f*=5) and between treatments (*B vs. B+As;* n=2; d*f*=1) at $p \leq 0.05$. F_0 parameters represent the minimal fluorescence yield of a dark adapted cell, *Ymax* parameters represent the photosynthetic capacity of the community, and *Yeff* parameters represent the photosynthetic efficiency.

Photosynthetic parameters	Time		Treatment (*B vs. B+As*)	
	F	*p*	F	*p*
Fo (general)	14.351	<0.001	27.910	0.006
Fo (cyanobacteria)	5.157	0.003	12.602	0.024
Fo (green algae)	11.103	<0.001	2.170	0.215
Fo (diatoms)	5.400	0.003	4.220	0.109
Ymax (general)	6.581	0.001	66.217	0.001
Ymax (diatoms)	1.509	0.231	127.755	<0.001
Ymax (cyanobacteria)	1.803	0.158	9.500	0.037
Yeff (general)	2.313	0.082	40.863	0.003
Yeff (diatoms)	0.276	0.921	1.290	0.320
Yeff (cyanobacteria)	0.961	0.465	75.072	0.001

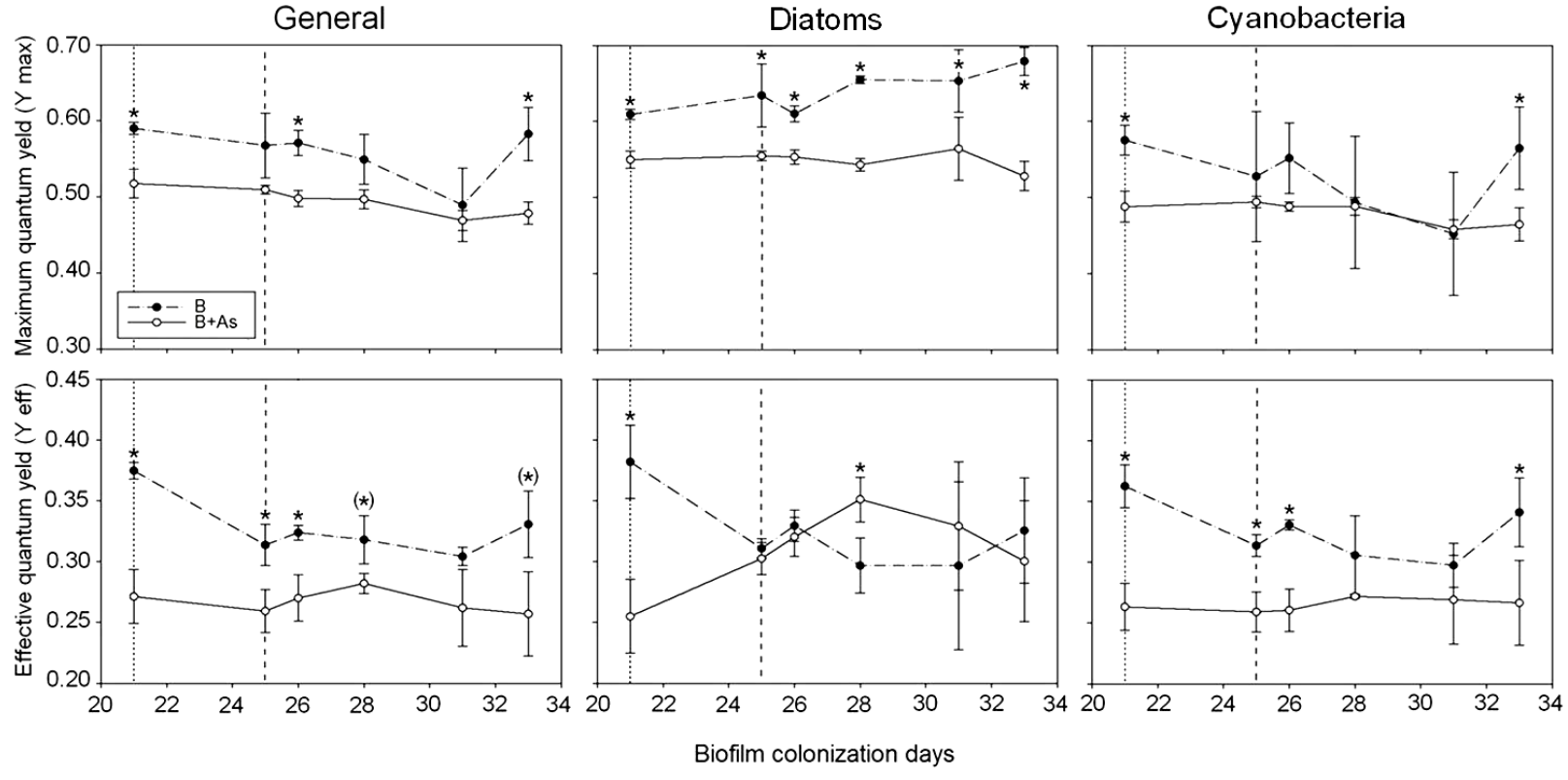


Figure 5 Evolution of Maximum quantum yield (*Ymax*) and Effective quantum yield (*Yeff*) of the algal groups together (General) and individual groups (Diatoms and Cyanobacteria) from the arsenic addition event until the end of the experiment. Vertical lines indicate arsenic addition (biofilm colonization day 21) and fish addition (biofilm colonization day 25). Statistical comparison between treatments (*B vs. B+As*) was done: stars indicate significant differences ($p \leq 0.05$) between treatments in each day; stars in brackets indicate marginal significance ($0.05 < p \leq 0.1$).

Diatom community identification and metrics

We identified 52 diatom taxa (Table 4), of which *Achnanthidium minutissimum* (Kützing) Czarnecki was the most abundant species, representing almost the 77% of the total abundance of diatoms (75% in *B* treatment and almost 79% in *B+As*). In general, the relative abundances of other species decreased when they were exposed to arsenic. Significant decreases were found in *Amphipleura pellucida* Kützing (p=0.007) and *Nitzschia dissipata* (Kützing) Grunow ssp. *dissipata* (p=0.004) whereas a significant proportion of diatom species (30%) increased in cell numbers, highlighting some *Fragilariaceae*, in particular *Ulnaria ulna* (Nitzsch) Compère (p=0.092).

Furthermore, arsenic effects on diatom species richness were marginally significant (p=0.051, Table 5).

Table 4 List of the all diatom taxa found at the end of the experiment

Achnanthidium minutissimum (Kützing) Czarnecki
Achnanthidium subatomoides (Hustedt) Monnier, Lange-Bertalot et Ector
Amphipleura pellucida Kützing
Amphora aff. *veneta* (Kützing)
Amphora inariensis Krammer
Amphora pediculus (Kützing) Grunow
Aneumastus stroesei (Ostrup) Mann & Stickle in Round Crawford & Mann
Caloneis sp.
Cocconeis placentula Ehrenberg var. *placentula*
Cyclotella meneghiniana Kützing
Cymbella affinis Kützing var. *affinis*
Cymbella cistula (Ehrenberg) Kirchner
Cymbopleura amphicephala Krammer
Denticula tenuis Kützing
Diploneis sp.
Encyonema minutum (Hilse in Rabhenhorst) D.G. Mann in Round Crawford & Mann
Encyonema prostratum (Berkeley) Kützing
Encyonopsis falaisensis (Grunow) Krammer
Encyonopsis microcephala (Grunow) Krammer
Eolimna minima (Grunow) Lange-Bertalot
Fragilaria capucina Desmazières var. *capucina*
Fragilaria capucina Desmazières var. *vaucheriae* (Kützing) Lange-Bertalot
Fragilaria gracilis Østrup
Fragilaria mesolepta Rabenhorst
Frustulia vulgaris (Thwaites) De Toni
Gomphonema lateripunctatum Reichardt & Lange-Bertalot
Gomphonema parvulum (Kützing) Kützing var. *parvulum* f. *parvulum*

Gomphonema truncatum Ehrenberg
Gyrosigma acuminatum (Kützing) Rabenhorst
Halamphora veneta (Kützing) Levkov
Mayamaea atomus (Kützing) Lange-Bertalot var. *atomus*
Melosira varians Agardh
Navicula aff. *saprophila* Lange-Bertalot & Bonik
Navicula capitatoradiata Germain
Navicula cryptotenella Lange-Bertalot
Navicula gregaria Donkin
Navicula menisculus Schumann var. *menisculus*
Navicula reichardtiana Lange-Bertalot var. *reichardtiana*
Navicula tripunctata (O.F.Müller) Bory
Nitzschia amphibia Grunow f. *amphibia*
Nitzschia dissipata (Kützing) Grunow ssp. *dissipata*
Nitzschia fonticola Grunow in Van Heurck
Nitzschia palea (Kützing) W.Smith
Nitzschia recta Hantzsch in Rabenhorst
Planothidium lanceolatum (Brebisson ex Kützing) Lange-Bertalot
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot
Sellaphora stroemii (Hustedt) Kobayasi in Mayama Idei Osada & Nagumo
Staurosira brevistriata (Grunow) Grunow
Staurosira construens Ehrenberg
Staurosira mutabilis (Wm Smith) Grunow
Staurosira venter (Ehrenberg) Cleve & Moeller
Ulnaria biceps (Kützing) Compère
Ulnaria capitata (Ehrenberg) Compère
Ulnaria ulna (Nitzsch) Compère

Diatom biovolume determination

Arsenic clearly reduced diatom average cell biovolume (p=0.003, see Table 5). Besides the global decrease in cell size, individual cell biovolume (or cell size) in some species, such as *Nitzschia fonticola,* was also reduced with arsenic exposure (p=0.066, Fig. 6a), although this result must be treated with caution because of data heteroscedasticity. There was a general trend in biovolume decrease (measured as total biovolume per sample surface unit, Table 5), which was statistically significant in one case, *Nitzschia dissipata* (p=0.040), and marginally significant in three cases, *Amphipleura pellucida* (p=0.051), *Nitzschia fonticola* (p=0.079) and *Nitzschia palea* (Kützing) W.Smith (p=0.073). In contrast, the biovolume of some *Fragilaria* species, such as *Fragilaria capucina* Desmazières var. *capucina* and *Fragilaria mesolepta* Rabenhorst, increased under arsenic exposure due to greater cell size and/or higher cell numbers in the arsenic treatment (Fig. 6b).

Table 5 Diatom metrics and biovolume data, with statistical results. Data are represented by the mean ± standard deviation. Three replicate samples were used for each datum (n=3). One-Way ANOVA was performed to analyze statistical differences between treatments (*B vs*. *B+A;* d*f*=1) at $p \leq 0.05$.

Treatment		Species Richness (*S*)	Shannon Diversity Index *(H)*	Species Eveness *(J)*	Density (cell cm^{-2})	Mean cell biovolume (μm^3)	Total diatom biovolume ($\mu m^3\ cm^{-2}$)
B		32.00 ± 4.36	1.19 ± 0.16	0.34 ± 0.04	$73.67 \times 10^6 \pm 28.36 \times 10^6$	153.41 ± 10.20	$2.20 \times 10^{12} \pm 1.40 \times 10^{12}$
B+As		24.67 ± 1.53	0.98 ± 0.15	0.31 ± 0.05	$70.67 \times 10^6 \pm 21.57 \times 10^6$	84.43 ± 15.17	$1.15 \times 10^{12} \pm 0.70 \times 10^{12}$
One-Way ANOVA	F	7.563	2.580	1.246	0.025	42.724	1.349
	p	0.051	0.183	0.327	0.882	0.003	0.310

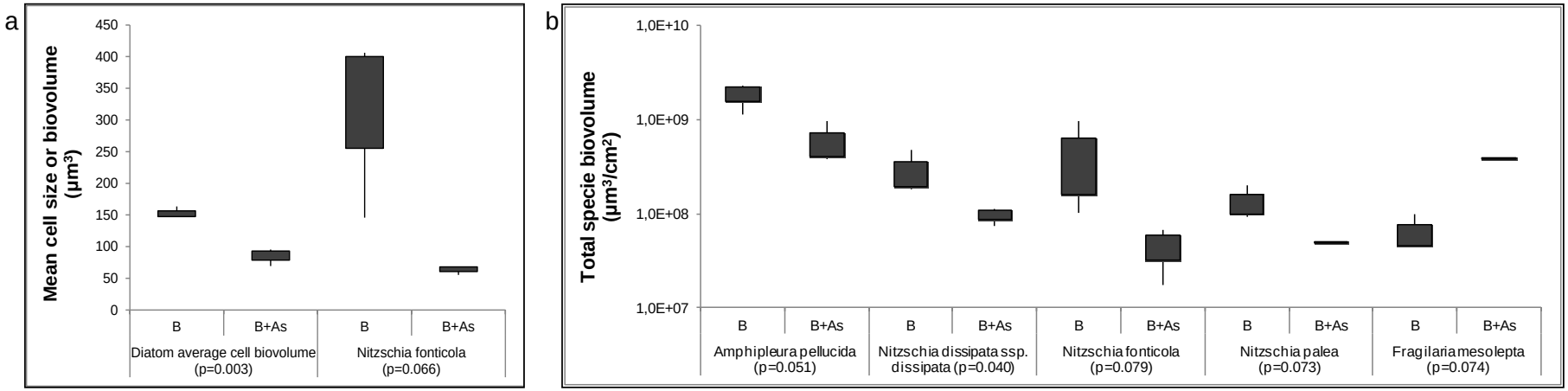


Figure 6 Boxplots representing changes in (a) average diatom cell size (μm^3) and (b) total diatom species biovolume ($\mu m^3\ cm^{-2}$), of significant and some marginal significant species. Y-axis is log transformed.

In addition, measured biovolumes were compared with theoretical biovolume data (http://hydrobio-dce.irstea.fr/cours-deau/diatomees/) for each species and were poorly correlated (R^2=0.039).

4. DISCUSSION

The arsenic concentration used in this experiment was low compared to the CMC (acute arsenic exposure) established by the US EPA (2014) in freshwater. Despite this, it affected biofilm structure and function. These effects were expected based on low measured phosphate concentrations, similar to the experiment of Rodriguez Castro *et al.* (2015). However, it does not agree with our expectation concerning the influence of fish on phosphate concentration.

After fish addition, higher dissolved phosphate concentrations were found in all treatments (about 13 µg L^{-1}), except in arsenic alone. However, these phosphate concentrations still remained limiting according to Dodds *et al.* (1998). Therefore, despite fish addition, the expected protection role of phosphate for algae was not fully achieved. Compared with Rodriguez Castro *et al.* (2015), final phosphate concentration was not high enough to protect all algae from arsenic toxicity. A possible explanation for this might be related to fish density, which was not high enough to provide enough nutrients via their excretion, and/or mineralization rates, which was not high enough to produce high phosphate concentration from organic matter (fish excretion) to overcome algal uptake.

Fish addition accelerated algal growth (Fig. 3), which corresponded to the higher phosphate concentration in water, which in turn was probably a result of fish metabolism (***Chapter 2***). Although phosphate is one of the most important determinants of algal production (Borchardt 1996), biofilm growth was delayed by arsenic exposure. Thus, it seems that arsenic prevented the uptake of phosphate by algal biofilm, as shown in Rodriguez Castro *et al.* (2015), which resulted in growth inhibition, caused also by the direct As-toxicity. The lower photosynthetic efficiency in P-limited conditions leading to lower algal growth has also been observed previously (Rodriguez Castro *et al.* 2015). During biofilm formation, algal succession usually begins with the emergence of diatoms, followed by cyanobacteria and finally filamentous green algae (Romaní 2010; Bonet 2013). However, arsenic impeded filamentous green algae growth and caused diatoms to dominate by the end of the experiment, leading to lower temporal variability (Fig. 4). Changes in *Ymax* at the end of the experiment indicated that important structural changes in photosystem II (PSII) occurred in biofilm exposed to arsenic (Fig. 5). Therefore, arsenic inhibits the potential maximum photosynthetic activity of algal biofilm in conditions of phosphate limitation, confirming the recent findings of Rodriguez Castro *et al.* (2015). In addition, the measures given by the PhytoPAM were in concordance with an increase of oxygen concentration in the water, which indicates that the main kind of photosynthesis in the system was an oxygenic photosynthesis. Therefore, arsenic caused biofilm to become less

phototrophic, what is also supported by the fact that, in contrast to algae, live bacterial cell densities did not decrease (chlorophyll-*a* concentration halved, Table 2). Thus, the proportion of biofilm consisting of algae decreased. Bacterial resistance to arsenic has already been documented (e.g. Davolos and Pietrangeli 2013). A general reduction of the *Yeff* parameter (Fig. 5) shows that arsenic caused a less efficient photosynthesis in algae (Corcoll *et al.* 2012a). However, diatoms were able to recover their photosynthetic efficiency at the end of the experiment, indicating adaptation of the diatom community to arsenic exposure.

Diatoms are cosmopolitan algae and predominate in fluvial biofilms. Diatom communities exposed to metals have variable capacities to tolerate the stress caused by the toxicant, both at the individual scale (with different levels of sensitivity among species) and at the community scale, where the biofilm acts as a coherent and protective matrix (Morin *et al.* 2012). Diatom taxonomical identification was carried out with samples taken on the last day of the experiment, when community structure was mature and expected to show the accumulated effects of 13 days of arsenic exposure. Besides the global shift in algal composition, the diatom community responded through a decrease in species richness, already documented as an effect of metal pollution (Morin *et al.* 2012). However, total diatom density remained relatively unaffected. Therefore, while total algal biomass was affected by arsenic, there was a delay in the expected replacement of diatoms by filamentous green algae due to succession, leading to similar values of diatom density at the end of the experiment. This was attributed to different processes (succession *vs.* selection pressure linked with arsenic exposure), which caused clear effects on cell size and slight changes in species composition. *Achnanthidium minutissimum*, a metal-tolerant species (see review in Morin *et al.* 2012), was the most abundant species found, representing almost the 77% of the total abundance of diatoms. *Achnantidium minutissimum* is also considered tolerant to nutrient limitation, and its small cell size is a key feature that allows maintenance of larger populations and broader regional distributions than larger species (Passy 2008). In addition, the shift towards its higher abundances in arsenic exposed communities (from 75% in the *B* treatment to almost 79% in *B+As*), highlighted its tolerance to arsenic. For other species found, 30% increased in cell numbers. In particular *Ulnaria ulna,* a species known for its resistance to metals (McFarland *et al.* 1997; Blanck *et al.* 2003; Tien 2004; Duong *et al.* 2008; Ferreira da Silva *et al.* 2009), achieved larger populations in the arsenic treatment.

In addition, arsenic clearly caused a global decrease in the average diatom cell size or cell biovolume (Table 5 and Fig. 6a), a phenomenon also observed in some individual species, such as *Nitzschia fonticola*. According to Morin *et al.* (2012), community size may be affected in several complementary ways: as a reduction of cell number, and/or a diminution of cell size within a given species. Reduction of cell size within taxa with metal exposure can be linked to the peculiar mitotic division during vegetative reproduction in diatoms, which is different to that of other algae. Each division results in two daughter cells, one of which is the same size as the mother cell, with the other being smaller. As a consequence, average cell size at the population level is reduced with each successive round of mitosis (Drebes 1977). Vegetative reproduction

is the dominant mode of multiplication in diatoms (Chepurnov *et al.* 2008), so this decrease in size could be a result of a higher cell division rate in organisms that live in stressed ecosystems (Gensemer 1995; Potapova and Snoeijs 1997). The decrease in size of many taxa in metal-contaminated environments has already been observed (Cattaneo *et al.* 1998; Cattaneo *et al.* 2004; Morin and Coste 2006; Luís *et al.* 2011). Moreover, it is known that in algae there is a positive richness-body size relationship (Passy 2012), which agrees with our results. Total diatom sample biovolume, a parameter dependant on both diatom abundances and cell size, decreased in several cases, such as *Amphipleura pellucida*, *Nitzschia dissipata* spp. *dissipata* and *Nitzschia fonticola,* and increased in others including *Fragilaria mesolepta* (Fig. 6b), highlighting the different strategies used to cope with arsenic contamination. An increase in cell volume in a diatom species, *Cylindrotheca fusiformis,* with copper exposure has also been attributed to a tolerance mechanism (Pistocchi *et al.* 1997). Summarizing, both higher *Achnanthidium minutissimum* relative abundances and greater abundance of smaller cell size diatoms were the two main changes favored under arsenic exposure. This supports the idea that large organisms are more sensitive to stress than short-lived and fast-reproducing small ones. This size-dependent sensitivity holds many implications for community functions: systems under stress would be dominated by smaller organisms with faster metabolism and flux rates. Thus, body size is a fundamental measured property of single organisms and whole communities. In addition, our results highlight the importance of taking cell biovolume real measures in water quality assessments or ecotoxicology studies based on diatoms.

The direct effects observed on biofilm function, structure and their dynamics (succession) could cause indirect effects on water chemistry. For example, a resultant increase in water conductivity may cause a decrease in the capacity of algae to take and hold solutes, which are necessary for photosynthesis; while a decrease in dissolved oxygen concentration reflects oxygen consumption by bacteria and the strong decrease in oxygenic photosynthesis activity (Table 1). A lower ability of biofilm to oxygenate the system could be therefore expected as an indirect effect of arsenic exposure.

Finally, it is necessary to highlight that this experiment with arsenic was very short (only 13 days), but still resulted in strong effects on biofilm and especially in diatoms. Furthermore, this experiment was a dynamic system with fish, making it more realistic than the classic short-term effect test with algae. Therefore, it is important to be aware that the long-term impact in a real polluted ecosystem would be different and probably much higher. In addition, the recovery would be more difficult since structural changes were also observed.

5. CONCLUSIONS

Knowing that chronic exposure of 130 µg As L^{-1} is commonly found in naturally As-enriched fluvial systems (Rosso *et al.* 2011), we conclude and highlight that short-term biofilm exposure to arsenic at environmentally realistic concentrations (130 µg L^{-1} during 13 days) under P-limited conditions, was sufficient to cause direct effects on algae. Using chlorophyll-a as a measure of algal biomass, and live bacteria number as an approximation of bacterial biomass, we conclude that a less phototrophic biofilm was developed, as algal growth and productivity were reduced. Moreover, arsenic impeded the algal succession process, causing changes in the algal community and specifically in diatoms: a loss of diatom species sensitive to arsenic and a significant decrease in cell size may allow diatoms to become more tolerant to the toxicant. Therefore, an important function of the system was lost, regarding to the decrease of primary production and the loss of biodiversity. All these changes have obvious ecological implications for freshwater environments, especially rivers. Considering how low arsenic concentration and exposure time were in this experiment compared with reality, the results call into question the limits of arsenic concentration established by the US EPA (2014) in freshwater based on acute arsenic exposure (340 µg L^{-1}).

The protection role of phosphate for algae exposed to arsenic was not fully achieved. Further experiments are needed to disentangle and better understand the complex set of processes contributing to arsenic and phosphate cycling by decomposers, primary producers and consumers.

Finally, we strongly support the use of biofilm and a multi-endpoint approach to measure effects of toxicants in freshwater ecosystems. This study also brings new arguments for the use of real measurements in the estimation of diatom biovolume (cell size), as well as for the use of multi-trophic studies to elucidate the real effects of toxicants.

CHAPTER 2

BEHAVIORAL AND PHYSICAL EFFECTS OF ARSENIC EXPOSURE IN FISH ARE AGGRAVATED BY AQUATIC ALGAE

Magellan K, Barral-Fraga L, Rovira M, Srean P, Urrea G, García-Berthou E, Guasch H. (2014). Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae. *Aquatic Toxicology,* 156:116-124.

doi: http://dx.doi.org/10.1016/j.aquatox.2014.08.006 (see Annex 2)

ABSTRACT

Arsenic toxicity on freshwaters depends on numerous interacting factors which makes effects difficult to estimate. The use of aquatic algae is often advocated for bioremediation of arsenic contaminated waters as they absorb arsenate and transform it into arsenite and methylated chemical species. Fish are another key constituent of aquatic ecosystems. Contamination in natural systems is often too low to cause mortality but sufficient to interfere with normal functioning. Alteration of complex, naturally occurring fish behaviors such as foraging and aggression are ecologically relevant indicators of toxicity and ideal for assessing sublethal impacts. We examined the effects of arsenic exposure in the invasive mosquitofish, *Gambusia holbrooki*, in a laboratory experiment incorporating some of the complexity of natural systems by including the interacting effects of aquatic algae. Our aims were to quantify the effects of arsenic on some complex behaviors and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas operculum movement decreased non-significantly and neither food capture efficiency nor consumption was notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomass. Possibly increased aggression facilitated food resource defense allowing fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioral and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

1. BACKGROUND

Arsenic (As) from both anthropogenic and natural sources has global impacts (Mandal and Suzuki 2002; Nordstrom 2002; Rahman and Hasegawa 2012; Rahman *et al.* 2012; Smedley and Kinniburgh 2002) and aquatic systems, including freshwaters, are major repositories for arsenic (Nordstrom 2002; Smedley and Kinniburgh 2002). Although some national and international standards are in effect, for example the World Health Organization safe limit for drinking water is 10 μg L^{-1} (Smith *et al.* 2002), the toxicity of arsenic is dependent on numerous interacting factors such as its source, concentration and bioavailability; environmental parameters; and organisms' resistance ability and detoxifying mechanisms (Mandal and Suzuki 2002; Rahman and Hasegawa 2012; Smedley and Kinniburgh 2002). A key factor is its chemical speciation. Inorganic arsenic (iAs) is generally more toxic than organic As,

while of the iAs species, arsenite (As^{III}) is more toxic than arsenate (As^{V}). However, the organic methylated species (dimethylarsenite, DMA^{III}, and monomethylarsenite, MMA^{III}) are more toxic than their iAs parent compounds (Rahman *et al.* 2012; Smedley and Kinniburgh 2002). Quantifying total arsenic in environmental and biological samples is therefore not synonymous with assessment of associated risks. The main chemical species in freshwaters are inorganic arsenics but methylated and other organic arsenic species are also found (Rahman and Hasegawa 2012; Rahman *et al.* 2012). Freshwater ecosystems are extensive and highly dynamic (Moss 1998) which together with the variable nature of arsenic toxicity makes effects difficult to estimate (Rahman *et al.* 2012; Smedley and Kinniburgh 2002; Smith *et al.* 2002). However, assessment and prediction are essential. In addition to providing water and nutrients for human consumption (Mandal and Suzuki 2002; Smith *et al.* 2002; Villéger *et al.* 2012), freshwater ecosystems may themselves suffer severe impacts from arsenic toxicity (e.g. Rahman and Hasegawa 2012; Rahman *et al.* 2012; Scott and Sloman 2004; Smedley and Kinniburgh 2002).

Biological activity plays a vital role in arsenic speciation, distribution and cycling in freshwaters (Rahman and Hasegawa 2012; Rahman *et al.* 2012). Organismal uptake of arsenic may be direct, through ingestion, inhalation and absorption, or indirect through the food chain (Mandal and Suzuki 2002; Moss 1998; Smedley and Kinniburgh 2002; Smith *et al.* 2002). Microalgae (and bacteria) have important functions in these processes through biotransformation of arsenic species (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). Algae mistake As^{V} for PO_4^{3-} and actively absorb it via the same pathways. Once inside the algal cells, As^{V} becomes toxic and algae can reduce it to As^{III}, methylate it and excrete it, which is thought to be a detoxifying mechanism (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). Several factors influence this process. Different algal species have different methylation abilities (Rahman and Hasegawa 2012) and tolerances to As^{V} (e.g. Favas *et al.* 2012; Levy *et al.* 2005; Wang *et al.* 2013), and not all algae excrete As^{III}. For example, both *Chlorella* sp. and *Monoraphidium arcuatum* take up As^{V} and reduce it to As^{III} but only *M. arcuatum* excretes it (Levy *et al.* 2005). Moreover, recent studies indicate that methylation may not be the primary mode of detoxification in freshwater algae. Instead, arsenic is taken up by cells using the phosphate transport system, reduced to As^{III} in the cell and then excreted into the growth medium, probably by an active transport system (Levy *et al.* 2005; Wang *et al.* 2013). For example, after exposing *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* to different arsenate concentrations, no methylated species could be detected (Wang *et al.* 2013). Similarly, arsenate and arsenite were the dominant species in the freshwater algae *Synechocystis sp.* and *C. reinhardtii* (Yin *et al.* 2011, 2012). This transformation reaction is suggested to be correlated with algal growth rate and P nutrient status, leading to almost complete methylation under P-limiting conditions and slower methylation and excretion of As^{III} into the media if P is in excess (Hellweger and Lall 2004). Nonetheless, these studies confirm that P has a key role in arsenate toxicity and that biotransformation of arsenic by algae is a central component of aquatic arsenic cycling. Indeed, the use of algae is often advocated for

bioremediation of arsenic contaminated water (e.g. Levy *et al.* 2005; Favas *et al.* 2012; Rahman and Hasegawa 2012; Rahman *et al.* 2012; Wang *et al.* 2013).

Fish are a key constituent of aquatic ecosystems and are involved in arsenic mobilization. They are an important component of the aquatic food chain (Agah *et al.* 2009; Kumar and Banerjee 2012; Zhang *et al.* 2013) and even small fish are a source of protein for human consumption (e.g. Moeller *et al.* 2003). Some fish are also used as bioindicators of various aquatic pollutants (Bhattacharya *et al.* 2007; Moeller *et al.* 2003; Moss 1998; Scott and Sloman 2004). Bioaccumulation of arsenic in fish occurs directly through absorption across the gills or skin and indirectly via consumption of prey (Rahman *et al.* 2012); and inorganic, methylated and other organic arsenicals are all found in various fish species (Rahman *et al.* 2012; Rahman and Hasegawa 2012). The effects of arsenic toxicity have been examined in numerous species worldwide. For example, bioaccumulation of arsenic has been recorded in fish from California (Moeller *et al.* 2003), sub-Saharan Africa (Ouédraogo and Amyot 2013), India (Kumar and Banerjee 2012), France (Noël *et al.* 2013), China (Zhang *et al.* 2013) and the Persian Gulf (Agah *et al.* 2009). However, most research has focused on parameters such as bioaccumulation, and physiological parameters such as growth (e.g. Kumar and Banerjee 2012) and metabolic and histopathological effects (e.g. Ahmed *et al.* 2013; Bhattacharya *et al.* 2007). One factor that has received much less attention is fish behavior (e.g. Scott and Sloman 2004; Weis and Candelmo 2012; Weis *et al.* 2001). Contamination in natural systems is often at concentrations well below those that cause mortality, but even low levels of toxicity may be sufficient to interfere with normal functioning. Fish behavior is ideal for assessing these sublethal impacts (Moss 1998; Scott and Sloman 2004; Weis and Candelmo 2012). Much of the current research focusses on direct behavioral responses to contaminants, for example, avoidance of contaminated sites, respiratory changes and behavior like body tremors associated with illness. However, alteration of complex, naturally occurring behaviors such as foraging and predation, agonistic interactions, shoaling and reproductive behaviors are more ecologically relevant indicators of toxicity (Scott and Sloman 2004; Sopinka *et al.* 2010; Weis *et al.* 2001). Various environmental toxicants have been shown to affect complex behaviors (reviewed in Atchison *et al.* 1987; Scott and Sloman 2004). Arsenic in particular reduces long-term memory in the zebrafish, *Danio rerio* (de Castro *et al.* 2009) and is part of a cocktail of chemicals that affects aggressive interactions in the round goby, *Neogobius melanostomus* (Sopinka *et al.* 2010). However, the effects of arsenic on fish behavior have received little attention to date: arsenic is not listed in Scott and Sloman's (2004) comprehensive review of contaminant effects on fish behavior. Given the global impacts of arsenic toxicity (e.g. Mandal and Suzuki 2002; Smedley and Kinniburgh 2002; Rahman *et al.* 2012) more work is needed in this field.

In this study, we examined the effects of arsenic on complex behaviors in the invasive mosquitofish, *Gambusia holbrooki*. This small fish has been introduced worldwide, primarily for mosquito control (Lever 1996; Pyke 2008). Although highly tolerant of a variety of stressors (e.g.

Evans *et al.* 2011; Staub *et al.* 2004; Uliano *et al.* 2010), *G. holbrooki* and the closely related *Gambusia affinis* have been used in toxicity studies (e.g. Tatara *et al.* 1999, 2001) and are known to be affected by arsenic (e.g. Moeller *et al.* 2003; Newman *et al.* 1989). Since behavior links physiological functions with ecological processes, an understudied field of research (e.g. Scott and Sloman 2004; Weis *et al.* 2001), we also included physiological parameters to assess interrelated effects of arsenic toxicity. Moreover, given the intricacies of the feedback and cycling interactions contributing to arsenic toxicity (e.g. Scott and Sloman 2004; Weis *et al.* 2011), field studies may be more general and realistic about environmental effects (Moss 1998), while laboratory studies allow more controlled quantification of effects, and both provide valuable insight (Weis and Candelmo 2012). Therefore, we also examined the interacting effects of naturally occurring algae, thus incorporating some of the complexity of natural systems in a laboratory experiment and disentangling some specific processes from whole ecosystem effects.

We addressed two main aims: first to quantify the effects of arsenic on *G. holbrooki*, and second to assess the interacting affects of algae on arsenic toxicity in this fish species. We examined one direct behavioral response to stress, opercular ventilation rate (Brown *et al.* 2005; Hawkins *et al.* 2004), predicting that operculum movement would increase in response to the stress of arsenic exposure; and two complex behaviors, aggression and foraging. Since both stress (Folkedal *et al.* 2012) and physiological effects of contaminants (Weis *et al.* 2001) can reduce feeding ability and motivation, we predicted that food capture and consumption would be decreased with arsenic exposure. For aggression the effects of toxicant exposure are more ambiguous, provoking both increases and decreases in aggression (Scott and Sloman 2004; Sopinka *et al.* 2010) so while we expected to see a difference with arsenic exposure we made no directional predictions. Then, for physical parameters, we predicted that fish would gain less weight (e.g. Kumar and Banerjee 2012) but increase bioaccumulation (Scott and Sloman 2004) in the presence of arsenic. Finally, given the various and interrelated influences on algal arsenic detoxification capacity we hypothesized that freshwater algal communities will affect As^{V} toxicity to fish, but the direction of effects is, *a priori*, difficult to predict.

2. METHODS

2. 1. Experiment

Mosquitofish were collected from the Ter (42.0451º N, 3.1960º E), Fluvià (42.1875º N, 3.0851º E) and Muga (42.2527º N, 3.0756º E) rivers and transported to the laboratory where they were placed in 60 L stock aquaria (60 cm × 30 cm × 32 cm) each containing a gravel substrate, conditioned water and a filtered air supply. *G. holbrooki* from all three rivers were housed together. Aquaria were maintained at 25 ± 1 ºC and a constant photoperiod (12:12 h light:dark cycle) using 6W bulbs. Fish were fed to satiation once per day with commercial food

flakes or frozen bloodworms (*Chironomus* spp.) and were able to acclimate to laboratory conditions for at least 6 months, with a further month to acclimate to experiment-specific environmental parameters (e.g. temperature: see below).

For the experiment, 12 independent sets of apparatus (experimental units) were set up (see Fig. 1 on ***Chapter 1***). A large (sump) tank (60 cm × 25 cm × 75 cm) was filled with 90 L of filtered water. A smaller (fish) tank (31.5 cm × 11 cm × 31.5 cm) containing 4 L of filtered water was placed on top, and above this was placed a channel (90 cm × 8.5 cm × 7.5 cm) containing sandblasted glass tiles (1 cm^2) to provide substrate for the algal biofilm. 10 g L^{-1} each of phosphate and silicate were added once per week to reproduce phosphate limiting conditions for algal growth, i. e. stationary growth phase (Hellweger and Lall 2004; Moss 1998; Rahman and Hasegawa 2012), and to facilitate diatom growth respectively. Water was pumped from the large tank to the head of the algal biofilm channel, passed through this channel into the fish tank, circulated in the fish tank then passed through the overflow back into the sump tank (see Fig. 1 on ***Chapter 1***). The overflow was covered with a fine mesh to prevent algae and fish from leaving via this route. Water levels were monitored throughout the experiment. Water pH was maintained at 7. 5 using a pH control system based on CO_2 addition (JBL Proflora m630: JBL, Ludwigshafen, Germany) to provide enough inorganic carbon for algal growth (Favas *et al.* 2012; Smedley and Kinniburgh 2002). Illumination (12 h light:12 h dark) was provided by 120W LED Grow Lights (Lightech, Girona, Spain) which produce light without heat, and temperature was maintained at 19.5 ± 5 ºC. This is quite a low temperature for mosquitofish, but well within their tolerance range (Evans *et al.* 2011), and was necessary for algal growth. The experimental units were left to condition for 1 week prior to the start of the experiment.

Natural algal inocula were obtained from the Llémena stream, a tributary of the Ter River, by scraping three cobbles from the upstream zone which has minimum human impact (see Serra *et al.* 2009). On day 1 of the experiment, and at weekly intervals during the following 19 days, the inocula were added to the channels of half of the experimental units so that biofilm was able to colonize the glass tiles. On day 20, 130 g L^{-1} of arsenate was added to the sumps of half of the experimental units. Arsenate was used as this is the most common arsenic species in freshwater and is the species that is taken up by aquatic algae (Hellweger and Lall 2004; Rahman and Hasegawa 2012; Rahman *et al.* 2012). This gave 3 replicates each of 4 conditions: control (C) with neither As^V nor biofilm, biofilm (B), arsenic (A) and biofilm with arsenic (B + A). On day 24, all fish were weighed to the nearest mg using a balance and total length (TL) was measured to the nearest mm using a ruler. Four fish were added to each experimental unit: 1 male (26.8 ± 2.89 mm TL; mean ± standard deviation) and 3 females (1 small: 28.6 ± 5.51 mm TL; 1 medium: 39.4 ± 1.78 mm TL; 1 large: 45.3 ± 2.96 mm TL). This sex ratio was chosen to reduce sexual harassment of females by males (Evans *et al.* 2011; Meffe and Snelson 1989) and as fish numbers were limited. Different sized females were used primarily to allow identification of individuals within a tank so any overlap in sizes between tanks was tolerated. Video recorded observations began on day 25 and continued for 9 days during

which arsenic was measured every day and phosphate was measured every 3 days (Table 1). The video camera was placed approximately 50 cm in front of the narrow sides of the fish tanks. Pilot observations showed that fish were not disturbed by the camera. Each day one 10-min video was taken of each tank. Immediately following this, five defrosted frozen bloodworms were added sequentially to each tank such that one prey was consumed before the next was added (also videoed). The order in which tanks were videoed was randomized daily. After observations, all fish were fed to satiation. Any excess food was removed after 1 h and fish were left until the following day. Any fish that died during the experiment (n = 4) were replaced immediately with a same sex, similar sized individual. This occurred only in the first three days of experiments and in all cases except one were males.

Table 1 Total arsenic and phosphate concentrations (μg L^{-1}: mean ± standard deviation) during the 9 days of observations. For As: n = 9 and P: n = 3.

Treatment	Arsenic	Phosphate
Control	1.92 ± 0.09	12.11 ± 4.10
Biofilm (B)	1.89 ± 0.11	12.28 ± 3.34
Arsenic (A)	127.96 ± 5.55	3.18 ± 1.17
B + A	124.20 ± 2.64	15.96 ± 4.14

After the final observations, all fish were euthanized using an overdose of anesthetic (clove oil) and weighed and measured as before. Liver and gills were dissected out of each female for analysis of tissue arsenic accumulation. These organs were selected as both are crucial sites of metabolic activity so are likely to accumulate arsenic (e.g. Ahmed *et al.* 2013; Kumar and Banerjee 2012). Only females were used for this analysis to avoid biases due to sex differences in bioaccumulation, and as it requires a minimum amount of tissue the single male in each tank was unlikely to be sufficient. To quantify the total amount of arsenic accumulated in fish, the dissected samples were frozen, then freeze-dried, then digested with 4 ml of concentrated HNO_3 (65% HNO_3, Suprapur, Merck, Germany) and 1 ml of H_2O_2 (31% H_2O_2, Suprapur, Merck, Germany). Next, a 75-times dilution with milliQ water and acidification (1%) of the samples was performed. Digested samples were analyzed following the procedure used for total arsenic in water. Bioaccumulation was expressed as dissolved arsenic per dry weight (g arsenic g DW^{-1}). Total dissolved arsenic concentration was measured by ICP-MS (7500c Agilent Technologies, Inc., Wilmington, DE). The detection limit for arsenic was 0.08 g L^{-1}. Rh was used as the internal standard. The accuracy of the analytical method was checked

periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

This work followed all national and institutional guidelines for animal experiments and every effort was made to ensure that suffering to the fish was minimized.

2. 2. Video and statistical analyses

Direct behavior

The frequencies of opercular movements were recorded for each individual by counting the number of times the operculum opened. Since opercula were not always visible, this variable was recorded for a total of approximately 1 min and converted to opercula beats per minute for analyses. In a few cases the fish remained hidden throughout the observation for that day so these observations were excluded from analyses. To assess differences in aggression between treatments, opercula beats min^{-1} were used in a generalized estimating equation (GEE: an extension of generalized linear models developed for situations where response variables are correlated rather than independent). Experimental unit was the between subjects factor and time (day) was the within subjects factor for the model. The fully factorial analysis included two independent factors, presence and absence of biofilm and arsenic, and time was included as a covariate.

Complex behaviors

We recorded the frequencies of aggressive interactions initiated for each fish. These included lunges (rapid movement towards another fish without physical contact), chases (prolonged movement towards another fish with the recipient individual swimming away from the attacker), and bites (as lunges but with physical contact). Since the largest female initiated almost all aggressive interactions in all tanks only these data were used for analyses. We then used the same model as above with number of attacks carried out by the largest female as the dependent variable. Two foraging parameters were obtained: the time required to locate and capture each food item (capture efficiency), quantified as the interval between the food item touching the surface of the water and the first fish grasping the food; and the interval between capture and when each food item was fully consumed (consumption). The means of each of these variables in each tank for each day were calculated and used in separate GEEs as above.

Physical parameters

We also recorded two physical parameters. First, the change in biomass was obtained by subtracting the weight of each fish at the beginning of the experiment from its weight at the end. Any fish that had replaced a deceased individual were excluded from this analysis. These data were used as the dependent variable in a GEE with experimental unit as the between

subjects variable and fish number within each tank as the within subjects variable. The final, factorial model included presence and absence of biofilm and arsenic as independent factors and total length of each fish as a covariate. Second, the tissue concentration of arsenic for the females in each tank was the dependent variable in a factorial generalized linear model (GLM) with the presence and absence of biofilm and arsenic, and the summed changes in biomass for all females in each tank (obtained from the previous analysis) as independent factors. Analyses were conducted using SPSS v. 20. All dependent variables were analyzed with normal distributions and identity link functions.

3. RESULTS

3. 1. Direct behavior

Operculum movement was highest in the control and lowest with just biofilm present. Arsenic produced a lesser decrease in operculum movement whether or not biofilm was present (Fig. 2). Opercular movements increased significantly over time (Table 2, Fig. 2) and there was a significant interaction between time and all other variables while the presence of biofilm and arsenic and their interaction were non-significant (Table 2).

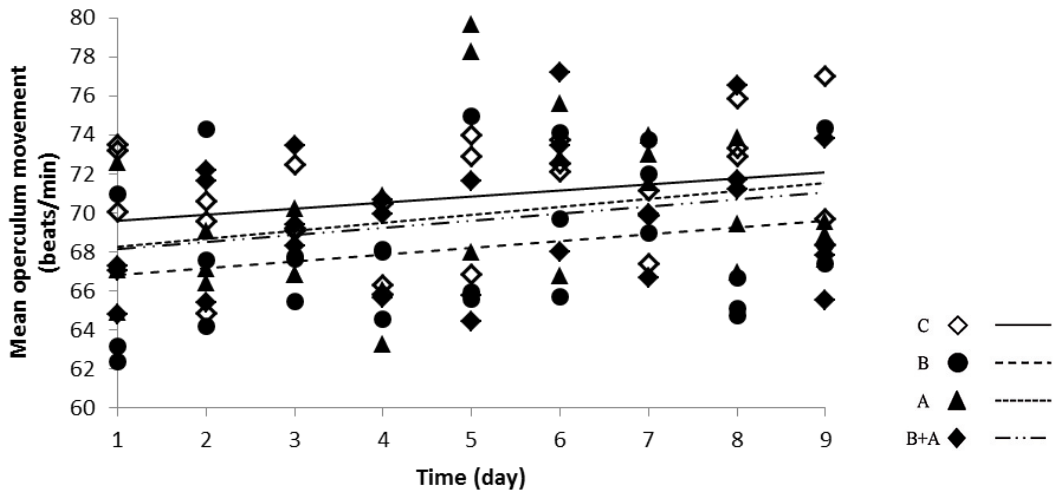


Figure 2 Mean opercular movements for all four fish in each tank. Trend lines illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

Table 2 Results for the generalized estimating equations for variations in operculum movement (beats min^{-1}) and aggression. Significant results are highlighted.

Variable	Operculum Movement			Aggression		
	Wald χ^2	***df***	***p***	**Wald χ^2**	***df***	***p***
Biofilm (B)	2.977	1	0.084	5.061	1	**0.024**
Arsenic (A)	0.025	1	0.876	11.898	1	**0.001**
Time (T)	110.179	8	**<0.001**	76.810	8	**<0.001**
B × A	2.121	1	0.145	1.102	1	0.294
B × T	242.592	8	**<0.001**	13.652	8	0.091
A × T	40.374	8	**<0.001**	18.053	8	**0.021**
B × A × T	207.470	8	**<0.001**	3.910	8	0.865

3. 2. Complex behaviors

Aggression was lowest in the control. Although biofilm presence initially induced an increase in aggression, this appeared to be returning to the same level as the controls (Fig. 3). Aggression increased almost linearly in the presence of arsenic, and was highest in the presence of both arsenic and biofilm (Fig. 3). The frequency of aggression increased significantly with all three independent factors (Table 2, Fig. 3); however, while the interaction between time and arsenic presence was significant, that between time and biofilm presence was marginally non-significant (Table 2). All other interactions were non-significant (Table 2).

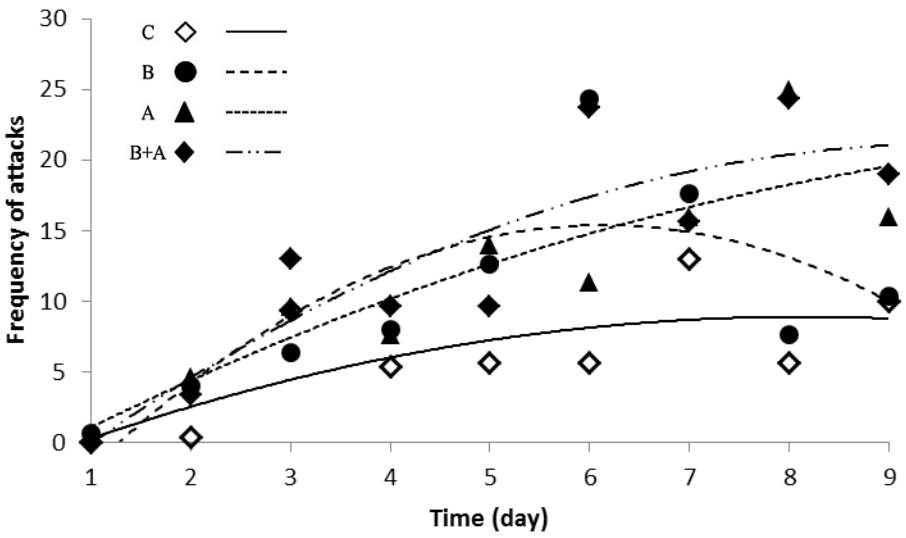


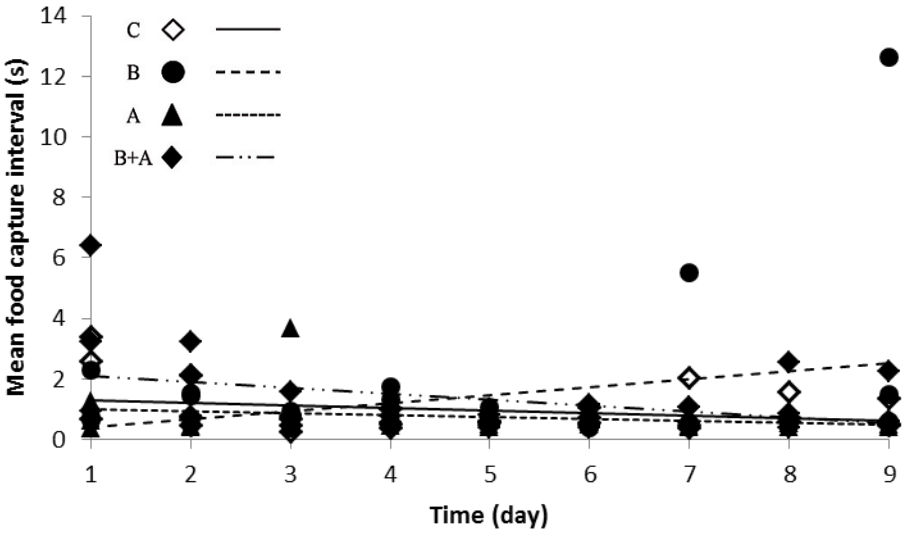
Figure 3 The frequency of attacks carried out by the largest female in each tank on each day. Best fit (quadratic) trendlines have been added to illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control (r^2 = 0.53); B = biofilm (r^2 = 0.63); A = arsenic (r^2 = 0.80); B+A = biofilm + arsenic (r^2 = 0.78).

Time had the greatest effect on both foraging variables with capture interval generally significantly decreasing and consumption interval generally significantly increasing over time (Table 3, Fig. 4). However, capture interval increased significantly in the presence of biofilm (Table 3, Fig. 4a), though this may be an artefact resulting from unusually high values in one tank towards the end of the experiment which may have been caused by external disturbance. We retained this outlier in analyses to maintain sample size. The interaction between time, biofilm and arsenic presence was also significant while all other variables and their interactions were non-significant (Table 3). For food consumption interval the only other significant interaction was between the presence of biofilm and the presence of arsenic (Table 3), though again this may reflect the later high values for biofilm presence in one tank (Fig. 4b).

Table 3 Results for the generalized estimating equations for variation in foraging parameters. Significant results are highlighted in bold.

Variable	Capture			Consumption		
	Wald χ^2	*df*	*p*	Wald χ^2	*df*	*p*
Biofilm (B)	5.816	1	**0.016**	2.759	1	0.097
Arsenic (A)	0.601	1	0.438	1.075	1	0.300
Time (T)	25.578	8	**0.001**	51.362	8	**<0.001**
B × A	0.013	1	0.909	6.611	1	**0.010**
B × T	10.303	8	0.244	7.205	8	0.515
A × T	8.315	8	0.403	6.690	8	0.570
B × A × T	20.873	8	**0.007**	13.325	8	0.101

a)



b)

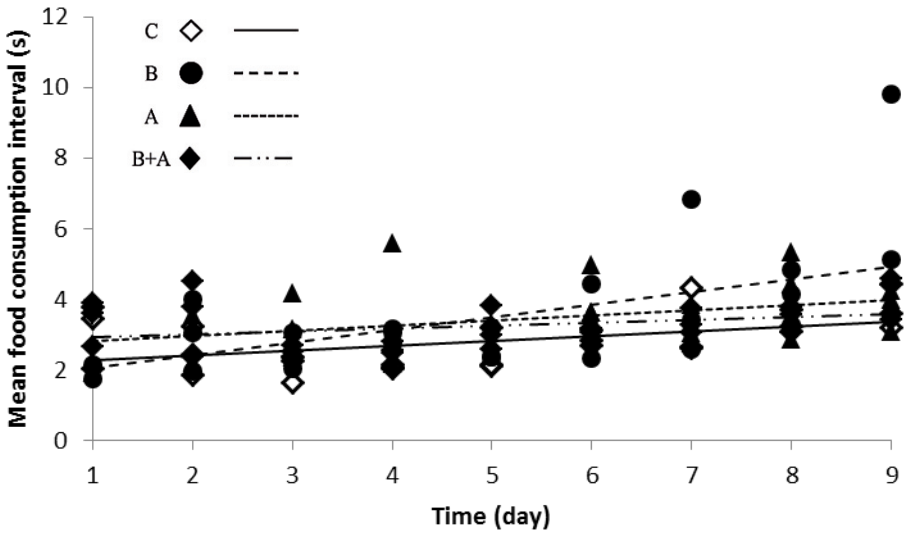


Figure 4. The mean time taken to a) capture and b) consume all five food items in each tank each day. Trendlines have been added to illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

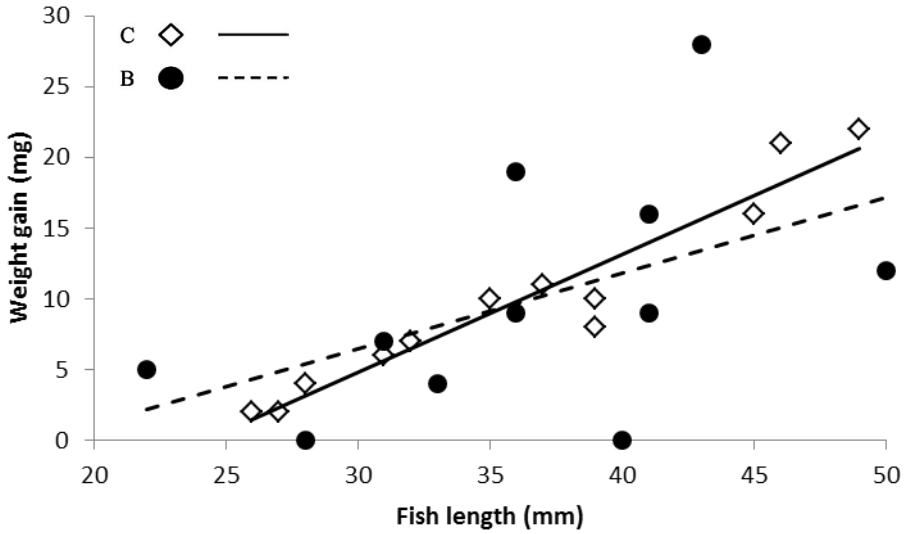
3.3 Physical parameters

All fish gained weight during the experiment (Fig. 5) and there was a significant positive relationship between weight gain and fish length (Table 4, Fig. 5). Biofilm alone showed no effect on weight gain (Table 4) though there was a significant interaction between these two variables (Table 4, Fig. 5a). However, the relationship is unclear. While weight gain increased with fish length, biofilm appears to affect smaller fish more than larger ones and the data is a widely scattered (Fig. 5a). Arsenic had a significant effect on weight gain and showed a significant interaction with both length and biofilm presence and the three-way interaction was likewise significant (Table 4). However, somewhat surprisingly weight gain increased in the presence of arsenic (Fig. 5b) and while the presence of biofilm to some extent appears to ameliorate this effect this is more apparent for smaller than larger fish (Fig. 5c).

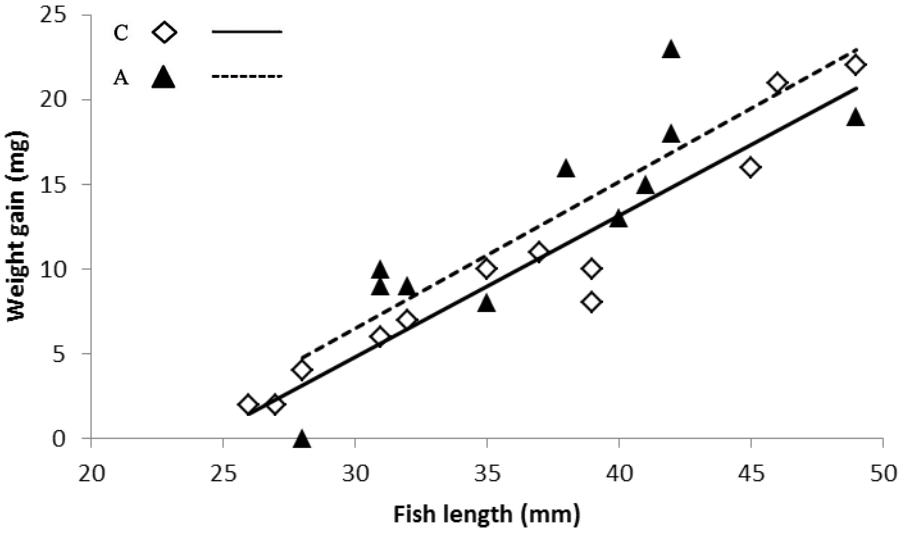
Table 4 Results for the generalized estimating equations for variations in physiological parameters. Significant results are highlighted in bold.

Change in Biomass				**Bioaccumulation**			
Variable	**Wald χ^2**	***df***	***p***	**Variable**	**Wald χ^2**	***df***	***p***
Biofilm (B)	13.208	1	0.349	**Biofilm (B)**	4.181	1	**0.041**
Arsenic (A)	0.876	1	**<0.001**	**Arsenic (A)**	5.138	1	**0.023**
Length (L)	639.187	1	**<0.001**	**Weight (W)**	6.490	1	**0.011**
B × A	15.094	1	**<0.001**	**B × A**	4.492	1	**0.034**
B × L	18.006	1	0.051	**B × W**	4.513	1	**0.034**
A × L	3.792	1	**<0.001**	**A × W**	7.784	1	**0.005**
B × A × L	13.494	1	**<0.001**	**B × A × W**	3.253	1	0.071

a)



b)



c)

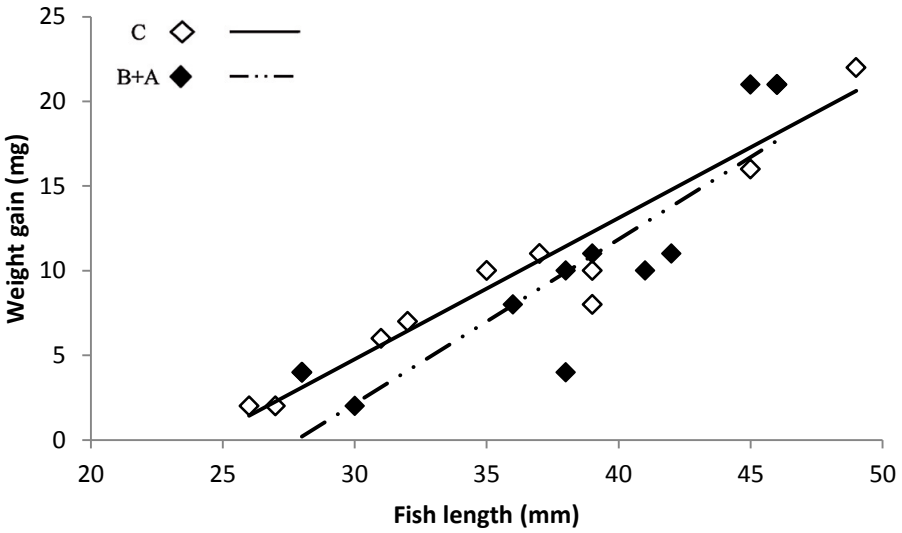


Figure 5 The change in weight between the start and end of the experiment for all fish. For clarity, each of the treatments is shown separately in comparison to the control: a) biofilm; b) arsenic; c) biofilm and arsenic. Trendlines have been added for illustration. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

For tissue arsenic bioaccumulation, all factors and their interactions were significant with the exception of the three-way interaction which showed just marginal significance (Table 4). Not surprisingly, bioaccumulation was higher when arsenic was added to the water and this increased with fish weight increase (Fig. 6). Biofilm presence alone decreased arsenic bioaccumulation, presumably by removing any naturally occurring arsenic in the water. However, when biofilm and arsenic were present together, tissue arsenic accumulation showed a dramatic increase, even above that shown with arsenic alone (Fig. 6).

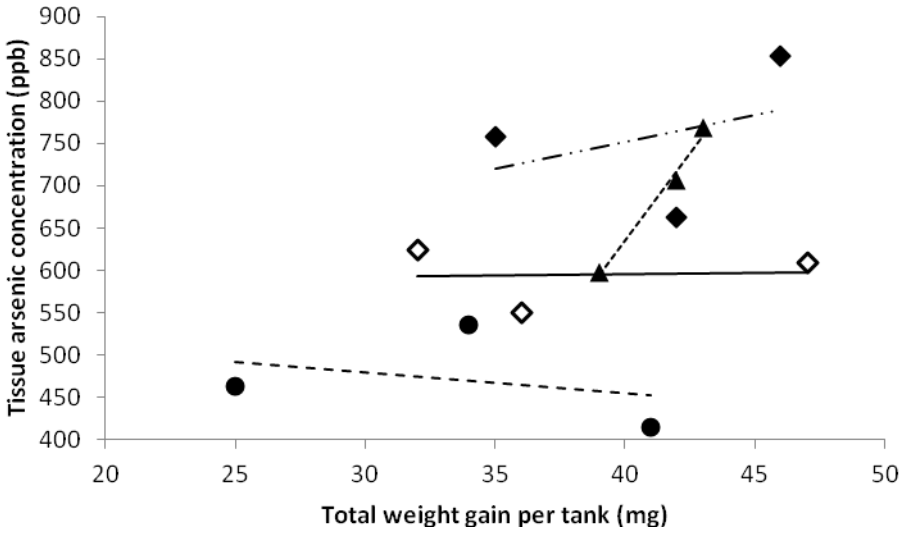


Figure 6 The differences in tissue arsenic concentration as a function of total weight gained in each tank and the presence and absence of biofilm and arsenic. Trendlines have been added to illustrate these relationships. C = control; B = biofilm; A = arsenic; B+A = biofilm + arsenic.

4. DISCUSSION

Arsenic produced some effects in mosquitofish, though not exactly as predicted. Aggression increased significantly in the presence of arsenic while for operculum movement and food capture efficiency and consumption rate time, rather than arsenic presence, was the major predictor. Aggression appears to be the major initial behavioral effect of arsenic exposure in this species and continued to increase with exposure duration. Of the behaviors measured, aggression may thus be a suitable biomarker for arsenic toxicity in mosquitofish (Moss 1998; Scott and Sloman 2004; Weis *et al.* 2001). Increased aggression may be induced through stress or related physiological changes due to arsenic exposure (e.g. Scott and Sloman 2004), which may increase the metabolic costs for an individual, thereby leading to increased stress and a potentially damaging feedback cycle. Aggression in some fish species increases with other toxicants. For example, bluegills, *Lepomis macrochirus*, exposed to copper for 96 h increased the frequency of agonistic acts (Henry and Atchison 1986), while round gobies from

contaminated sites increased their rate of assessment displays compared to fish from a reference site (Sopinka *et al.* 2010). In both these cases, dominance status played a role with more dominant bluegills increasing aggression over subordinates (Henry and Atchison 1986) and reduced dominance establishment in contaminant site gobies (Sopinka *et al.* 2010). In the present study, almost all agonistic acts were initiated by the largest, presumably dominant, female which may explain the lack of notable effects on foraging parameters. One of the major functions of aggression is resource defense, mainly defense of mates, shelter or food (Huntingford and Turner 1987; Magellan and Kaiser 2010). If the largest female was monopolizing most of the food resources, competition for the remaining food by the other individuals may mask any effects of arsenic exposure. However, foraging efficiency was only recorded for the first few food items, after which fish were fed to excess, so later effects may have been overlooked. Time had the greatest effect on foraging, the faster capture efficiency probably being due to fish learning to anticipate food and the slower consumption rate reflecting reduced motivation to feed as they gained weight. However, other factors cannot be ruled out. The concomitant increase in operculum rate over time suggests variation in oxygen demand or efficiency of oxygen uptake which may be induced by the build-up of other chemicals, such as nitrogen, naturally excreted by fish.

These behavioral results can be integrated with the physical results. All fish gained weight during the nine days of observations, probably because the few fish per tank were fed to excess each day so were released from the competition they would have experienced in the stock aquaria, which reflects the foraging results above. Larger fish gained the most weight in all treatments, although unexpectedly arsenic promoted weight gain. The reasons for this result are unknown. The accepted view is that contaminant load should cause a loss of condition (e.g. Kumar and Banerjee 2012; Scott and Sloman 2004; Weis *et al.* 2011). Increased size has been shown in grass shrimps, *Palaemonetes pugio*, from contaminated sites but this is explained by reduced predation from fish at these locations (Weis *et al.* 2011). In this study, predation was not a factor although it is interesting that weight gain and aggression varied in parallel, which may imply some effect of resource defense. Increase in fish biomass and bioaccumulation also showed similar patterns, the obvious explanation being that greater weight gain allows more arsenic to be assimilated and fixed in tissues. However, it may also be that fish that gain more weight have characteristics, such as increased aggression and therefore resource holding potential (e.g. Magellan and Kaiser 2010), that also contribute to arsenic bioaccumulation. Although we provided daily uncontaminated food, mosquitofish also consume algae and diatoms (García-Berthou 1999). The algae present in the biofilm treatments, some of which dropped into the fish part of the experimental units, were likely to be heavily contaminated with arsenic, which may have promoted bioaccumulation. Finally, small fish such as these mosquitofish, which have a large surface area to volume ratio, are particularly susceptible to absorption of toxins through the skin (Moeller *et al.* 2003; Rahman *et al.* 2012), which may be another contributing factor.

Surprisingly, the presence of algae appeared to aggravate, rather that ameliorate, the effects of arsenic exposure in mosquitofish. In terms of increase in fish biomass, although algae acted antagonistically with arsenic, this resulted in a reduction in weight gained which is not likely to be advantageous. This effect is particularly apparent in smaller fish. For bioaccumulation the effects of algae were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic, although in this case the interaction was not significant. One plausible explanation concerns the biotransformation of arsenic by algae as described in the section 1 of this chapter. The exact nature of this transformation depends on algal growth and P nutrient status in the environment (Hellweger and Lall 2004; Levy *et al.* 2005; Rahman *et al.* 2012). Under P-limiting conditions, when algal growth is slow, algae excrete DMA^{III}. Under P-replete conditions with fast algal growth, PO_4^{3-} assimilation is up-regulated and As^{V} uptake increases in parallel. Since the transformation of As^{V} to As^{III} is faster than that of As^{III} to DMA^{III}, As^{III} builds up within algal cells and is consequently excreted into the environment to keep intracellular As^{III} at low levels and allow reductase activity (Hellweger and Lall 2004; Levy *et al.* 2005; Rahman *et al.* 2012). The phosphate concentration in our system was selected to simulate P-limiting conditions (Hellweger and Lall 2004; Moss 1998; Rahman and Hasegawa 2012) so should have limited algal growth and consequent arsenic uptake. However, as a recent study showed (Wang *et al.* 2013), even in P-limiting conditions algal As^{V} uptake may increase as cells synthesize more P transporters to compensate for the lack of phosphate in the environment. More importantly, however, fish metabolism produces waste, especially ammonia and phosphate. N and P recycling rates vary between species (Vanni *et al.* 2002; Villéger *et al.* 2012) and while the exact rate of N and P excretion by fish in this experiment was not quantified, stress is known to strongly stimulate urea (N) excretion in mosquitofish (Uliano *et al.* 2010). It is therefore likely that the presence of mosquitofish stimulated P-replete conditions and accelerated the biotransformation of arsenic by algae. A further consideration is algal growth. Nutrient supply, in particular phosphorus and nitrogen, is the most important determinant of algal production (Moss 1998; Rahman and Hasegawa 2012; Villéger *et al.* 2012). Algal growth, nutrient concentration, and arsenic are thus intricately linked. Research has shown a positive correlation between As^{III} concentration and primary productivity (Rahman and Hasegawa 2012) and the presence of fish is likely to contribute to this effect. Other elements such as oxygen (Smedley and Kinniburgh 2002; Wang *et al.* 2013) and iron (Senn and Hemond 2002) also influence arsenic speciation. Whatever the exact mechanisms here, it is evident that these various processes interacted to promote biotransformation of arsenic by algae. The end products of this transformation, in particular As^{III}, are less toxic to algae, but more toxic to fish (Rahman *et al.* 2012; Smedley and Kinniburgh 2002), so even if the overall aquatic arsenic concentration is reduced by algae, this may be counterproductive at an ecosystem scale.

For mosquitofish, the effects of arsenic exposure are overall detrimental. Despite the increased biomass seen here with arsenic, bioaccumulation of arsenic is harmful (de Castro *et al.* 2009; Moeller *et al.* 2003; Sopinka *et al.* 2010) and increased aggression may increase the

chance of physical damage (e.g. Huntingford and Turner 1987) and exacerbate physiological effects of arsenic exposure (e.g. Scott and Sloman 2004). Moreover, in addition to, or as a consequence of, the effects documented here other functions and interactions are likely to be disrupted. For example, both mate recognition (e.g. Fisher *et al.* 2006) and predator recognition (e.g. Mandrillon and Saglio 2007) are compromised by alteration of the chemical environment. The mechanisms underlying the behavioral changes demonstrated in this study may involve sensory, hormonal, neurological and metabolic systems (Scott and Sloman 2004) all of which may also affect other behaviors including locomotory behaviors like predator avoidance or swimming performance. The increase in aggression and lack of effects on feeding behavior in this study suggest locomotory functions were not affected. However, the exposure treatments here were neither particularly acute nor chronic and increased exposure concentrations or durations are likely to lead to more serious impacts. Finally, here we used an invasive, highly tolerant fish as a model. The effects of arsenic exposure on potentially endangered native species would be both more difficult and more critical to evaluate.

5. CONCLUSION

In conclusion, we have shown here that changes in complex behaviors are practical, ecologically relevant measures of toxicological effects. Aggression in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behavior. In common with other authors, we also highlight interacting effects of contaminant exposure, both through integration of behavioral and physical mechanisms and consideration of different taxa together. Especially, toxicant responses in multi-trophic, natural ecosystems are often found to be different from single-species laboratory studies. Multi-trophic studies are therefore crucial to elucidate the real effects of toxicants. An important finding in this respect from the current study is the aggravating influence of algae on the impacts of arsenic exposure in fish. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. Such multidisciplinary, cross-taxon research is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

CHAPTER 3

MUTUAL INTERACTION BETWEEN ARSENIC AND BIOFILM IN A MINING IMPACTED RIVER

Barral-Fraga L, Martiñá-Prieto D, Barral MT, Morin S, Guasch H.

Mutual interaction between arsenic and biofilm in a mining impacted river.

In prep.

ABSTRACT

Gold mining activities in fluvial systems may cause arsenic (As) pollution, as is the case in the Anllóns River (Galicia, NW Spain), where high concentrations in surface sediments (up to 270 mg kg^{-1}) were found. A 51 day-long biofilm translocation experiment was carried out in this river, moving colonized substrata from upstream (less As-polluted) to downstream the mine area (more As-polluted site with also more easily extractable As), to explore the effect of arsenic on benthic biofilms and the role of these biofilms on arsenic retention and speciation in the water-sediment interface. Eutrophic conditions (high total dissolved phosphorus and total nitrogen) were detected in water at both sites, while sediments were not considered P-polluted. Translocated biofilms accumulated more arsenic and showed higher potential toxicity (higher As/P ratio) than non-translocated ones. In concordance, their growth was reduced to half that observed in those non-translocated. Moreover, they became less nutritive (less N content) and with higher bacteria and dead diatom densities than the non-translocated biofilms. Methylated As-species (DMA^V) were found in the intracellular biofilm compartment and also in the river water, suggesting a detoxification process by biofilm (methylation) and its contribution to arsenic speciation in the water-benthic biofilm interface. Since most arsenic in sediments and water was arsenate (As^V), the high amount of arsenite (As^{III}) detected in the biofilm extracellular compartment may be attributed to As^V reduction by biofilms. Our study provides new arguments to understand microorganism contribution to arsenic biogeochemistry in freshwater environments.

1. BACKGROUND

Microorganisms constitute the majority of all living matter on Earth, most of them living in the form of multicellular aggregates commonly referred to as biofilms (Mora-Gómez *et al.* 2016). The modification of microbial composition and activity may have ecological consequences on local, regional and global scales (Huang 2014). In rivers, biofilms are the first to interact with dissolved substances from the surrounding environment, such as pollutants, being able to actively influence their sorption, desorption and transformation (Behra *et al.* 2002; Guasch *et al.* 2010). For all these reasons, fluvial biofilms provide an outlook of community ecotoxicology and allow a high degree of ecological realism either in ecotoxicological studies in micro/mesocosms or in the field by controlling the simultaneous exposure of many species and investigating direct and indirect toxic effects after acute and chronic exposure (Guasch *et al.* 2010). By carrying out studies in the field, the effects of pollution may be evaluated under real exposure conditions, using a set of biofilm parameters (i.e., endpoints) together with the analysis of water chemistry and the prevailing environmental conditions (Guasch *et al.* 2010; 2016). For instance, *biofilm translocation* experiments in fluvial systems using biofilm developed on artificial substrates are considered an active biomonitoring approach to assess the effects of metal pollution on these natural communities (Bonet *et al.* 2014; Morin *et al.* 2016). As a major component of benthic biofilms, diatoms (microscopic, unicellular brown algae) are considered

good indicators of environmental conditions due to their quick response to environmental changes and their cosmopolitanism in aquatic systems, making them widely used in ecotoxicological studies (Morin *et al.* 2012).

Another interesting aspect of biofilms is that they allow the coexistence of microniches of different physiological requirements, allowing the simultaneous, but spatially separated occurrence of opposite redox processes in the same biofilm environment (Huang 2014). For instance, in biofilms, there might be interestingly simultaneous arsenic (As) oxidation and reduction (Huang 2014) due to existing niches for both arsenate (As^{V}) respirers and arsenite (As^{III}) oxidizers (Kulp *et al.* 2004). This characteristic contributes to the fact that biofilms play a major role in driving biogeochemical cycles (Huang 2014). For instance, autotrophic and heterotrophic communities in biofilms may drive, directly or indirectly, a complex interplay of arsenic mobilization, sequestration and transformation processes that determine the fate of this metalloid in the environment (Huang 2014; Wang *et al.* 2015).

Several parts of the world have been affected by arsenic due to its poisoning in soils, sediments and water through natural events and anthropogenic activities, especially mining (Smedley and Kinniburgh 2002; Wang and Mulligan 2006; Inam *et al.* 2011; Battogtokh *et al.* 2013). Originally, more than 200 minerals containing arsenic are found in nature, forming primary minerals in the Earth's crust, most of which are in close association with metals, such as gold (Au) (Drewniak and Sklodowska 2013). Thus, arsenic may be mobilized during gold mining activities as gold- and arsenic-bearing minerals coexist (Garelick *et al.* 2009). In river systems, and as a result of weathering and the hydrothermal alteration of these primary minerals, arsenic is predominantly bound to sediments, which may contain high amounts of this element, especially in mining areas (Drewniak and Sklodowska 2013). In these areas, arsenic concentrations can reach up to hundreds or thousands of mg kg^{-1} in sediment (Smedley and Kinniburgh 2002; Rubinos *et al.* 2011b), thus exceeding the Severe Effect Level, set at 33 mg As kg^{-1} by the Canadian Sediment Quality Guidelines (Persaud *et al.* 1993), and above which a sediment is considered to be heavily polluted and likely to affect the sediment-dwelling organisms. Furthermore, mean arsenic concentration of 137.17 µg L^{-1}, and even up to 7900 µg L^{-1}, have been found in river waters close to mining areas (Smedley and Kinniburgh 2002), clearly exceeding the concentration limit of chronic arsenic exposure in freshwaters set at 150 µg L^{-1} (Criteria Continuous Concentration, CCC) by the Aquatic Life Criteria (US EPA 2014).

Sediments may act as a sink for pollutants that can subsequently be released (Magbanua *et al.* 2013) upon changes in environmental conditions. Arsenic mobility in soils and sediments depends on different processes (oxidation/reduction, complexation/precipitation, adsorption/desorption and dissolution of As-bearing phases). Moreover, changes in water chemistry, such as the introduction of high concentrations of phosphorus (P), may promote the release of arsenic from solid phases through competitive ligand-exchange reactions due to the chemical similarity between phosphate (PO_4^{-3}) and arsenate (AsO_4^{-3}), since both form oxyanions in the +V state and have similar p*Ka* values (Rubinos *et al.* 2011b).

Both the total arsenic concentration and the chemical form (arsenic species) have to be considered in toxicological analyses, since a key factor in the arsenic mobility and toxicity is its chemical speciation. Specifically, arsenic may occur in the environment in four oxidation states: +V (arsenate), +III (arsenite), 0 (arsenic) and -III (arsine). In natural waters and soils, it is mostly found in inorganic form (iAs) as arsenate (As^{V}) and arsenite (As^{III}) (Oremland and Stolz 2003; Sharma and Sohn 2009). Eh (oxidation/reduction potential measurement) and pH are considered the most important abiotic factors controlling arsenic speciation (Smedley & Kinniburgh 2002). Arsenate (As^{V}) is the thermodynamically stable state under oxic conditions, while arsenite (As^{III}) is the predominant species in anoxic conditions (Smedley and Kinniburgh 2002).

Usually, the form As^{III} is more toxic for the environment than As^{V}, but algae are more sensitive to the As^{V} than to the reduced form As^{III} (Levy *et al.* 2005; Wang *et al.* 2015). Moreover, biofilms may perform other biotransformations, such as arsenic methylation (Prieto *et al.* 2016a), and produce other more complex organic compounds, such as arsenosugars and arsenolipids (Huang 2014). According to Hellweger *et al.* (2003), arsenic biotransformation depends on phosphorus availability: in P-deficient conditions, As^{V} is uptaken by algae, reduced to As^{III} and further methylated as final products; in contrast, under P-enriched conditions, As^{V} is uptaken and reduced, but not all of it is methylated, resulting in As^{III} excretion into the environment. All these transformations have an enormous impact on the environmental behavior of arsenic, since the different chemical forms of arsenic exhibit different toxicity towards higher organisms: usually methyl As^{III} > As^{III} > As^{V} > methyl As^{V} (Huang 2014).

The implications of arsenic biogeochemistry on the ecology of freshwaters have been poorly addressed, despite having proved the key role of microorganisms, in particular microalgae and bacteria, on the arsenic detoxification and fate in aquatic environments as in mining impacted rivers. With the aim of assessing the fate and effects of the arsenic pollution on a freshwater ecosystem, we carried out a translocation experiment with benthic biofilms in the Anllóns River (Galicia, Spain), where high arsenic concentrations in soils and river bed sediments caused by old gold-mining activities were found (Rubinos *et al.* 2003; Devesa-Rey *et al.* 2008; Rubinos *et al.* 2010; Costas *et al.* 2011). Arsenic fractionation has indicated that most arsenic 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). However, arsenic mobility increased upon changes in environmental conditions, such as high salinity, alkaline pH, high phosphorus concentrations and high liquid:solid ratio (Rubinos *et al.* 2010, 2011). Moreover, arsenic biogeochemistry in the Anllóns River is also affected by the biological status of the river sediments, covered by biofilms (Devesa-Rey *et al.* 2009) mainly constituted by diatoms, which represent more than 86% of the total algal abundance in the superficial sediments (Martiñá Prieto *et al.* 2016). In fact, epipsammic biofilms from the Anllóns River have proved that they have a great effect on arsenic retention, mobilization and speciation (Prieto *et al.* 2013; 2016a; 2016c).

We intended to study the influence of benthic biofilms on arsenic retention, transformation and mobilization at the water-solid interface of this fluvial polluted-system. Therefore, we proceed from the assumption that the fluvial biofilm plays a crucial role in the mobilization of arsenic from the sediment to other compartments. We expected to find a causal link between the presence of arsenic in mobile forms in river sediments and their accumulation in the biofilm, causing changes in its structure and function. We also hypothesized that biofilms may transform and excrete into the water the bioadsorbed or bioaccumulated arsenic by transforming it into other inorganic and organic arsenic species (biospeciation), modulating arsenic toxicity in the environment. To test these hypotheses a set of analyses was carried out to study the ecology of the Anllóns river in two sites, upstream and downstream the mine area, focusing on the relationship between environmental conditions, especially arsenic exposure, and the structure and function of the biofilm community.

2. MATERIAL AND METHODS

2.1 Study site

This experiment was carried out in the Anllóns River (Fig. 1), located in Galicia (northwest Spain). The river is 71 km long, with a mean slope of 7.6 %, and an average annual flow of 10.77 m^3 s^{-1}. The basin covers an area of 516 km^2 mostly dedicated to forestry, agriculture and cattle farming. Gold mining activities were active in the area during the Roman Empire and also between the years 1895 and 1910. Gold in this area is associated with pyrite and arsenopyrite (Nespereira 1978). Concentrations of arsenic up to 4000 mg kg^{-1} have been detected in superficial soil horizons due to the presence of arsenopyrite mineralizations associated with gold ores in hydrothermal quartz veins (Boixet *et al.* 2007). High total arsenic contents were also detected in the sediments of the Anllóns River, downstream of the gold-mining area (Rubinos *et al.* 2003), which were attributed to natural geogenic arsenic enrichment exacerbated by mining activities (Devesa-Rey *et al.* 2008). Recently, the possibility of exploiting this mineralized zone again was considered, causing social concern among locals. Concerning water quality, there are two important sources of P pollution near the main town of Carballo, located upstream of the gold-mining area: a wastewater treatment plant and a seafood canning factory (Rubinos *et al.* 2003). According to EU Directive 75/440/CE (Díaz-Fierros 2003). the Anllóns River has been classified as A2 (potabilization requires physical and chemical treatments) with the exception of a stretch of the river, downstream of Carballo, which is classified as A3 (physical, intense chemical and disinfection treatments).

In this research, we carried out a translocation experiment between two sites with similar lithology (alkaline gneiss) and physicochemical conditions, but with different arsenic concentrations in the sediments. We selected one site upstream of the mining area referred to as Upstream site or Up (43.222149° N, -8.782352° W); and a second one located just downstream of the mining area, with a higher arsenic concentration, referred to as Downstream site or Down (43.230118° N, -8.831897° W).

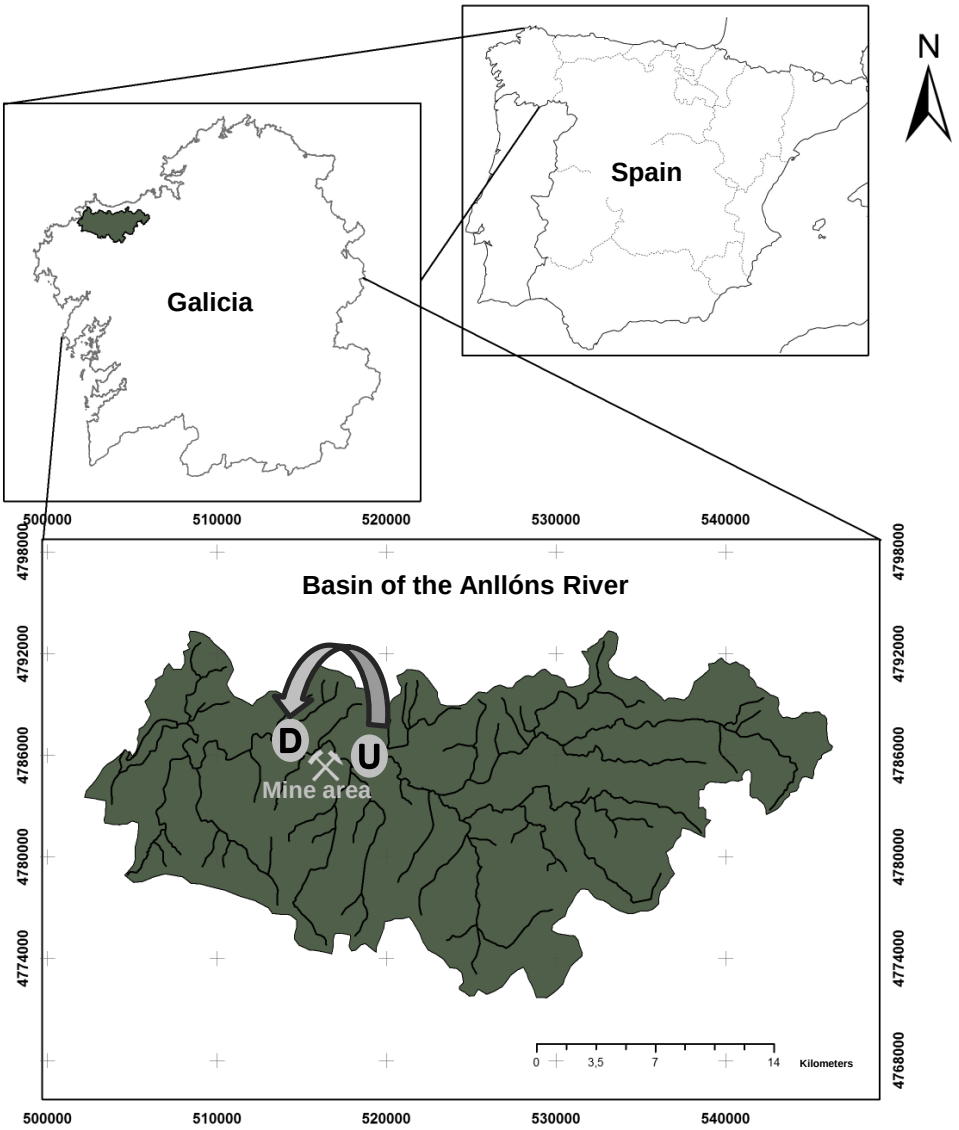


Figure 1 Study site in the Anllóns River (Galicia, NW Spain). "U" (in gray circle), corresponding to the Upstream sampling site (located upstream of the mining area); "D" (in grey circle), corresponding to the Downstream sampling site (located downstream of the mining area). The grey arrow indicates the biofilm translocation from the Upstream to the Downstream sampling site.

2.2 Experimental set-up

Small (8.5 × 2 cm) and large (12 × 9 cm) sandblasted glass tiles, fixed to cement cobbles with neutral silicone sealant (Quilosa, Madrid, Spain), were placed horizontally on the riverbed in the Upstream site for natural biofilm colonization and growth (Fig. 2). The use of artificial glass substrates reduces the heterogeneity that occurs on natural substrates (Cattaneo *et al.* 1997), making quantitative sampling easier, and allows to control the maturity degree of the biofilm for the analyses. Translocation was carried out after 5 weeks of biofilm colonization: half of the artificial substrates were moved from the Up site to the Down site. The experiment

started at that moment and several samplings were carried out at both sites to simultaneously assess the fate and effects of arsenic on biofilms. The experiment lasted 51 days, from July 8th until August 28th 2014.

Figure 2 Experimental setup in the Anllóns River. Artificial substrates before the biofilm colonization (a) and after 5 weeks of colonization (b), when the biofilm was translocated from the Upstream to the Downstream site. Artificial substrates on the bottom of the river in the Upstream site (c).

2.3 Sampling

Biofilm and river water were sampled 5 times during the experiment: on days 2, 4, 7, 22 and 51 after translocation. Two samplings were carried out for dissolved arsenic concentration: after 22 days of translocation in the Downstream site, and at the end of the experiment (day 51 after translocation) at both sampling sites. Sediments were sampled twice: on days 7 and 51 after translocation.

River Water sampling

Triplicate water samples (1 L) were collected and immediately filtered using GF/F glass microfiber filters (Whatman, ~0.7 µm of pore size) for nutrients analyses, including total dissolved phosphorus (TP), total dissolved nitrogen (TN) and total dissolved organic carbon (DOC). In the laboratory, some samples were filtered through 0.45-µm cellulose nitrate membrane filters NCS 045 47 BC (Albet LabScience, Dassel, Germany) for soluble reactive phosphorus (SRP); and some were filtered with sterile 0.45-µm Whatman Puradisc 25AS™ syringe filters (GE Healthcare Europe GmbH, Barcelona) for DOC determination. For the analysis of dissolved arsenic water was filtrated through 0.45 µm nylon membrane filters (Whatman). All river water samples were frozen until analysis. The GF/F filters were also kept for dry weight (DW) determination of river water suspended solids (SS).

Sediment sampling

Complex sediment samples were collected using a small plastic shovel from the top 5 cm at various points from each site, mixed in situ and taken to the laboratory in hermetic plastic containers topped up to prevent oxidation. In the laboratory, sediment samples were centrifuged (3000 rpm, 15 minutes). Solid sediment samples were freeze-dried and sieved (<2 mm). Only some organic debris were eliminated by sieving, so the fraction <2 mm practically represented the bulk sediment. This fraction was used to determine the particle size distribution using sieves of 2, 1, 0.5, 0.25, 0.1 and 0.05 mm, as well as the easily-extractable arsenic concentrations (extracted with phosphate buffer, following Gleyzes *et al.* 2002) in the sediment, and arsenic speciation in these extracts. A representative aliquot of the <2 mm fraction was milled and sieved (<50 µm) for the determination of total arsenic concentration (by X-ray fluorescence analysis), total phosphate (TP, through the molybdenum blue method of Murphy and Riley 1962), total Kjeldhal nitrogen (TN, by Guitián and Carballas 1976) and percentage of total organic matter (OM, following UNE-EN 13039, AENOR, 2012), from which total carbon (TC) was then calculated.

Bioavailable arsenic in sediment was also measured *in situ,* using diffusive gradient in thin films (DGT) devices (DGT Research Ltd., Lancaster, UK). On the translocation day, the DGT devices were placed on the surface of the riverbed sediments in the Down site, to evaluate arsenic bioavailability at two sampling times: after 7 and 51 days of translocation (that is, at the beginning and at the end of the experiment).

Biofilm sampling

Colonized artificial substrates were sampled at random and in triplicate in the field, and the biofilm scraped into falcon tubes or glass vials. For bacterial density and diatom analyses (relative abundance and quantification of live diatom community), samples were preserved in glass vials with 5 mL of filtered river water with 37% formaldehyde (Panreac, Spain) (dilution 0.5:4.5), with 3 replicates for each one. All samples were kept in dark conditions in the fridge

until they were analyzed in the laboratory. In turn, biofilm from large substrata was scraped into falcon tubes and then some fresh samples were used to determine the concentration of Total bioaccumulated arsenic and its species distribution in the different biofilm compartments, while other samples were freeze-dried to determine total biofilm dry weight biomass (DW) and elemental composition. Non-scraped samples from small artificial substrates were transported in triplicate to the laboratory, in dark conditions and in an oxygenated box with river water, for *in vivo* fluorescence analyses.

2.4 Sample analysis

Environmental endpoints

Environmental light intensity (µmol photons s^{-1} m^{-2}) was measured *in situ* with a light sensor (LI-COR Inc., Lincoln, Nebraska, USA) at each sampling time in both sites. Light measurements were carried out on submerged biofilm in order to measure light reaching benthic biofilm, as well as below the riparian zone and below a nearby zone without vegetation to calculate the cover (%) of the sampled river sections.

River water analyses

Water temperature, dissolved oxygen, pH and electrical conductivity were measured in the field at each sampling time using multiparametric probes (WTW METERS, Weilheim, Germany). Redox potential was determined with a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, Eibar, Spain). Eh values obtained with the Pt-Ag/AgCl electrode were corrected to refer them to the standard hydrogen electrode by adding 245 mV.

Suspended solids (SS) were analyzed in river water during the experiment (on days 2, 7, 22 and 51 after translocation) at both sampling sites, according to APHA (1995). The solids were separated by filtration of 1 liter of river water through pre-weighed GF/F glass microfiber filters (~0.07µm, Whatman). Filters were then left to dry at 103-105 °C to a constant weight to obtain the dry weight (DW) of SS.

Total dissolved phosphorus was determined by means of an acid digestion of the filtered samples (~0.7µm) with 1 ml of H_2SO_4 31% and 0.4 g of $(NH_4)_2S_2O_8$ at 121 °C during 30 min (APHA 2005). Phosphorus in the extracts was determined using the phosphomolybdenum blue method described by Murphy and Riley (1962) and soluble reactive phosphorus was determined colorimetrically in 0.45 µm filtered samples following the same method. Total dissolved nitrogen was determined by digestion of filtered samples (~0.7µm) with H_2SO_4 and 3% H_3PO_4 using the Kjeldhal method (UNE-EN 25663: AENOR, 1994). Dissolved organic carbon was determined in 0.45 µm filtered samples using a Total Organic Carbon Analyser Model TOC-5000 (Shimadzu, Kyoto, Japan), by subtracting the inorganic carbon (IC) concentration from the total carbon (TC) concentration.

Arsenic concentration in river water was analyzed in samples taken at translocation day 22 and 51. Total arsenic concentration was determined in 0.45-µm filtered samples by Inductively Coupled Plasma Spectrometry (ICP-MS, Varian 820MS), equipped with collision reaction interface (CRI) technology to reduce polyatomic interferences. For arsenic speciation (As^{V}, As^{III}, DMA^{V}, MMA^{V} and As-Bet), High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Spectrometry (HPLC-ICP-MS) was used. A Varian Prostar 230 HPLC was employed, equipped with a guard column and an anion exchange column Hamilton PRP-X100 (4.1 x 250 mm and 10 µm). For the determination of arsenic species, a 13 minute 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 were used. 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 As-Bet, respectively.

Sediment sample analysis

The pH and Eh were analysed *in situ* with a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, S.L., Eibar, Spain. Particle size distribution was determined by dry sieving. Total phosphate was determined in previously ignited (450 °C for 1h) samples (<50 µm), using the molybdenum blue method (Murphy and Riley 1962) after acid digestion (concentrated HF + H_2SO_4 + HCl 10:1:10, 220 °C). Total Kjeldhal nitrogen was determined by wet digestion of samples (<50 µm) with H_2SO_4, following the Kjeldahl method as described in Guitián and Carballas (1976). The determination of % OM was carried out following the UNE-EN 13039 standard (AENOR, 2012), by calcination at 450°C for 2 h of 5 g dried samples. Total carbon was calculated using the equation %C = %OM/1.724.

Total arsenic concentration in the sediment was determined by X-ray fluorescence (XRF) spectrometry (custom built, equipped with a Philips high-voltage generator and a Mo anode of 2.2 Kw as X-ray source), following the considerations described by Devesa-Rey *et al.* (2008): 1g of sample was placed in a cylindrical container (2.5 cm diameter × 2 cm height) covered by a film (Prolene®). The accuracy of the XRF measurement of total arsenic was checked by using the certified reference material BCR CRM-277b. For this reference material, the arsenic concentration (mg kg^{-1}) measured was 45.4 ± 4.1 (certified value 47.3 ± 1.6).

The easily-extractable arsenic concentration (total arsenic and arsenic speciation) was obtained using a phosphate buffer, following the method of Gleyzes *et al.* (2002). Arsenic extraction was carried out on 1g of freeze-dried sediment samples (<2 mm fraction) during 1 hour, after addition of 20 ml of buffer KH_2PO_4/K_2HPO_4 0.1 M and 1% of hydroxylamine to preserve the redox state of the arsenic species. The extracts were then filtered through a sterile 0.45 µm filter (Whatman Puradisc 25AS™ syringe filters, GE Healthcare Europe GmbH, Barcelona), and the total arsenic concentration as well as the arsenic speciation (As^{V}, As^{III}, DMA^{V}, MMA^{V} and Arsenobetaine, As-Bet) were determined by ICP-MS (Varian 820MS) and by

HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS), respectively. The detection limits were similar to those determined for water analysis.

Bioavailable concentrations of arsenic in the Down site were measured twice (days 7 and 51 after translocation) with in situ DGT devices placed at the interface water-sediment. Once collected and moved to the laboratory, the devices were rinsed with Milli-Q water and opened for the removal of the resin gels, which were then eluted with 1 mL of 7.2 M HNO_3 for at least 24 h, allowing a complete extraction of the arsenic from the resin. An aliquot was pipetted and filtered through 0.45 µm, and then diluted with Milli-Q water prior to analysis using ICP-MS (Varian 820MS) to ensure that the acid concentration was below 10%. The mass of arsenic in the resin gel (M), the time-averaged DGT concentrations (C_{DGT}) and the flux (F) of arsenic measured by DGT were calculated according to Zhang and Davison (1995), and DGT® technical documentation.

Biological endpoints

In vivo fluorescence measurements

Several small biofilm-colonized artificial substrates (8.5 × 2 cm^2) were used to analyze the *in vivo* fluorescence in the laboratory, using a MINI-PAM fluorometer (HeinzWalz, Effeltrich, Germany), at a constant temperature (20 °C) and from a constant distance between the light-emitting diode and the samples (5 mm). First, the minimum fluorescence yield (F_o) was given by the fluorometer in dark adapted samples and then a saturation pulse was applied to obtain the maximum PSII quantum yield (Y_{max}). After 15 minutes of light adaptation, a saturation pulse of actinic light was applied to the samples to obtain the effective PSII quantum yield (Y_{eff}). According to Corcoll *et al.* (2012a), the F_0 parameter can be used as an estimation of algal biomass. The Y_{max} is defined as a measurement of the photosynthetic capacity of the community, whereas the Y_{eff} is a measurement of the community photosynthetic efficiency.

Biofilm elemental composition

Total dry weight biomass (DW) was obtained by weighing freeze-dried samples of biofilm, from which nutrient stoichiometry (C, N, P content and molar ratios) was analyzed. Carbon and N biogenic elements were determined using an elemental analyzer (PerkinElmer 2400). In contrast, TP biogenic element was determined after digestion of samples using an oxidation reagent at high pressure. Digestion transformed all organic P forms into inorganic forms and, then, total dissolved P content was obtained according to the method detailed in the SRP protocol. Quantification of the C, N and P elements of the benthic biofilm were performed on a dry weight basis, and C/N and N/P biofilm molar ratios were then calculated (Sterner and Elser 2002; Muñoz *et al.* 2009; Scharler *et al.* 2015).

Bacterial density

Bacterial density was determined by flow cytometry, adapted from Amalfitano *et al.* (2009) and Perujo *et al.* (2015) for biofilm samples. Scraped biofilm was preserved in formaldehyde solution (37%) and diluted with filtered river water to a final volume of 5 mL (with 3 replicates for each one). Samples (1 ml) were placed in a glass vial for sonication with 4 ml of detaching solution (1/5 sample dilution), added to help separate cells and avoid their aggregation. Detaching solution consists of NaCl (130 mM), Na_2HPO_4 (7 mM), NaH_2PO_4 (3 mM), formaldehyde (37%), sodium pyrophosphate decahydrate 99% (0.1% final concentration), and Tween®20 (0.5% final concentration). After sedimentation of larger biofilm cells (and possibly some sediment particles), a sample purification process was carried out with Nycodenz® (Sigma-Aldrich, USA) to ensure the elimination of those larger particles and cells in the supernatant (1/10 diluted sample). Then, 400 µL of sample (1/10 diluted) were stained with Syto13 (4 µl Fisher, 5 µM solution) and incubated in dark conditions for 15–30 min (1/100 final sample dilution). To normalize fluorescence data, bead solution (10 µl of 10^6 beads ml^{-1}, Fisher 1.0 µm) was added to the samples in a known concentration. Bacterial density was measured using flow cytometry (FACSCalibur, Becton–Dickinson) with a selected size of ~ 0.1 µm (corresponding to most mean heterotrophic bacterial size). Results are referred to as bacteria cm^{-2}.

Quantitative estimates of live diatom community

The quantitative estimates of live diatoms were carried out according to Morin *et al.* (2010). Scraped biofilm was preserved in formaldehyde solution (37%) and diluted with filtered river water to a final volume of 5 mL (with 3 replicates for each one). After ultra-sonication of samples (7 min) to separate the aggregated cells without destroying the frustules, 125 µL of each sample were pipetted into a Nageotte counting chamber to count the total number of diatom cells in 10 microscope fields (1.25 µL each, 0.5 mm depth) selected at random, using light microscopy at a 10x magnification (photomicroscope Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). Data were recorded as cells per unit area of sampled substrate (cells cm^{-2}). Counting was separated into 2 types: empty cells that were considered 'dead' and cells occupied by chloroplasts were considered 'alive'.

Relative abundances of the diatom species

Samples for diatom community identification were prepared in permanent slides as recommended by Leira and Sabater (2005), after digestion with 35% HCl and 30% H_2O_2. Diatom identification to the lowest taxonomic level possible was carried out following standard references and recent nomenclature updates: i.e., Krammer and Lange-Bertalot (1986-1991) and Coste and Rosebery (2011). About 400 frustules were counted per slide, as far as possible, using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of 1000x. Additionally, diatom species richness (*S*), Shannon-Wiener index of diversity (*H*), and species evenness (*J*) were calculated (Shannon and Weaver 1949; Pielou 1975).

Biofilm arsenic content

Extracellular and intracellular arsenic extraction was carried out in translocated biofilms of the Down site on days 7 and 51 after translocation, following the procedure of Levy *et al.* (2005) for extracellular arsenic, and Myashita *et al.* (2009) for intracellular arsenic. For the extractions, 2 g of fresh biofilm sample were used. A rinse solution was obtained by washing the samples with river water and was then analyzed for arsenic, as it can extract soluble arsenic slightly associated to the matrix of extracellular polymeric substances (EPS) and cell surfaces. The extracellular fraction was determined in the supernatant after addition of 20 ml of buffer KH_2PO_4/K_2HPO_4 0.1 M, at pH 5.95. The suspensions were stirred for 30 s and allowed to stand for 20 min. The eluates of two washes were combined and frozen (-80 ºC) until arsenic analysis. The remaining solid phases were gently washed with Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity), centrifuged (3000 rpm, 15 min) and frozen (-20 ºC) for the analysis of intracellular arsenic. Then, 10 mL of methanol:water solution (dilution 1:1) were added and the suspensions were sonicated for 10 min and centrifuged at 3000 rpm for 15 min. The extraction was repeated twice and the supernatants were combined and evaporated (Büchi Rotavapor R-200, BÜCHI Labortechnik GmbH, Essen, Germany). Intracellular arsenic was determined after re-dissolution of dried extracts in a known volume of Milli-Q water (18.2 $M\Omega.cm^{-1}$ resistivity). All aliquots of the rinse solution, extracellular and intracellular solutions were filtered through sterile 0.45 µm Whatman Puradisc 25AS™ syringe filters (GE Healthcare Europe GmbH, Barcelona, Spain), and maintained frozen (-80 ºC) until analysis. The total arsenic concentration in each solution, as well as the arsenic speciation (As^V, As^{III}, DMA^V, MMA^V and As-Bet) were determined by ICP-MS (Varian 820MS) and by HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820 MS), respectively. The detection limits were similar to those determined for water analysis.

Total amount of arsenic in the biofilm was also analyzed, in freeze-dried biofilm samples, after digestion in 4 ml of concentrated HNO_3 (65% Suprapure, Merck, Germany) and 1 mL of H_2O_2 (33% Suprapure Merck), using a high performance microwave digestion unit (Milestone, Ethos Sel, Sorisole (BG), Italy). Then, samples were diluted to 15 mL with milli-Q water and dissolved total arsenic concentration was measured using ICP-MS (7500c Agilent Technologies, Inc. Wilmington, Denmark).

2.5 Data analysis

Homogeneity of variances and normality of data were checked prior to statistical analyses, using SPSS v19.0 software. Before being included in the analysis, variables (except pH and Eh) were transformed: most of them were ln(x+1)-transformed but proportions and percentages were “arcsine square root” transformed. In the cases where sample size were too small (n=2), only mean values were calculated.

Changes in biofilm biomass during both colonization (5 weeks) and experiment period

under arsenic exposure (51 days) were fit to a 3-parameter log-normal curve using SigmaPlot v.11.0. We tested for differences between the estimated parameters using the following equation:

$$y = a\ e^{-0.5\left(\frac{x-x_0}{b}\right)^2}$$

where **y** = periphyton biomass, a is associated with the peak of the curve, b is the rate of inhibition after the peak, x_0 is the time required to reach the maximum or peak value and x is time in days.

For other biofilm metrics and light measurements, on the one hand, a Two-Way Repeated Measures ANOVA was carried out with all the variables that were analyzed in triplicate every sampling day. That is, the riparian cover percentage, the light irradiance reaching biofilm communities, and the biofilm parameters, such as photosynthesis (*Fo*, *Ymax* and *Yeff*), live diatom quantification, biogenic elements (%C, %N and %P) and their ratios (C/N and N/P), as well as the total arsenic concentration accumulated and also the As/P ratios in biofilms. The time variable (expressed as *translocation days*) was the within-subject continuous variable, while the treatment (the Downstream and Upstream sites) was the between-subject variable. On the other hand, the arsenic in different biofilm compartments (total arsenic and species) and bacterial density were checked by Two-Way ANOVA, since samples had been taken twice during the experiment, while One-Way ANOVA was applied to diatom diversity indices (*S, H, J*). Finally, post hoc Bonferroni's tests were applied to locate significant differences. All these analyses were carried out using SPSS v19.0 software.

Diatom relative abundance (≥1%) of the last day (translocation day 51) was represented in a Non-Metric Multidimensional Scaling plot (NMDS), to show possible variations of community composition between sites. Multi-response permutation procedures (MRPP) were used to test for inter-site *versus* intra-site heterogeneity in diatom community structure (Zimmerman *et al.* 1985) based on Bray Curtis distance (same as for the NMDS). PC-ORD software (version 6.08; McCune and Mefford, 2011) was used to perform the analyses.

The effect of the environmental factors on the biological responses was studied using redundancy data analysis (RDA), using variables taken at both sites (Up, Down) and every sampling day (2, 4, 7, 22 and 51). Previously, variables with a strong inter-correlation were eliminated to avoid collinearity, and then, two principal component analyses (PCA) were carried out to select the variables with the highest explained variance between sampling sites and time: one PCA using the explanatory variables (mainly physico-chemical) and the other one using the response variables (mainly biological variables). Finally, the RDA was carried out on the explanatory variables that best explained the variation of the response matrix (Borcard *et al.* 2011), corresponding to the light cover percentage, river water velocity and conductivity, river water nutrients (TP, TN, SRP), pH and Eh of sediment, and the biofilm As/P ratio. Biofilm As/P ratio was considered an explanatory variable due to the fact that it may determine the activity and metabolism of the biofilm, since it is the best proxy to estimate arsenic toxicity in cells. The

response variables comprised some biofilm metrics as DW, TP and TN content and C/N ratio, as well as photosynthetic parameters (*Fo* and *Yeff*) and live diatom density. Correlation analyses were carried out using SPSS v19.0 software. The "vegan" package of R statistical software (version 3.2.2 for Windows; www.r-project.org) was used to carry out both PCAs and the final RDA.

The dynamics and distribution of arsenic species (mean %) in the different environmental and biofilm compartments of the Downstream site (sediment, river water, biofilm rinse solution, extra- and intracellular biofilm) were represented in cumulative bar charts.

3. RESULTS

3.1 Site conditions

Site physicochemical data

Physical and chemical conditions during the experiment are summarized in Tables 1.a, 1.b and 2. Riparian cover was high at both sites, but especially in the Down site (Table 1a), causing slightly differences on light conditions between both sampling sites. Low light irradiance in biofilms was detected on days 7 and 22 (<50 µmol photons s^{-1} m^{-2}), especially in the Down site on day 22 (4.90 ± 0.24 µmol photons s^{-1} m^{-2}). In contrast, high irradiances (>1000 µmol photons s^{-1} m^{-2}) enough to cause photo-inhibition were measured on day 2, proving high and significantly temporal light variability. River water (Table 1a) showed a well oxygenated status, neutral pH and low mineralization. High concentrations of TN and TP were detected, especially at the end of the experiment, when high SRP concentrations were also observed. Conversely, higher concentrations of suspended solids were found at the beginning of the experiment. Low arsenic concentrations were detected in river water samples (ranging from 0.56 in the Up site to 1.83 µg L^{-1} in the Down site).

The sediments had neutral pH and Eh values corresponding to a suboxic state (100mV < Eh < 400 mV) at this pH. A higher percentage of OM was observed in the sediments of the Up site and especially at the beginning of the experiment (in July), coinciding also with high TN concentrations. These values strongly decreased during the experiment, resulting in high values of the C/N ratio, especially in the Up site at the end (Table 1b). Regarding the total amount of arsenic in sediments, considerably high concentrations (ranging from 31 to 100 mg kg^{-1}) were detected at both sites, and especially at the Down site (Table 1b), where the highest concentrations of the easily-extractable arsenic (ranging from 1.77 to 2.23 mg kg-1) were also found (Table 1b). Moreover, the arsenic time-averaged DGT concentration measured in the Down site, which is an indicative of the average arsenic concentration released from the sediment, was of 0.36 µg L^{-1} and, although the total arsenic retained by DGT devices increased with time (0.05 µg on day 7 and 0.25 µg on day 51), the arsenic time-averaged concentration

decreased over the experiment (0.44 ± 0.00 µg L^{-1} after 7 days of translocation; 0.29 ± 0.00 µg L^{-1} after 51 days, at the end of the experiment). Finally, sediment samples showed grain size differences between sites (Table 2), with a higher percentage of fine sand in the Down than in the Up site.

Benthic biofilm analyses

The evolution of the biofilm after the translocation of artificial substrates and until the end of the experiment is reflected in Table 3, showing significant differences between the translocated and non-translocated biofilms for the following biofilm metrics: the minimum fluorescence yield (*Fo*), the density of live diatoms and total bacteria, the amount of accumulated arsenic and the As/P ratio, as well as the elemental composition (% C, % N and % P) and the C/N and N/P ratios.

In the non-translocated biofilms (Upstream site), *Fo* was higher (mean value 345.95 ± 145.90) than in the translocated ones (195.14 ± 45.70). Furthermore, the *Fo* parameter was significantly fitted to a curve (Fig. 3) showing how the growth of the translocated biofilms located in the Downstream site reached half of those non-translocated (Up site). The percentages of C, N and P were generally higher in the non-translocated biofilms (Up site, with mean values of 23.27 ± 4.35 for % C, 3.97 ± 0.83 for % N and 0.19 ± 0.03 for % P), than in those translocated in the Down site (showing values of 18.27 ± 5.99 for % C, 2.68 ± 0.89 for % N and 0.12 ± 0.03 for % P). However, C/N and N/P molar ratios were higher in the translocated biofilms (7.08 ± 0.84 of C/N and 22.09 ± 5.41 of N/P) than in those non-translocated (5.97 ± 0.17 of C/N and 20.50 ± 2.64 of N/P).

Live diatom density was, in correlation with the *Fo* parameter, higher in the non-translocated biofilms ($492.99\times10^3 \pm 193.35\times10^3$ cells cm^{-2}) than in those translocated ($4.92\times10^3 \pm 2.75\times10^3$ cells cm^{-2}). In contrast, a higher density of bacteria was found in the translocated biofilms (Down site), especially at the end of the experiment.

During the experiment, the arsenic concentration was generally higher ($p=0.017$) in the translocated biofilms (mean values of 15.20 ± 6.85 µg As g^{-1} biofilm in the Down site) than in the non-translocated ones (mean values 8.82 ± 2.24 µg As g^{-1} biofilm in the Up site). Differences were more apparent in the As/P ratio ($p<0.001$), reaching mean values of $8.75\times10^{-3} \pm 5.10\times10^{-3}$ in the translocated biofilms, while $2.27\times10^{-3} \pm 1.17\times10^{-3}$ were found in the non-translocated biofilms.

Summarizing, the biofilms in the Up site (non-translocated biofilms) contained higher nutrient content (lower C/N and C/P ratios), as well as higher algal biomass (*Fo*) and had more live diatoms than in the Down site, where the biofilms had a higher bacterial density. The arsenic content of the biofilms and the As/P ratio were also higher in the Down site, evidencing higher potential arsenic toxicity.

Table 1a Physico-chemical properties: environmental light measurements (riparian cover percentage and light reaching biofilms) and physico-chemical properties of river water in the Upstream (Up) and Downstream (Down) sampling sites of the Anllóns River, on days 2, 4, 7, 22 and 51 (corresponding to the last day of the experiment) after the translocation. Single values or mean values are shown (n=3 for riparian cover, light irradiance reaching biofilm and total As on 22Down; n=2 for DOC, nutrients and total As; n=1 for pH, Eh, conductivity, oxygen and temperature). nd= no data. Statistical results of riparian cover for effects on time (F=151.741, p<0.001; degrees of freedom, df=4) and sites (F=1593.701, p<0.001; df=1) were achieved by Two-Way Repeated Measures ANOVA and Bonferroni's test, as well as results of light irradiance reaching biofilm for effects on time (F=1192.900; p<0.001; degrees of freedom, df=4) and sites (F=7.093; p=0.056; df=1). *Significant differences (p≤0.05) between sampling sites at a specific date (from Bonferroni's test). [a]Values of pH and Eh of sediments (n=1) are indicated in brackets with the river water pH and Eh values, respectively.

	ENVIRONMENTAL LIGHT		**RIVER WATER[a]**											
Transloc. Date & Site	**Riparian Cover**	**Light reaching biofilm**	**pH**	**Eh**	**Electrical Cond.**	**O_2**	**T**	**Water Velocity**	**SS**	**DOC**	**TP**	**SRP**	**TN**	**Total As**
	(%)	(µmol photons s^{-1} m^{-2})		(mV)	(µS cm^{-1})	(mg L^{-1})	(°C)	(m s^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(µg L^{-1})
2Up	90.09±0.81*	1023.00±113.32*	7.07 (7.59)	415 (215)	141	8.88	17.60	0.98	3.60	nd	0.07	0.03	1.07	nd
2Down	98.62±0.14*	1454.67±32.96*	7.16 (7.16)	493 (232)	142	9.28	17.70	0.38	12.60	nd	0.08	0.06	0.81	nd
4Up	83.82±0.19*	156.20±3.40*	7.05 (7.24)	431 (240)	145	9.41	16.50	0.93	nd	nd	0.08	0.03	1.03	nd
4Down	95.00±0.64*	40.90±1.44*	7.19 (7.24)	470 (241)	145	9.02	17.80	0.35	nd	nd	0.09	0.05	1.09	nd
7Up	80.89±2.22*	31.01±0.49*	7.00 (7.38)	431 (191)	149	8.77	17.60	0.87	0.60	nd	0.08	0.04	0.50	nd
7Down	97.66±0.24*	42.69± 0.58*	7.22 (7.30)	461 (329)	146	8.98	18.70	0.34	3.10	nd	0.11	0.04	0.36	nd
22Up	93.30±0.95*	45.02±0.34*	6.81 (7.55)	420 (224)	153	8.53	19.10	0.67	1.90	4.45	0.09	0.01	3.65	nd
22Down	91.53±0.26*	4.90±0.24*	7.06 (7.13)	459 (250)	152	8.92	19.20	0.55	1.90	3.39	0.09	0.05	1.94	1.83
51Up	71.20±2.51*	155.43±7.21*	6.83 (6.80)	519 (253)	158	8.00	18.90	0.49	1.20	2.92	0.38	0.07	2.15	0.56
51Down	88.38±1.12*	52.51± 2.13*	7.13 (6.86)	464 (281)	161	8.86	18.70	0.20	0.50	2.64	0.19	0.06	3.15	0.79

Table 1b Physico-chemical properties of sediments in the Upstream (Up) and Downstream (Down) sampling sites of the Anllóns River, on day 7 after the translocation and day 51 (the end of the experiment). Mean values are shown (n=2). Values of pH and Eh are shown in Table 1a.

	SEDIMENT						
Translocated Date & Site	**OM**	**TC**	**TN**	**C/N**	**TP**	**Total As (XRF)**	**Easily-extractable As**
	(%)	(mg kg^{-1})	(mg kg^{-1})		(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
7Up	4.91	28.50×10^{3}	1169.73	24	363.25	31	1.32
7Down	2.71	15.70×10^{3}	604.21	25	381.75	54	2.23
51Up	2.33	13.50×10^{3}	327.22	41	402.80	51	0.66
51Down	2.13	12.40×10^{3}	379.68	32	253.10	110	1.77

Table 2 Grain size distribution (%) of the sediments of the Anllóns River, in the Upstream and Downstream sampling sites, on 7 days and 51 days after the translocation.

Sampling site and date / **Grain size fraction**	**Upstream**		**Downstream**	
	7 days	**51 days**	**7 days**	**51 days**
<50 µm	11.9	8.1	6.9	4.5
50-100 µm	2.1	2.5	3.4	2.9
100-250 µm	22.4	18.4	46.6	42.4
250-500 µm	15.0	13.6	37.0	41.7
500-1000 µm	16.1	18.6	5.0	5.7
1-2mm	32.5	38.8	1.1	2.8

Table 3 Biofilm metrics of the non-translocated and translocated biofilms located Upstream (Up) and Downstream (Down) of the mine area, respectively, sampled at several times after the translocation: on days 2, 4, 7, 22 and 51 (corresponding to the last day of the experiment). Value units may refer to the surface of the colonized artificial-glass substrates (cm^2) or the biofilm freeze-dried mass (g). Mean values±standard deviations (n=3) are shown. nd= no data. Except for Bacteria density, all statistical results (F and *p*) for effects on time (degrees of freedom, *df*=4) and treatment (*df*=1) were achieved by Two-Way Repeated Measures ANOVA and Bonferroni's test. For Bacteria density, statistical results (F and *p*) for effects on time (*df*=1) and treatment (*df*=1) were achieved by Two-Way ANOVA and Bonferroni's test. Significant differences in time or site effects are set at $p \leq 0.05$. *Significant differences ($p \leq 0.05$) between sampling sites at a specific date (from Bonferroni's test). (*)Marginal significance ($0.05 < p \leq 0.1$) between sampling sites at a specific date (from Bonferroni's test).

Translocated Date & Site	**BIOFILM**												
	Fo	***Ymax***	***Yeff***	**Live Diatoms (×10³ cells cm⁻²)**	**Bacteria (×10⁶ cells cm⁻²)**	**DW (mg cm⁻²)**	**C (%)**	**N (%)**	**P (%)**	**C/N**	**N/P**	**As (µg g⁻¹)**	**As/P**
2Up	271 ±112.46	0.46 ±0.14	0.28* ±0.05	4.96 ±1.27	0.12 ±0.04	0.40 ±0.31	13.96 ±4.26	2.30 ±0.79	0.13 ±0.02	6.13 ±0.24	17.39 ±3.23	10.42 ±2.27	0.004* ±0.002
2Down	179.33 ±55.89	0.48 ±0.28	0.35* ±0.18	9.97 ±8.21	0.08 ±0.04	1.24 ±1.25	12.84 ±5.98	1.48 ±0.38	0.10 ±0.03	8.36 ±1.96	16.04 ±7.07	17.73 ±6.43	0.013* ±0.006
4Up	147.67* ±15.31	0.54 ±0.06	0.40 ±0.02	7.66 ±10.96	nd	0.66 ±0.29	27.23 ±8.32	4.70(*) ±1.60	0.21 ±0.08	5.85* ±0.25	23.11 ±1.77	7.76 ±2.16	0.002 ±0.001
4Down	228.00* ±18.73	0.58 ±0.04	0.34 ±0.03	2.06 ±1.24	nd	1.23 ±0.61	14.87 ±5.52	2.21(*) ±0.93	0.09 ±0.03	6.80* ±0.30	23.92 ±2.93	24.51 ±18.55	0.014 ±0.010
7Up	354.33 ±206.36	0.28 ±0.14	0.23 ±0.13	0.95* ±0.28	nd	3.30 ±1.51	16.22 ±4.41	2.57 ±0.74	0.14 ±0.04	6.34 ±0.17	18.66 ±3.37	7.89 ±3.42	0.002 ±0.002
7Down	306.67 ±34.65	0.25 ±0.19	0.22 ±0.17	1.88* ±0.45	nd	1.10 ±1.29	20.12 ±2.71	3.20 ±0.48	0.14 ±0.02	6.3 ±0.19	23.21 ±4.18	12.60 ±3.16	0.005 ±0.002
22Up	434.33 ±84.05	0.47 ±0.06	0.30 ±0.08	12.20* ±3.62	nd	1.10 ±0.46	26.49 ±0.40	4.51 ±0.15	0.25 ±0.01	5.87 ±0.11	17.91 ±0.85	8.78 ±1.56	0.002 ±0.000
22Down	231.67 ±103.16	0.51 ±0.06	0.40 ±0.06	5.79* ±1.12	nd	1.43 ±0.56	25.26 ±9.74	3.81 ±1.78	0.15 ±0.03	6.88 ±0.94	25.18 ±7.46	8.80 ±1.07	0.004 ±0.001
51Up	522.43* ±311.31	0.48 ±0.08	0.40 ±0.04	2439.22* ±950.64	0.11* ±0.16	1.47 ±0.54	32.44 ±4.36	5.75(*) ±0.87	0.23 ±0.00	5.65* ±0.09	25.43 ±3.97	9.26 ±1.78	0.001* ±0.001
51Down	179.20* ±16.04	0.55 ±0.02	0.47 ±0.01	27.41* ±6.35	1.97* ±1.24	1.15 ±1.08	23.45 ±9.11	3.77(*) ±1.61	0.15 ±0.04	6.34* ±0.41	23.91 ±4.88	12.38 ±5.05	0.008* ±0.006
TIME EFFECT	F=1.449	F=2.984	F=2.735	F=35.045	F=2.942	F=1.630	F=4.669	F=5.711	F=5.170	F=3.547	F=2.547	F=0.888	F=1.826
	p=0.264	*p*=0.051	*p*=0.066	*p*<0.001	*p*=0.125	*p*=0.215	*p*=0.011	*p*=0.005	*p*=0.007	*p*=0.030	*p*=0.080	*p*=0.494	*p*=0.173
SITE EFFECT	F=10.936	F=0.063	F=0.312	F=8.323	F=6.312	F=2628.636	F=7.357	F=17.822	F=25.761	F=8.129	F=17.413	F=15.417	F=145.397
	p=0.030	*p*=0.814	*p*=0.606	*p*=0.045	*p*=0.036	*p*=0.127	*p*=0.053	*p*=0.013	*p*=0.007	*p*=0.046	*p*=0.014	*p*=0.017	*p*<0.001

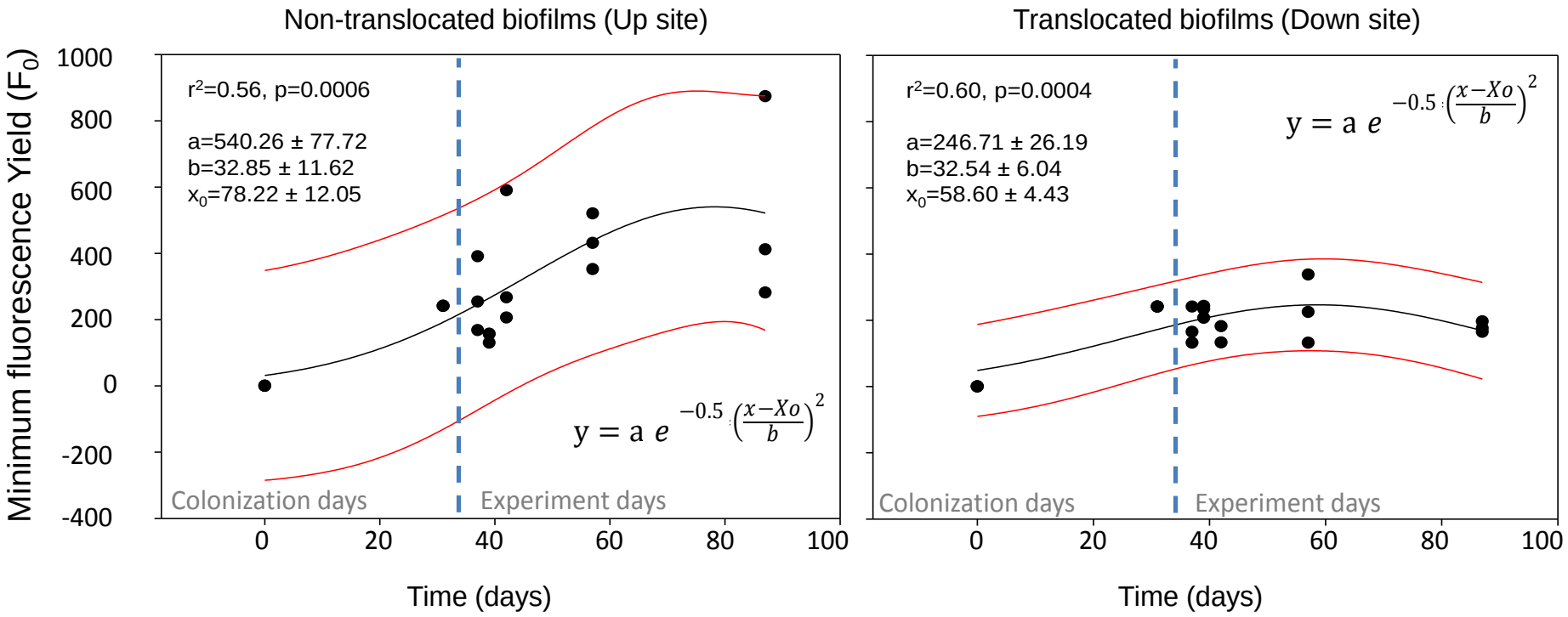


Figure 3 Changes in biofilm biomass during the "biofilm colonization days" *versus* the "experiment days" in the Upstream site (plot on the left) and the Dowstream site (plot on the right). Fitting of *Fo* parameter over time (days) follows a 3-parameter log-normal curve, assuming growth inhibition at the end of the experiment, and where the **a** parameter is associated with the peak of the curve; the ***b*** parameter is the rate of inhibition after the peak; the x_0 parameter is the time required to reach the maximum or peak value and ***x*** is time in days. Vertical dotted lines indicate the translocation day (before that time, all samples were located exclusively in the Upstream site, corresponding to the colonization period). Differences between the parameters for each sampling site were tested with two-way ANOVA in order to check if changes in the growth of the communities were statistically significant. Significance was set at $p \leq 0.05$. Red lines indicate 95% confidence band.

Diatom community composition

At the end of the experiment, 34 species of diatoms were identified and 4 more only at genus rank (Table 4). The most abundant species identified were *Cocconeis placentula* Ehrenberg var. *placentula* (55.37%) and *Mayamaea atomus* (Kützing) var. *atomus* (18.34%). Regarding sampling sites, *C. placentula* var. *placentula* (67.60 %) and *C. pediculus* Ehrenberg (14.16%) were the most abundant species in the Up site (non-translocated biofilms), whereas *M. atomus* (33.48%) dominated in the Down site (translocated biofilms), together with *C. placentula* var. *placentula* (43.21%). No significant differences were found in the diatom diversity indices between the translocated and non-translocated biofilms at the end of the experiment *(S, H* and *J)*. The NMDS ordination showed slight differences in the diatom assemblages between the samples at this moment (Fig. 4), suggesting natural variability between communities of the sampled sites (inter – replicates). However, less variability of species composition was found within than between sites (MRPP value: A=0.14, p=0.12).

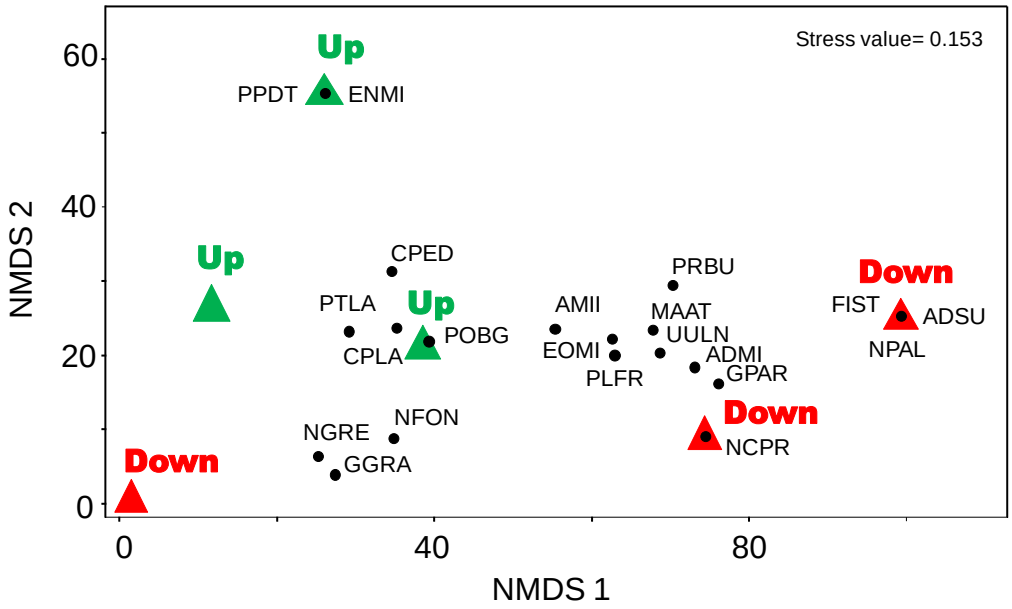


Figure 4 Nonmetric dimensional scaling (NMDS) plot showing sampling sites ordination according to their diatom species composition at the end of the experiment (day 51 after biofilm translocation to Xavarido). Species abbreviation: ADMI, *Achnanthidium minutissimum* (Kützing) Czarnecki; AMII, *Achnanthidium minutissimum* (Kützing) Czarnecki f. *inconspicuum* (Østrup) Compère & Riaux-Gobin; ADSU, *Achnanthidium subatomus* (Hustedt) Lange-Bertalot; CPED, *Cocconeis pediculus* Ehrenberg; CPLA, *Cocconeis placentula* Ehrenberg var. *placentula*; ENMI, *Encyonema minutum* (Hilse in Rabhenhorst) D.G. Mann in Round, Crawford & Mann; EOMI, *Eolimna minima* (Grunow) Lange-Bertalot; GGRA, *Gomphonema gracile* Ehrenberg; GPAR, *Gomphonema parvulum* (Kützing) Kützing var. *parvulum* f. *parvulum*; MAAT, *Mayamaea atomus* (Kützing) Lange-Bertalot var. *atomus*; NCPR, *Navicula capitatoradiata* Germain; NGRE, *Navicula gregaria* Donkin; NFON, *Nitzschia fonticola* Grunow in Van Heurck; NPAL, *Nitzschia palea* (Kützing) W.Smith var. *palea*; PLFR, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot; PTLA, *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot; PPDT, *Planothidium pseudotanense* (Cleve-Euler) Lange-Bertalot; PRBU, *Planothidium robustius* (Hustedt) Lange-Bertalot; POBG, *Psammothidium oblongellum* (Østrup) Van de Vijver; UULN, *Ulnaria ulna* (Nitzsch) Compère; FIST, *Fistulifera* sp. Species representing ≥1% in at least one sample.

Table 4 List of the diatom taxa found at the end of the experiment in both sampling sites (Up and Down) of the Anllóns River

Achnanthidium minutissimum (Kützing) Czarnecki
Achnanthidium minutissimum (Kützing) Czarnecki f. *inconspicuum* (Østrup) Compère & Riaux-Gobin
Achnanthidium pfisteri Lange-Bertalot
Achnanthidium straubianum (Lange-Bertalot) Lange-Bertalot
Achnanthidium subatomus (Hustedt) Lange-Bertalot
Amphora oligotraphenta Lange-Bertalot
Amphora veneta Kützing
Cocconeis pediculus Ehrenberg
Cocconeis placentula Ehrenberg var. *placentula*
Cocconeis pseudolineata (Geitler) Lange-Bertalot
Cymbella sp.
Encyonema minutum (Hilse in Rabhenhorst) D.G. Mann in Round, Crawford & Mann
Eolimna minima (Grunow) Lange-Bertalot
Eunotia sp.
Fistulifera sp.
Gomphonema gracile Ehrenberg
Gomphonema lateripunctatum Reichardt & Lange-Bertalot
Gomphonema parvulum (Kützing) Kützing var. *parvulum* f. *parvulum*
Mayamaea atomus (Kützing) Lange-Bertalot var.*atomus*
Melosira varians Agardh
Navicula capitatoradiata Germain
Navicula cryptotenelloides Lange-Bertalot
Navicula gregaria Donkin
Navicula lanceolata (Agardh) Ehrenberg
Nitzschia amphibia Grunow f.*amphibia*
Nitzschia fonticola Grunow in Van Heurck
Nitzschia palea (Kützing) W.Smith var. *palea*
Nitzschia pura Hustedt
Planothidium dubium (Grunow) Round & Bukhtiyarova
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot
Planothidium lanceolatum (Brebisson ex Kützing) Lange-Bertalot
Planothidium pseudotanense (Cleve-Euler) Lange-Bertalot
Planothidium robustius (Hustedt) Lange-Bertalot
Psammothidium oblongellum (Østrup) Van de Vijver
Rhoicosphenia abbreviata (C.Agardh) Lange-Bertalot
Surirella angusta Kützing
Tabelaria sp.
Ulnaria ulna (Nitzsch) Compère

The influence of environmental parameters on the biofilm responses

Redundancy Analysis (RDA) plot (Fig. 5) shows the influence of environmental parameters (in grey) on the responses attributed to the biofilm (parameters in italics and black),

in every sampling day (2, 4, 7, 22 and 51) and sampled site (D for Down site, U for Up site). Based on this analysis, 72.17% of the total variance attributed to all physicochemical parameters is by the riparian cover, the SRP, TP and TN of the river water, the conductivity and velocity of the river water, the pH and Eh of sediments, and the biofilm As/P ratio as a parameter of potential toxicity.

Almost half of the total variance (49.16%) is expressed by the first axis, which arranges the sampling sites according to the N and P contents, live diatoms and algal biomass (*Fo*), all of which are higher in the biofilms located at the Up site, where environmental conditions are described by higher water TN, TP and electrical conductivity. In turn, the C/N ratio was higher in the translocated biofilms at the Down site, where a higher As/P biogenic ratio was also detected, as well as the highest riparian cover. Therefore, sampling site distribution on axis 1 followed a gradient related to canopy cover, water chemistry and arsenic pollution, separating most Downstream (on the left) from Upstream samples (on the right of the plot).

The second axis explains 23.01% of the total variance and shows an event that happened on day 7 in both sampling sites, related to an increase in water velocity and water turbidity, causing an increase in biofilm DW and a decrease in the *Yeff*, and illustrating the effects of a previous rain event.

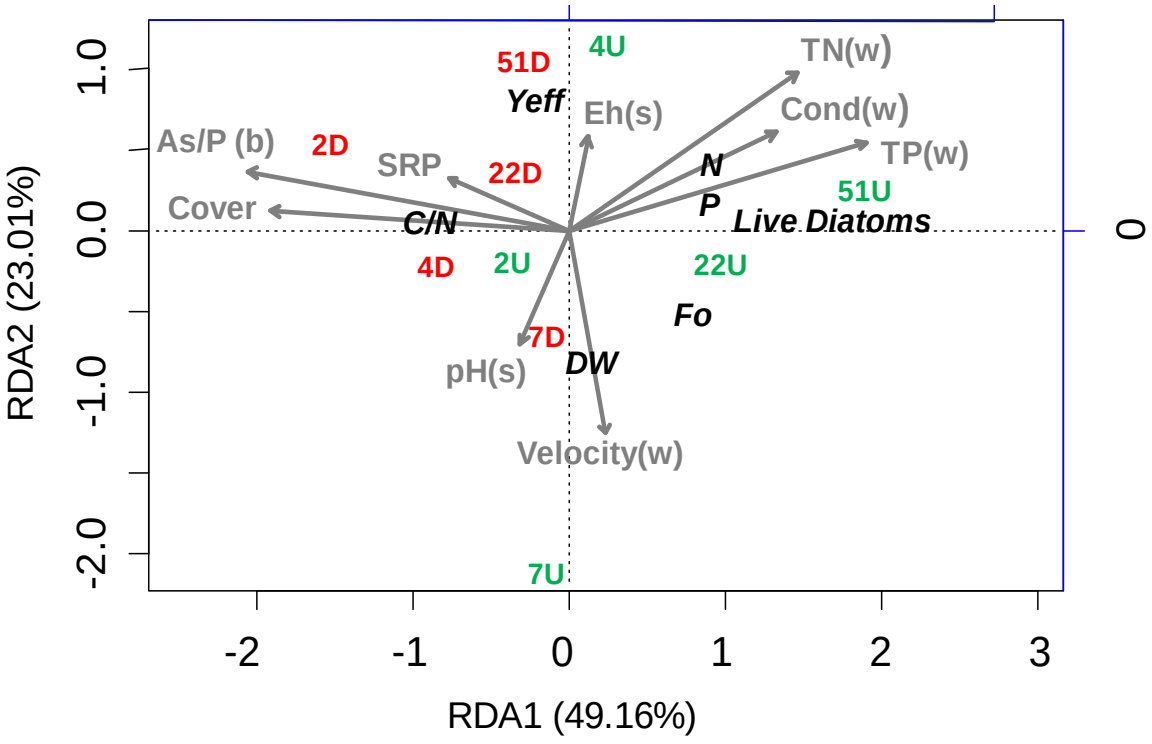


Figure 5 Plot of redundancy data analysis (RDA) to analyze the effect of the environmental factors on the biological responses. The explanatory variables that best explained the variation of the response are represented in the plot. The environmental compartment (water or sediment) where variables were measured are specified in parentheses (w and s, respectively).

3.2 Arsenic speciation

In river water (Table 5), As^{V} was the dominant species (99% in the Down site on day 22 and 72.5% on day 51; and almost 89% in the Up site on day 51). At the end of the experiment, As^{III}, DMA^{V} and As-Bet percentages were higher in the Down site, where these species represented all together more than 27 % of the total arsenic, but only accounted for 11 % in the Up site. In contrast, only inorganic arsenic species were found in the sediment samples: As^{V} was the predominant arsenic species in both sites, and especially in the Down site, where it represented more than 86 % of the total arsenic (Table 5).

Table 5 Percentages of arsenic speciation (referring to the total arsenic concentration analyzed) in samples of sediment and river water from the Upstream and Downstream site are shown (n=2). n.d.= not detected

Translocation Date & Site	**ARSENIC SPECIATION IN RIVER WATER (% of Total As)**				
	As^{V}	**As^{III}**	**DMA^{V}**	**MMA^{V}**	**As-Bet**
22Down	99.39	n.d.	0.61	n.d.	n.d.
51Up	88.80	2.45	2.98	n.d.	5.77
51Down	72.50	8.40	7.00	n.d.	12.10
	ARSENIC SPECIATION IN SEDIMENT (% of Total As)				
	As^{V}	**As^{III}**	**DMA^{V}**	**MMA^{V}**	**As-Bet**
7Up	61.11	38.90	n.d.	n.d.	n.d.
7Down	88.14	11.86	n.d.	n.d.	n.d.
51Up	64.65	35.35	n.d.	n.d.	n.d.
51Down	86.06	13.95	n.d.	n.d.	n.d.

As was previously mentioned, the translocated biofilms accumulated more total arsenic than the non-translocated biofilms. For this reason, arsenic speciation was focused on the translocated biofilms (Down site), in the extracellular and intracellular biofilm compartments (Table 6). The total arsenic in the rinse solution, which contains soluble arsenic slightly associated to cell surfaces and *EPS* matrix, showed the same proportion of As^{V} and As^{III} on day 7, but As^{V} dominated at the end of the experiment. A low amount of DMA^{V} was also detected in this rinse solution (around 2% on day 7 and 0.6% on day 51). Arsenic in the extracellular compartment, where is more strongly adsorbed to cell surfaces and *EPS* matrix, showed a species distribution very similar to that of the rinse solution, but with higher DMA^{V} concentrations at the end of the experiment. Finally, in the intracellular compartment at the end

of the experiment As^{V} species predominated by, followed by an important amount of DMA^{V} (around 25% on day 7 and 18% on day 51) and As^{III}. Low percentages of As-Bet (ranging from 0.13 to 1.35%) appeared in all the compartments at the end of the experiment. No MMA^{V} was detected in any sample. In summary, while in river water and sediment As^{V} was the predominant species, other arsenic species were relevant in the biofilm, namely As^{III} in the rinse solution and extracellular compartment (particularly on day 7), and DMA^{V} in the intracellular fraction.

Table 6 Total arsenic concentration and percentage of arsenic species in translocated biofilms (the rinse solution, the extracellular and the intracellular compartments). Mean values ± standard errors are shown (n=3 in samples on day 7; n=5 in samples on day 51). Statistical results (F and *p*) for effects on time (degrees of freedom, *df*=1) and biofilm compartment (*df*=2) were achieved by two-way ANOVA. Significant differences are set at $p \leq 0.05$. n.d.= not detected

BIOFILM COMPARTMENT						
Translocation Date & Site	**TOTAL ARSENIC**	**ARSENIC SPECIATION IN BIOFILM (% of total As)**				
	(µgAs g^{-1})	**As^{V}**	**As^{III}**	**DMA^{V}**	**MMA^{V}**	**As-Bet**
RINSE SOL.						
7Down	0.97±0.35	48.33±3.89	49.66±4.15	2.01±0.40	n.d.	n.d.
51Down	1.48±0.63	78.06±5.36	19.97±4.46	0.62±0.62	n.d.	1.35±0.83
EXTRACEL.						
7Down	1.96±0.60	49.48±4.90	50.52±4.90	n.d.	n.d.	n.d.
51Down	1.99±0.63	83.73±5.88	11.61±4.14	4.54±1.68	n.d.	0.13±0.13
INTRACEL.						
7Down	1.18 ±0.52	60.27±9.70	14.39±7.62	25.34±4.21	n.d.	n.d.
51Down	2.91±0.55	77.95±6.66	3.54±2.01	18.26±4.58	n.d.	0.25±0.25
BIOFILM COMPARTMENT EFFECT	F=1.564 *p*=0.237	F=0.403 *p*=0.674	F=18.585 *p*<0.001	F=30.522 *p*<0.001		F=7.526 *p*=0.004

4. DISCUSSION

4.1. Site characterization

Several environmental factors measured in the sediments and river water resulted to be similar in both sampling sites, attributed in particular to the similar lithology and intense farming activities in the basin. Thus, eutrophic conditions were detected in the water at both sites during the whole experiment (experimental ranges: 0.07-0.38 mg TP L^{-1} and 0.36-3.15 mg TN L^{-1}), especially at the end, as most values correspond to eutrophic conditions set at 0.075 mg TP L^{-1} and 1.5 mg TN L^{-1} by Dodds *et al.* (1998). Furthermore, high concentrations of SRP, commonly analyzed as a measurement of the immediately available orthophosphate (PO_4^{-3}) in water and effectively used for predicting algal production (Dodds 2006; Allan and Castillo 2007), confirmed these eutrophic conditions (experimental range: 0.01-0.07 mg SRP L^{-1}, usually 0.01-0.03 mg SRP L^{-1}, according to Mainstone and Parr 2002). Regarding DOC, the values corresponded to typical concentrations in running water (Thurman 1985; Allan and Castillo 2007) in both sites. For the heterotrophic component of the biofilms, DOC is usually a major C source, and particularly under low light conditions (Romani *et al.* 2004; Allan and Castillo 2007). With respect to sediments, and regarding nutrient concentrations, they were not considered polluted (the values detected were below the threshold values of 600 mg TP kg^{-1} and 550 mg TN kg^{-1}, according to Persaud *et al.* 1993), except for TN at the beginning of the experiment and especially in the Up site. More organic matter (% OM and C concentration) was also found in the sediments of the Up site. High C/N ratio values in the sediments suggested an allochthonous origin of this organic matter, probably coming from terrestrial plants, since C/N ratios >12 are indicative of OM rich in lignin and cellulose, and are attributable to terrestrial origin (Lamb *et al.* 2006). Insufficiently treated water from the collectors of the wastewater and sewage treatment plant, as well as effluents from the canning factory, both located upstream in the town of Carballo, might also contribute to the allochthonous organic matter.

With regard to arsenic concentrations, the river water was not contaminated in any of the sampling sites, since concentrations were far below 150 µg As L^{-1}, which is the limit for a chronic exposure in freshwater systems (Aquatic Life Criteria, US EPA 2014). However, sediment arsenic concentrations were high. According to the Canadian Sediment Quality Guidelines (Severe Effect Level set at 33 mg kg^{-1} by Persaud *et al.* 1993), the sediments in both sites may be considered heavily arsenic-polluted and, thus, likely to affect the sediment-dwelling organisms, especially in the Down site. In this site, the generic reference level for soils in this region, set at 50 mg kg^{-1} by Macías-Vázquez and Calvo de Anta (2009), was exceeded twice on day 51. Arsenic was also more easily extractable in the Down site, indicating that it can be easily transferred from sediments to other compartments as water and biota, and, consequently, be more available. Inputs of dissolved phosphate may favour arsenic mobilization, as shown by Rubinos *et al.* (2010, 2011).

4.2. Arsenic fate

According to the higher arsenic concentration and mobility in the sediments at the Down site, the bioaccumulated arsenic concentration and As/P ratio were always higher in the translocated biofilms than in the non-translocated ones. Focusing on arsenic distribution and speciation in the Down site, biofilms accumulated high arsenic concentrations, which were equally distributed among the intracellular and extracellular compartments. The total arsenic concentration in the biofilms exceeded the easily-extractable arsenic in the sediments, thus suggesting arsenic accumulation over time and confirming that biofilm is a major sink for arsenate (López *et al.* 2016). This indicates that biofilms growing on rock surfaces and granular sediments are able to accumulate the arsenic released from the sediment, which is the main source of arsenic since the water concentration is very low (below the environmental limits established). The arsenic time-averaged DGT concentration measured in the Down site supports these results. Average arsenic concentration accumulated in this device was below the arsenic concentration in the river water in this polluted site, suggesting that there is a contribution of arsenic from the sediment.

With regard to arsenic mobility, exchanges between the water and sediment may be modified due to inputs of dissolved phosphate (Rubinos *et al.* 2011b), leading to arsenic release from the sediment to the water column. In this scenario, arsenic retention and biotranformation in fluvial biofilms might have important implications for the ecosystem health. Previous laboratory studies which analyze arsenic mobilization from As-polluted sediments from the Anllóns River have also shown arsenic accumulation by biofilms and similar arsenic distributions among intracellular and extracellular compartments (Prieto *et al.* 2016c). Biofilm also retained arsenic from As-polluted waters, but in this case it was mostly accumulated in the extracellular compartment (Prieto *et al.* 2016a).

4.3 Biofilm responses

The most significant differences in the biological properties between the sampling sites were the inhibition of algal growth (although photosynthesis was not clearly affected), the increase of diatom mortality, the development of higher bacterial density and the loss of nutritional elements (biofilm with less N and more C content). Besides the high arsenic exposure, the higher amount of DOC in the Down site could contribute to the development of a less nutritive biofilm, which would be reflected in the strong decrease in live diatoms and the increase in bacterial density. These changes in the structure of the biofilm may be attributed to the arsenic toxicity, since some of them were already detected in previous As- exposed biofilms (e.g. Rodriguez-Castro *et al.* 2015; Tuulaikhuu *et al.* 2015). A selective decrease in diatom abundance, measured as % *Fo* for brown algae, was already detected in chronic arsenic exposure in biofilms developed on artificial substrates in channels including a sediment

compartment (Tuulaikhuu *et al.* 2015).

However, effects caused by other environmental factors, mainly light availability, have to be considered. Light is the first energy source for the autotrophic component of biofilm, modulating not only biofilm structure and its function but also biofilm response to pollutants as metals (Corcoll *et al.* 2012b; Guasch *et al.* 2016). At the Down site, the higher riparian cover could contribute to a lower algal growth and, in combination with DOC availability, may favour the growth of heterotrophic bacteria (Romani *et al.* 2004; Allan and Castillo 2007). Turbidity, favored by the finer particle size of the sediment and evidenced by the higher SS in water, may influence biofilm communities (Magbanua *et al.* 2013), enhancing also heterotrophic organisms at the Down site. Light regime is usually strongly variable for fluvial biofilms in space and time (Guasch and Sabater 1998), high irradiances being >1000 µmol photons s^{-1} m^{-2} (Hill 1996). Light intensity controls photosynthesis, and many authors have reported that there is a range of intensities over which photosynthesis is highly efficient (30-400 µmol s^{-1} m^{-2}), with an inhibitory effect above 500 µmol s^{-1} m^{-2} (Villeneuve *et al.* 2010). In this study, the light available to the biofilms was very variable during the experiment. Particularly at the Down site, light may have been a limiting factor, especially during the last days of the experiment, but may also have caused photoinhibition at the beginning, when very high irradiance was detected at both sites. Effects of light stress in combination with metal toxicity were already found in biofilms for which metal exposure caused a magnification of light inhibitory effects on algae and a reduction in relative contribution of diatoms (Corcoll *et al.* 2012b). Taking into account the higher arsenic amount in the Down site and the higher potential arsenic toxicity in these translocated biofilms (> As/P), this magnification of light inhibitory effects could partially explain the observed lower algal growth and higher diatom mortality. Therefore, arsenic may affect algal growth, but a direct or combined effect of light conditions should not be discarded.

4.4 Evidence of arsenic speciation by biofilm

Arsenate was the predominant species in all the studied compartments (Fig. 6), particularly in the river water, as predicted by the chemical theory and observed by Prieto *et al.* (2016a; 2016c) for this river. Arsenite was very scarce in water but increased in sediment and also in the rinse solution and extracellular compartment of the biofilm, to again diminish in the intracellular compartment (Fig. 6).

Our results point to an effect of the biofilm on arsenic speciation by, first, reducing As^{V} to As^{III}. This biotransformation may occur extracellularly (adsorbed As^{V}), or intracellularly (uptaken As^{V}) followed by As^{III} excretion (Oremland and Stolz 2003; Rahman and Hassel 2014). Excretion of As^{III}, which typically occurs under P-enriched conditions (Hellweger *et al.* 2003), does not seem to be relevant in this case, as only low amounts of As^{III} were detected in the water. Inside the cells, As^{III} might be transformed into the less toxic methylated species (DMA^{V}), which were then also excreted, probably indicating active arsenic detoxification by the biofilm.

Although As-methylation was suggested to occur mainly under P-limiting conditions by the model proposed by Hellweger *et al.* (2003), this transformation took place in the apparent eutrophic conditions of this study. Accordingly, an intensification of biomethylation process in eutrophic conditions was also previously detected (Baker and Wallschläger 2016; Yan *et al.* 2016).

The As-Bet ("Fish-As") detected in the river water was unexpected, since this species is almost absent in freshwater organisms (Caumette *et al.* 2012). It was tentatively attributed to the contribution of the seafood canning factory located upstream of the sampled sites. This type of pollution was not considered in the design of this study, thus requiring further investigations.

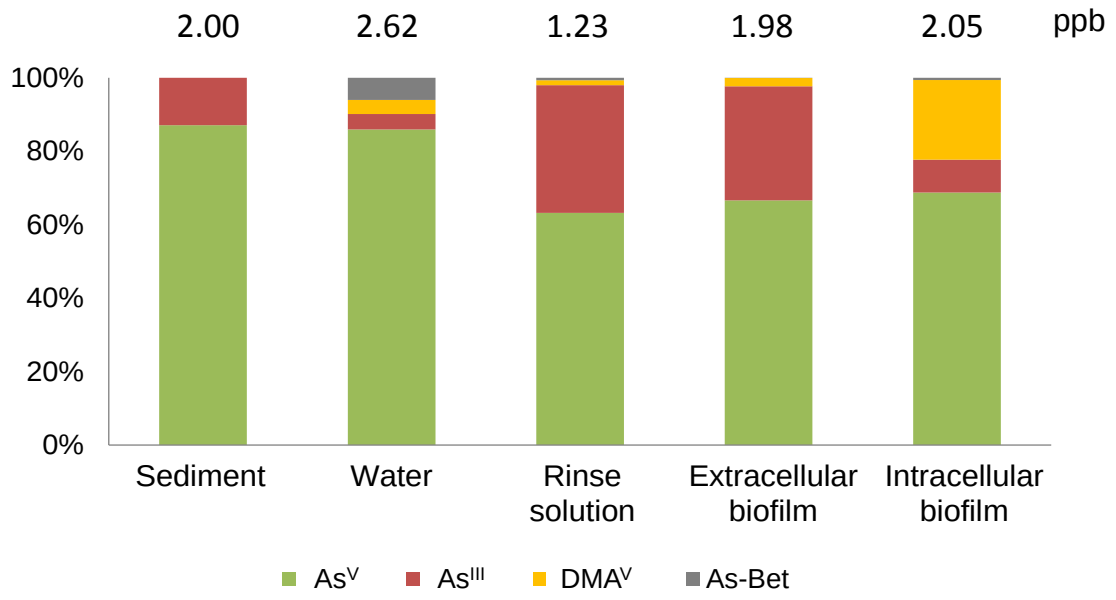


Figure 6 Arsenic speciation in the Downstream site. Stacked bar plots show the evolution of arsenic speciation in the Downstream site through the different analyzed compartments: sediment (the easily-extractable As), river water, and biofilm (rinse solution, extracellular and intracellular fractions), with mean values calculated from samplings on day 7 after translocation (except for river water, which was on day 22) and at the end of the experiment (51 days after translocation). Total mean arsenic concentrations are also shown above each bar expressed in ppb (concretely, in µg As g^{-1} for sediment and biofilm; while in µg As L^{-1} for river water). As^{V} = arsenate; As^{III} = arsenite; DMA^{V} = dimethylarsenate; As-Bet = arsenobetaine.

Overall, the results point to a relevant contribution of benthic biofilms to arsenic biogeochemistry in freshwater environments. The mutual interactions between arsenic and biofilm are tentatively represented in Figure 7. The As^{V} is deposited in the sediments, where it is available for microorganisms (epipsammon), which may transform and excrete arsenic into the water-sediment interface of the river. Moreover, the fate of arsenic (As^{V}) in the Anllóns River system is very dependent on the inputs of dissolved phosphate, acting as an As-displacing ligand in the sediments (Rubinos *et al.* 2011b), which promotes the exchanges between the water and sediment. This dissolved As^{V} can then be available for microorganisms, namely for

the benthic biofilms growing on rock surfaces (epilithic biofilms composed by bacteria and, mostly, microalgae), represented in this work by those developed on artificial substrates. Once on the biofilm surface, As^{V} may accumulate in the EPS matrix, adsorb to cells or even be uptaken by microorganisms, which in turn may modify arsenic speciation (through arsenate reduction and methylation) and excrete it into the water as the more toxic As^{III} and/or as organic species such as DMA^{V} (and, less uncertain, As-Bet), thus becoming available to the aquatic food web.

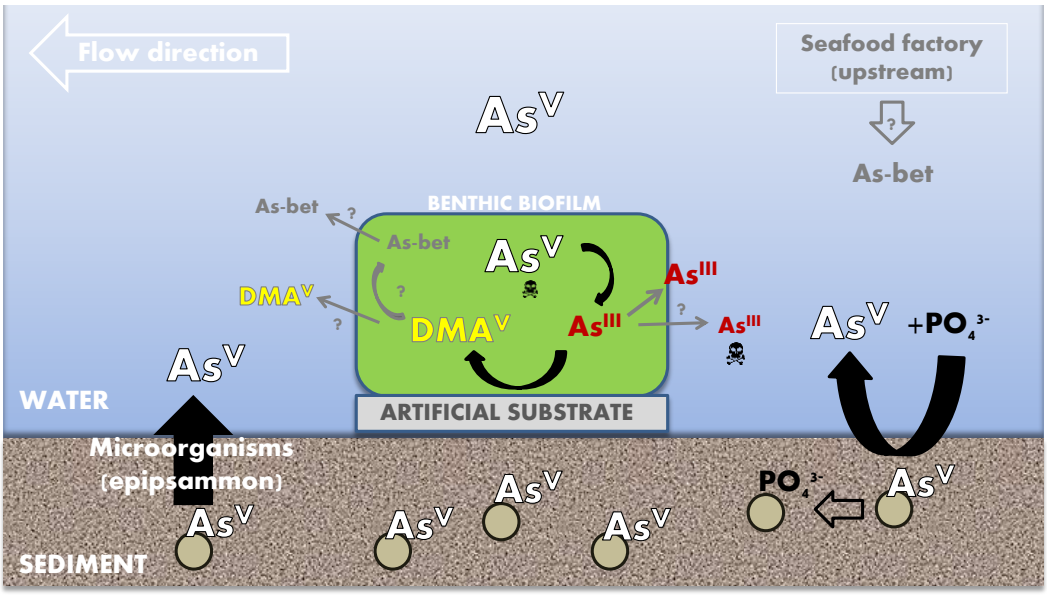


Figure 7 Hypothesized scenario of arsenic fate in the Downstream site, based on obtained results and literature, focusing on the contribution of the benthic biofilms to the arsenic biogeochemistry in this freshwater environment. Arsenate (As^{V}) may be mainly released from the sediment to the water by epipsammic biofilm (see it on the left side) or by exchange with phosphate (PO_4^{-3}), coming from external inputs (see it on the right side). Once in the water, arsenate may be uptaken by the benthic biofilm, which may also transform it to other As-species (arsenite, As^{III}; and dimethylarsenate, DMA^{V}) mainly by reduction and further methylation reactions, More complex organoarsenicals were found in water, specifically the arsenobetaine (As-Bet), which could come from the seafood factory located upstream the sampling sites, or be produced by the biofilm itself, what would be less probably.

5. CONCLUSIONS

In this experiment, we have proved the mutual interaction between arsenic and benthic biofilms in a mining impacted river, where the toxicant was mostly associated to sediments. Biofilms growing at the site with higher arsenic concentrations in sediments, accumulated higher arsenic concentrations. This arsenic exposure may partially explain the inability of algae to grow and the increase in bacterial and dead diatom density. Methylated As-species (DMA^{V}) were found in the intracellular biofilm compartment and even under eutrophic conditions, suggesting that detoxification processes (methylation) occurred within the biofilm. Our study provides valuable information to understand the contribution of benthic biofilms to arsenic biogeochemistry in freshwater environments and, specifically, in the water-benthic biofilm interface.

Laura Barral Fraga

O Anllóns!!

4. General Discussion

5. General Conclusions

1. Short-term biofilm exposure to environmentally realistic arsenic concentrations (130 µg As L^{-1}) and under P-limited conditions may cause important toxic effects to biofilms, becoming **less phototrophic** after being reduced the algal growth and productivity. Moreover, arsenic may inhibit the algal succession process in biofilms, causing changes in the algal community. A **loss of diatom species (those sensitive to arsenic)** and a **significant decrease in their cell size** may allow diatoms to become **more tolerant** to the toxicant than the other algal groups.

2. Similar effects may be observed in epilithic biofilms growing in a **mining impacted river**, even being the toxicant mostly associated to sediments. These biofilms **accumulate** high arsenic concentrations, resulting in a **inability of algae to grow** and in an **increase in bacterial and dead diatom density**. Therefore, **the release of arsenic** (through phosphate replacement or microbial activity) from sediments to other compartments such as water and/or biofilms should be contemplated in such mining areas, especially in rural regions where **phosphate inputs** are important. Other environmental factors in field experiments (such as nutrients, DOC, temperature or light availability) must be also taken in consideration when analyzing the arsenic effects in freshwater ecosystems.

3. **Methylated As-species (especially, DMA^V)** may be found within arsenic affected biofilms, suggesting arsenic detoxification (methylation) by microorganisms, even under eutrophic conditions, what agree with other field studies but not with some laboratory studies and suggested theoretical models, contributing thus to the lack of consensus about the role of nutrients (mainly P) on arsenic uptake and speciation by microorganisms.

4. Further experiments are needed to disentangle and **better understand the complex set of processes contributing to arsenic and phosphate cycling by microorganisms.** Considering the stoichiometry of P in relation to other elements like N could allow a better understanding of the dynamics among P and arsenic uptake and toxicity in microorganisms.

5. We strongly support the use of biofilm and a **multi-endpoint approach** to analyse effects of toxicants in freshwater ecosystems, especially including the measure of the **chlorophyll-a fluorescence** in biofilms and the **diatom biovolume (cell size)**. Regarding fish endpoints, **changes in complex behaviors** are practical, ecologically relevant measures of toxicological effects, and **aggression** in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behavior. Also, the analysis of arsenic speciation in the **extracellular** and **intracellular** part of the biofilm is highly recommended and contribute to the understanding of the arsenic cycle in freshwaters.

6. **Multi-trophic studies** are crucial to better elucidate the **real** effects of toxicants. Such **multidisciplinary**, cross-taxon research should therefore be considered for understanding the impacts of arsenic toxicity on aquatic ecosystems. An important finding in this respect from this thesis is the **aggravating influence of fluvial biofilms on the impacts of arsenic exposure in fish**.

7. Exploring **new endpoints** along with the traditional taxonomical parameters can greatly enhance the evaluation of fluvial ecosystem quality for biomonitoring practices using diatoms. In this sense, the **easiness**, **quickness**, **cheapness**, **global acceptation** and **no especial training in diatom taxonomy** should be the main characteristics of these new endpoints. Moreover, progress in diatom classification will come from the combination of **molecular techniques** with microscopic observations, especially in the case of complicated species complexes such as the case of cryptic species.

8. The results obtained in this thesis about the arsenic effects in fluvial systems **call into question the limits** of arsenic concentration established by the US EPA (2014) for freshwater systems. Also, the **difference in thresholds** between environmental and human health should be considered and **updated**, recognizing the strong consequences of the actual thresholds on the ecosystem functioning and, indirectly, on human health.

9. This thesis provides valuable information to understand **the contribution of benthic biofilms to arsenic biogeochemistry** in fluvial environments, and specifically in the water-biofilm interface.

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Annex 1

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RESEARCH ARTICLE

Short-term arsenic exposure reduces diatom cell size in biofilm communities

Laura Barral-Fraga[1] · Soizic Morin[2] · Marona D. M. Rovira[1,3] · Gemma Urrea[1] · Kit Magellan[1] · Helena Guasch[1]

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Abstract Arsenic (As) pollution in water has important impacts for human and ecosystem health. In freshwaters, arsenate (As^V) can be taken up by microalgae due to its similarity with phosphate molecules, its toxicity being aggravated under phosphate depletion. An experiment combining ecological and ecotoxicological descriptors was conducted to investigate the effects of As^V (130 μg L^{-1} over 13 days) on the structure and function of fluvial biofilm under phosphate-limiting conditions. We further incorporated fish (*Gambusia holbrooki*) into our experimental system, expecting fish to provide more available phosphate for algae and, consequently, protecting algae against As toxicity. However, this protection role was not fully achieved. Arsenic inhibited algal growth and productivity but not bacteria. The diatom community was clearly affected showing a strong reduction in cell biovolume; selection for tolerant species, in particular *Achnanthidium minutissimum*; and a reduction in species richness. Our results have important implications for risk assessment, as the experimental As concentration used was lower than acute toxicity criteria established by the USEPA.

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✉ Laura Barral-Fraga
laura.barral.fraga@gmail.com

[1] Institute of Aquatic Ecology, University of Girona, E-17071 Girona, Spain

[2] Irstea, UR EABX, Bordeaux, France

[3] Bio-Protection Research Centre, Lincoln University, PO Box 85084, Lincoln, New Zealand

Keywords Arsenate · Fluvial biofilm · Fish · Phosphate · Laboratory streams · Ecotoxicology · Multi-trophic studies

Introduction

Arsenic (As) is a widely distributed metalloid in natural ecosystems, and it is considered a priority pollutant, being the second most common inorganic contaminant in the original National Priority List (NPL), created by the United States Environmental Protection Agency (USEPA) (Davis et al. 2001). The Aquatic Life Criteria (USEPA 2014) establishes the limits of arsenic concentration in freshwaters: the Criteria Maximum Concentration (CMC), which refers to acute arsenic exposure, is 340 μg L^{-1} while the Criteria Continuous Concentration (CCC; refers to chronic arsenic exposure) is set at 150 μg L^{-1}.

In rivers, water contaminated with As have baseline concentrations ranging between 0.1 and 2.1 μg L^{-1}, with an average of 0.8 μg L^{-1} (Smedley and Kinniburgh 2002; Rahman et al. 2012). Naturally occurring arsenic enrichment from geothermal sources or groundwaters can range from 10 up to 370 μg L^{-1}. Concentrations from 0.75 up to 30 μg L^{-1}, and occasionally as high as 1100 μg L^{-1}, have been measured in industrial or sewage effluents (Smedley and Kinniburgh 2002).

A key factor in arsenic toxicity is its chemical speciation, and biological activity plays a major role in arsenic biogeochemistry (speciation, distribution, and cycling) in freshwaters (Smedley and Kinniburgh 2005; Rahman et al. 2012). The pentavalent arsenate oxyanion (As^V) is the stable and predominant arsenic species in well-oxygenated aquatic environments such as river and lake waters and oxic seawater (Smedley and Kinniburgh 2005). Little is known about As^V toxicity in algae, especially in rivers, although some studies have found that

ANNEX 2

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Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae

Kit Magellan*, Laura Barral-Fraga, Marona Rovira, Pao Srean, Gemma Urrea, Emili García-Berthou, Helena Guasch

Institute of Aquatic Ecology, University of Girona, E-17071 Girona, Spain

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ABSTRACT

Arsenic contamination has global impacts and freshwaters are major arsenic repositories. Arsenic toxicity depends on numerous interacting factors which makes effects difficult to estimate. The use of aquatic algae is often advocated for bioremediation of arsenic contaminated waters as they absorb arsenate and transform it into arsenite and methylated chemical species. Fish are another key constituent of aquatic ecosystems. Contamination in natural systems is often too low to cause mortality but sufficient to interfere with normal functioning. Alteration of complex, naturally occurring fish behaviours such as foraging and aggression are ecologically relevant indicators of toxicity and ideal for assessing sublethal impacts. We examined the effects of arsenic exposure in the invasive mosquitofish, *Gambusia holbrooki*, in a laboratory experiment incorporating some of the complexity of natural systems by including the interacting effects of aquatic algae. Our aims were to quantify the effects of arsenic on some complex behaviours and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas operculum movement decreased non-significantly and neither food capture efficiency nor consumption were notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomass. Possibly increased aggression facilitated food resource defence allowing fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioural and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

1. Introduction

Arsenic (As) from both anthropogenic and natural sources has global impacts (Mandal and Suzuki, 2002; Nordstrom, 2002; Rahman and Hasegawa, 2012; Rahman et al., 2012; Smedley and Kinniburgh, 2002) and aquatic systems, including freshwaters, are major repositories for arsenic (Nordstrom, 2002; Smedley and Kinniburgh, 2002). Although some national and international standards are in effect, for example the World Health Organization safe limit for drinking water is 10 $\mu g\,L^{-1}$ (Smith et al., 2002), the toxicity of As is dependent on numerous interacting factors such as its source, concentration and bioavailability; environmental parameters; and organisms' resistance ability and detoxifying mechanisms (Mandal and Suzuki, 2002; Rahman and Hasegawa, 2012; Smedley and Kinniburgh, 2002). A key factor is its chemical speciation. Inorganic As (iAs) is generally more toxic than organic As, while of the iAs species, arsenite (As^{III}) is more toxic than arsenate (As^{V}). However, the organic methylated species (dimethylarsinous acid, $DMAA^{III}$, and monomethylarsonous acid, $MMAA^{III}$) are more toxic than their iA parent compounds (Rahman et al., 2012; Smedley and Kinniburgh, 2002). Quantifying total arsenic in environmental and biological samples is therefore not synonymous with assessment of associated risks. The main chemical species in freshwaters are inorganic arsenics but

* Corresponding author. Tel.: +34 972 418 369; mobile: +34 666 785 381.
E-mail addresses: kit8x@hotmail.com, kit.magellan@udg.edu (K. Magellan).

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concentration is reduced by algae, this may be counterproductive at an ecosystem scale.

For mosquitofish, the effects of arsenic exposure are overall detrimental. Despite the increased biomass seen here with arsenic, bioaccumulation of arsenic is harmful (de Castro et al., 2009; Moeller et al., 2003; Sopinka et al., 2010) and increased aggression may increase the chance of physical damage (e.g. Huntingford and Turner, 1987) and exacerbate physiological effects of arsenic exposure (e.g. Scott and Sloman, 2004). Moreover, in addition to, or as a consequence of, the effects documented here other functions and interactions are likely to be disrupted. For example, both mate recognition (e.g. Fisher et al., 2006) and predator recognition (e.g. Mandrillon and Saglio, 2007) are compromised by alteration of the chemical environment. The mechanisms underlying the behavioural changes demonstrated in this study may involve sensory, hormonal, neurological and metabolic systems (Scott and Sloman, 2004) all of which may also affect other behaviours including locomotory behaviours like predator avoidance or swimming performance. The increase in aggression and lack of effects on feeding behaviour in this study suggest locomotory functions were not affected. However, the exposure treatments here were neither particularly acute nor chronic and increased exposure concentrations or durations are likely to lead to more serious impacts. Finally, here we used an invasive, highly tolerant fish as a model. The effects of arsenic exposure on potentially endangered native species would be both more difficult and more critical to evaluate.

In conclusion, we have shown here that changes in complex behaviours are practical, ecologically relevant measures of toxicological effects (e.g. Scott and Sloman, 2004; Weis et al., 2001). Aggression in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behaviour. In common with other authors, we also highlight interacting effects of contaminant exposure, both through integration of behavioural and physical mechanisms (e.g. Scott and Sloman, 2004; Weis et al., 2001) and consideration of different taxa together (e.g. Scott and Sloman, 2004; Weis et al., 2011). In particular, toxicant responses in multi-trophic, natural ecosystems are often found to be different from single-species laboratory studies. Multi-trophic studies are therefore crucial to elucidate the real effects of toxicants. An important finding in this respect from the current study is the aggravating influence of algae on the impacts of arsenic exposure in fish. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. Such multidisciplinary, cross-taxon research is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

Conflict of interest

The authors declare no conflict of interest.

Contributors

Concept: HG, KM, EGB; experimental design: HG, KM, EGB; field collection: EGB, KM, HG; carried out experiments: KM, LBF, MR, GU, HG; video analyses: KM, PS; biochemical analyses: LBF, MR, PS, GU, HG; statistical analyses: KM; wrote the paper: KM; edited, revised, critiqued and wrote small sections of the manuscript: HG, EGB, MR, LBF, GU.

All authors have approved the final article.

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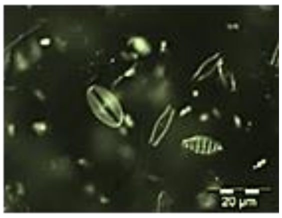

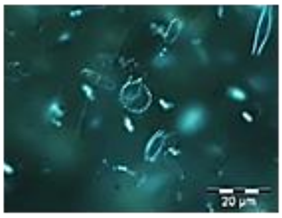

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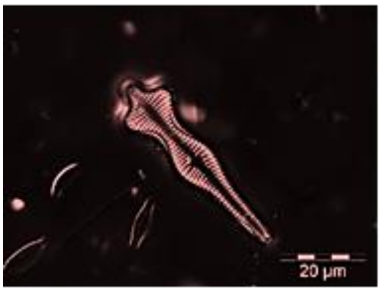

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