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INFLUENCES OF TOXICANTS ON FRESHWATER BIOFILMS AND FISH: FROM EXPERIMENTAL APPROACHES TO STATISTICAL MODELS

Baigal-Amar Tuulaikhuu

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DOCTORAL THESIS

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Baigal-Amar TUULAIKHUU

2016

Universitat de Girona

Doctoral Thesis

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2016

DOCTORAL PROGRAM IN WATER SCIENCE AND TECHNOLOGY

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In the Doctoral School of the University of Girona

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WE DECLARE THAT:

The thesis entitled "**Influences of Toxicants on Freshwater Biofilms and Fish: From Experimental Approaches to Statistical Models**", presented by Baigal-Amar Tuulaikhuu has been completed under our supervision and meets the requirements to obtain a philosophy doctoral degree at University of Girona.

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For everybody, who believed in, and trusted me
who taught me with patience
who encouraged me, and listened to me
at both professional and personal level

Many thanks

Мэргэжлийн ч хувь хүний нь ч хувьд
надад итгэж найддаг,
урам өгдөг, намайг сонсдог
тэвчээртэйгээр зааж сургасан
хүн бүхэнд

Баярлалаа

Acknowledgements

I would like to express my deep gratitude to Helena Guasch and Emili García-Berthou, my research supervisors, for their patient guidance, enthusiastic encouragement and useful comments on this research work.

My grateful thanks are also extended to all the colleagues at University of Girona, particularly Dr. Berta Bonet, for teaching me analyzing antioxidant enzyme activities, Dr. Anna Romaní, teaching for analysis extracellular enzyme activities, Dr. Kit Magellan for providing the experimental fish and giving recommendations for maintaining and measuring them, Laura Barral for helping lab works and Dr. Pao Srean for his assistance in R programming language.

I would also like to extend my thanks to the technicians of the laboratory of the Institute of Aquatic Ecology and staffs, Lluïsa Matas and Imma Arrom from the Technical Research Services of the University of Girona during laboratory analyses.

I am very grateful to the colleagues and the director of the School of Ecology and Technology Development of Mongolian State University of Agriculture (named before 2014) for refereeing me to the doctoral program in University of Girona, and all other research staffs in my previous colleagues from Hovsgol GEF/WB Project, Institute of Biology, Eco-Asia Institute we were working together and directed me to be a researcher.

I thank that I benefitted for a doctoral fellowship from the Techno 2 Program of the European Union Erasmus Mundus partnership.

Finally, I wish to thank my family for their support and encouragement throughout my study and life.

According to the Agreement of the Executive Committee of the School of Doctoral Studies of the University of Girona, in session 10/2012, of 12 December 2012, the thesis was written as a compendium of the following research articles:

Article 1

Baigal-Amar Tuulaikhuu, Anna M. Romaní, Helena Guasch, 2015. Arsenic toxicity effects on microbial communities and nutrient cycling in indoor experimental channels mimicking a fluvial system. *Aquatic Toxicology*, 166, 72-82. doi:10.1016/j.aquatox.2015.07.005.

Journal quality index for Aquatic toxicology:

Source Normalized Impact per Paper (SNIP): 1.275

SCImago Journal Rank (SJR): 1.377

Impact Factor: 3.451

Article 2

Baigal-Amar Tuulaikhuu, Berta Bonet, Helena Guasch, 2016. Effects of low arsenic concentration exposure on freshwater fish in presence of fluvial biofilms. *Science of the Total Environment*, 544, 467–475 doi:10.1016/j.scitotenv.2015.11.126

Journal quality index for Science of the Total Environment:

Source Normalized Impact per Paper (SNIP): 1.733

SCImago Journal Rank (SJR): 1.437

Impact Factor: 4.099

Article 3

Baigal-Amar Tuulaikhuu, Helena Guasch, and Emili García-Berthou, Submitted. Examining predictors of chemical toxicity in freshwater fish using the Random Forest technique.

LIST OF ABBREVIATIONS

AEAs	: Antioxidant enzyme activities
AFDW	: Ash-free dry weight
ANCOVA	: Analysis of covariance
ANOVA	: Analysis of variance
APX	: ascorbate peroxidase
As	: Arsenic
As(III)	: Arsenite
As(V)	: Arsenate
ATP	: Adenosine triphosphate
C	: Carbon or Control
CAS	: Chemical abstracts service
CAT	: Catalase
CCC	: Criterion continuous concentration
chl-a	: Chlorophyll-a
DDT	: Dichlorodiphenyltrichloroethane
DMA	: Dimethylarsenite
DNA	: Deoxyribonucleic acid
DW	: Dry weight
EC50	: Effective concentration to 50% of test organisms
ECOSAR	: Ecological structure activity relationships
EINECS	: European inventory of existing commercial chemical substances
Eu	: Experimental units
F0	: Minimal fluorescence yield
GR	: Glutathione reductase
GSH	: Tripeptide glutathione
GST	: Glutathione S transferase
iAs	: inorganic arsenical
ICP-MS	: Inductively coupled plasma mass spectroscopy
L/D	: Live/dead
LC_{50}	: 50% lethal concentration
LED	: Light-emitting-diodes
LOEC	: Lowest observed effect concentration

log *P*	: Octanol-water partition coefficient
MMA	: Monomethylarsenite
MoA	: Mode of action
MUF	: Methylumbelliferone
N	: Nitrogen
NADPH	: Nicotinamide adenine dinucleotide phosphate
NADPHO	: Nicotinamide adenine dinucleotide phosphate-oxidase
NOEC	: No observed effect concentration
OASIS	: Optimized Approach based on Structural Indices Set
OECD	: Organisation for Economic Co-operation and Development
P	: Phosphorus
PAM	: Pulse amplitude modulated
PCA	: Principal component analysis
PDH	: pyruvate dehydrogenase
POC	: Particulate organic carbon
PSII	: Photosystem II
QSARs	: Quantitative structure-activity relationships
RE	: Random - effect
RF	: Random forests
ROS	: Reactive oxygen species
SOD	: Superoxide dismutase
U.S. EPA	: United States Environmental protection agency
WST-1	: Water soluble tetrazolium-1
WW	: Wet weight
Yeff	: Photosynthetic efficiency
Ymax	: Maximal PSII quantum yield

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SUMMARY

Aquatic ecosystems are vulnerable to various kinds of pollutants such as organic compounds, heavy metals, radioactive substances, petroleum and its products, thermal pollution, and domestic wastes. While toxicological effects of single toxic chemicals on aquatic organisms have largely been investigated in the laboratory, real effects are difficult to predict since their behaviour in the environment is influenced by many factors. The main aims of this thesis are: i) to evaluate arsenic toxicity in two major, interacting components of the freshwater ecosystem, biofilm and fish, to provide information on environmentally realistic exposures and on biotic interactions, such as nutrient cycling that modulate toxicity; and ii) to rank predictors of toxicity to fish and quantify the differences in sensitivity among fish species. These are key aspects needed to link chemical pollution data with their real effects on wild fish populations and communities.

In the **first article,** a simplified fluvial experimental system including fish, periphyton and sediment was used to investigate the fate and effects of environmentally realistic concentrations of arsenic on biofilm growth and nutrient cycling. Total dissolved arsenic concentration decreased exponentially from 120 µg/L to 28.0 ± 1.5 (mean ± SD) µg/L during the experiment (60 days), mostly sinking to the sediment and a smaller percentage accumulating in the periphytic biofilm. Most phosphorus and nitrogen, which was provided by fish, were also retained in the epipsammic biofilm (growing on sediment grains). We conclude that exposure to this concentration of arsenic under oligotrophic conditions is changing the quality and quantity of the base of the aquatic food chain and its respective contribution to nutrient cycling, and normal functioning of the ecosystem: as lowering the total biomass of biofilm and its potential ability to use organic phosphorus (i.e. phosphatase activity), inhibiting algal growth,

especially diatoms, decreasing nitrogen contents and making the epipsammic biofilm more heterotrophic, thus reducing its ability to oxygenate the aquatic environment.

The purpose of the **second article** was to evaluate if environmentally-realistic arsenic exposure causes toxicity to fish. In the same experimental system used in the first article, four different treatments (control (C), biofilm (B), arsenic (+As) and biofilm with arsenic (B+As)) were applied to test the interactive effects of biofilm and arsenic on fish toxicity. Average arsenic exposure of Eastern mosquitofish (*Gambusia holbrooki*) was 40.5 ± 7.5µg/L for +As treatment and 34.4 ± 1.4 µg/L for B+As treatment for 56 days. Fish were affected directly and indirectly by this low arsenic concentration since exposure did not only affect fish but also the function of periphytic biofilms. Arsenic effects on the superoxide dismutase (SOD) and glutathione reductase (GR) activities in the liver of mosquitofish were ameliorated in the presence of biofilms at the beginning of exposure (day 9). Moreover, fish weight gain was only affected in the treatment without biofilm. After longer exposure (56 days), effects of exposure were clearly observed. Fish showed a marked increase in catalase (CAT) activity in the liver but the interactive influence of biofilms was not further observed since the arsenic-affected biofilm had lost its role in water purification. Our results highlight the interest and application of incorporating some of the complexity of natural systems in ecotoxicology and support the use of criterion continuous concentration (CCC) for arsenic lower than 150 µg/L and closer to the water quality criteria to protect aquatic life recommended by the Canadian government which is 5 µg As/L.

In the **third article** the random forest technique was used to examine the factors that best predict toxicity in a set of widespread fishes and analyses of covariance were used to further assess the importance of differential sensitivity among fish species. Among 13 variables the five best predictors of toxicity with random forests were, by order of

importance: the chemical substance (i.e. CAS number), octanol-water partition coefficient (log *P*), pollutant prioritization, ECOSAR classification, and fish species for 50% lethal concentrations (LC_{50}); and the CAS number, fish species, log *P*, ECOSAR classification, and water temperature for no observed effect concentrations (NOEC). Fish species was a very important predictor for both endpoints and with the two contrasting statistical techniques used. Different fish species displayed very different relationships with log *P*, often with different slopes and with as much importance as the latter predictor. Therefore, caution should be exercised when extrapolating toxicological results or relationships among species and further research of species-specific sensitivities and the mechanisms that cause them is needed.

Protecting aquatic organisms and their normal functioning in their natural habitat is challenging to environmental conservation. Therefore, our research highlights that incorporating some of the complexity of natural systems in ecotoxicology may not be sufficient. Monitoring (field sampling) is still needed to provide information on the real effects of pollution, having always in mind that effects may occur at lower concentrations than expected, since effects of toxicants like arsenic on fish, biofilm and their interactions depend on other water conditions such as nutrient availability. In addition, sensitiveness differs markedly among species and toxicants' effects can be more severe under the influence of biotic interactions.

RESUMEN

Los ecosistemas acuáticos son vulnerables a varios tipos de contaminantes tales como compuestos orgánicos, metales pesados, sustancias radiactivas, petróleo y sus derivados, la contaminación térmica y residuos domésticos. Mientras que los efectos toxicológicos de las sustancias tóxicas en los organismos acuáticos han sido ampliamente investigados en el laboratorio, los efectos reales son difíciles de predecir, ya que su comportamiento en el medio ambiente se ve influido por muchos factores. Los objetivos principales de esta tesis doctoral son: i) evaluar la toxicidad del arsénico en dos elementos clave que interactúan en el ecosistema acuático, biofilm y peces, para proporcionar información sobre los efectos de niveles de contaminación realistas a nivel ambiental y sus interacciones con otros factores que modulan la toxicidad, tales como el reciclado de los nutrientes; y ii) clasificar predictores de toxicidad para los peces y cuantificar las diferencias de sensibilidad entre las especies. Estos son aspectos clave para vincular los datos de contaminación química con sus efectos reales sobre las poblaciones y comunidades de peces en sistemas naturales.

En el primer **artículo** se utilizó un sistema fluvial experimental simplificado (formado por peces, perifiton y sedimento), para investigar el destino y los efectos de concentraciones de arsénico realistas a nivel ambiental en el crecimiento del biofilm y el ciclo de los nutrientes. La concentración total de arsénico disuelto se redujo de forma exponencial de 120 μg/L a 28.0 ± 1.5 μg/L a lo largo del experimento (60 días), reteniéndose mayoritariamente en el sedimento y, en menor medida, en el biofilm perifítico. La mayor parte del fósforo y el nitrógeno procedentes de los peces, también fueron retenidos en el biofilm episámico (que crece entre los granos de sedimento). Llegamos a la conclusión de que la exposición a esta concentración de arsénico, en condiciones oligotróficas, modifica la calidad y cantidad de organismos situados en la

base de la red trófica y su respectiva contribución al reciclado de los nutrientes, afectando también al funcionamiento normal del ecosistema. Disminuye su cantidad (biomasa total de biofilm) y su potencial capacidad de utilizar fósforo orgánico disuelto (es decir, la actividad de la fosfatasa alcalina), se inhibe el crecimiento de las algas, especialmente de las diatomeas, disminuye el contenido de nitrógeno y provoca que el biofilm episámico sea más heterotrófico, reduciendo así su capacidad para oxigenar el medio acuático.

El propósito del segundo artículo fue evaluar si dicha exposición al arsénico era tóxica para los peces. En el mismo sistema experimental utilizado en el primer artículo, se aplicaron cuatro tratamientos (control (C), biofilm (B), arsénico (+ As) y biofilm con arsénico (B + As)) para estudiar los efectos interactivos de biofilm y arsénico sobre la toxicidad para los peces. La exposición promedio al arsénico de las gambusias (*Gambusia holbrooki*) durante los 56 días de exposición fue de 40.5 ± 7.5 μg/L para el tratamiento + As y de 34.4 ± 1.4 μg/L para el tratamiento B + As. El arsénico, a pesar de su baja concentración, afectó directa e indirectamente a los peces ya que la exposición no sólo afectó a los peces, sino también a la función del biofilm perifítico. Los efectos del arsénico en las actividades superóxido dismutasa (SOD) y glutatión reductasa (GR) en el hígado de los peces se vieron aliviados en presencia de biofilm al inicio de la exposición (día 9). Además, los efectos sobre el incremento de peso de los peces se observaron únicamente en el tratamiento sin biofilm. Después de una exposición más prolongada (56 días), se observaron sus efectos. Los peces mostraron un marcado incremento en la actividad de la catalasa (CAT) en el hígado, pero la influencia interactiva del biofilm desapareció ya que dicho biofilm, afectado por el arsénico, había perdido su función en la depuración del agua. Nuestros resultados ponen de manifiesto el interés y la aplicación de la incorporación de parte de la complejidad de los sistemas

naturales en ecotoxicología, y apoyan el uso de valores de exposición crónica ("continuos concentration criterion", CCC) para el arsénico inferiores a 150 μ/L y más cercanos a los criterios de calidad del agua para proteger la vida acuática recomendados por el gobierno canadiense, que son de 5 μg As/L.

En el tercer artículo se utilizó la técnica denominada "Random Forests" para examinar los factores que mejor predicen la toxicidad en un conjunto amplio de peces y el análisis de la covarianza para evaluar la importancia de la sensibilidad diferencial entre de las especies. Entre las 13 variables, cinco de los mejores predictores de la toxicidad fueron, por orden de importancia: la sustancia química (es decir, número CAS), coeficiente de partición octanol-agua (log *P*), priorización de contaminantes, clasificación ECOSAR y la especie de pez para la concentración letal al 50% (LC_{50}); y el número CAS, especie de pez, log *P*, clasificación ECOSAR y temperatura del agua para las concentraciones sin efectos observados (NOEC). La especie de pez fue un predictor muy importante para ambos criterios de valoración y con las dos técnicas estadísticas utilizadas. Diferentes especies de peces muestran relaciones muy diferentes con log *P*, a menudo con diferentes pendientes y con tanta o más importancia de la especie como predictor. Por lo tanto, se debe tener precaución en extrapolar los resultados o las relaciones entre especies y es necesaria una mayor investigación de las sensibilidades toxicológicas de distintas especies y los mecanismos que las generan.

La protección de los organismos acuáticos y su normal funcionamiento plantean un enorme reto para la conservación. Los resultados de esta tesis ponen de manifiesto que la incorporación de parte de la complejidad de los sistemas naturales en ecotoxicología puede ser insuficiente. El monitoreo (muestreos de campo) es aún imprescindible para proporcionar información sobre los efectos reales de la contaminación, teniendo siempre en cuenta que la toxicidad puede ocurrir a niveles muy bajos de contaminación,

ya que los efectos de tóxicos tales como el arsénico en los peces, biofilm y sus interacciones se ven modulados por las condiciones ambientales (tales como la disponibilidad de nutrientes) y la composición biológica (ya que las distintas especies de peces difieren en su sensibilidad a los tóxicos). Además, la toxicidad puede agravarse si tenemos en cuenta las interacciones biológicas.

RESUM

Els ecosistemes aquàtics són vulnerables a diversos tipus de contaminants com ara la matèria orgànica, els metalls pesants, les substàncies radioactives, el petroli i els seus derivats, la contaminació tèrmica i les deixalles domèstiques. Mentre que els efectes toxicològics dels contaminants sobre els organismes aquàtics han estat àmpliament investigats al laboratori, els efectes reals són difícils de predir, ja que el seu comportament en el medi està influït per molts factors. Els objectius principals d'aquesta tesi doctoral són: i) avaluar la toxicitat de l'arsènic en dos components clau que interactuen a l'ecosistema aquàtic, el biofilm i els peixos, per tal de proporcionar informació sobre els efectes de nivells de contaminació realistes (a nivell ambiental) sobre biofilm, peixos i les seves interaccions biològiques (a través del reciclat dels nutrients) que en modulen la toxicitat; i ii) classificar predictors de toxicitat per als peixos i quantificar les diferències de sensibilitat entre les diferents espècies. Aquests aspectes són claus per vincular les dades de contaminació química amb els seus efectes reals sobre les poblacions i les comunitats de peixos en el medi.

En el primer article es va utilitzar un sistema fluvial experimental simplificat (amb peixos, perífiton i sediments) per investigar el destí i els efectes de nivells baixos de concentració d'arsènic, sobre el creixement del biofilm i el cicle dels nutrients. La concentració d'arsènic dissolt total va disminuir de manera exponencial de 120 μg/L a 28.0 ± 1.5 μg/L al llarg de l'experiment (60 dies), essent retingut majoritàriament pel sediment, i acumulant-se també (però en menor percentatge) en el biofilm perifític. Per altra banda, la major part del fòsfor i el nitrogen provinents dels peixos van ser retinguts en el biofilm episàmic (que creix en els grans de sediment). S'arriba a la conclusió de què l'exposició a aquesta concentració d'arsènic, en condicions oligotròfiques, provoca canvis en la qualitat i quantitat dels organismes que es troben a la base de la xarxa

tròfica, i la seva contribució al cicle dels nutrients, alterant el funcionament natural de l'ecosistema: fent que disminueixi la quantitat de biofilm (biomassa total) i el seu potencial per utilitzar el fòsfor orgànic dissolt (és a dir, l'activitat fosfatasa alcalina), inhibint-se també el creixement algal, especialment de les diatomees; provocant també una disminució en els continguts de nitrogen i fent que el biofilm episàmic esdevingui més heterotròfic, reduint així la seva capacitat per oxigenar el medi aquàtic.

En el segon article es volia avaluar si els nivells d'arsènic utilitzats eren tòxics pels peixos. En el mateix sistema experimental utilitzat en el primer article, es van aplicar quatre tractaments (control (C), biofilm (B), arsènic (+ As) i biofilm amb arsènic (B + As)) per tal d'estudiar els efectes interactius de la presencia de biofilm i arsènic sobre la toxicitat pels peixos. Les gambúsies (*Gambusia holbrooki*) van ser exposades a 40.5 ± 7.5 μg As/L (tractament + As) i 34.4 ± 1.4 μg As/L (tractament B + As) al llarg de l'experiment (56 dies). Els peixos es van veure tan directa com indirectament afectats per aquesta baixa concentració d'arsènic ja que l'exposició no només va afectar als peixos, sinó també a la funció del biofilm perifític. Els efectes de l''arsènic sobre les activitats superòxid dismutasa (SOD) i glutatió reductasa (GR) al fetge de peixos es van veure alleugerits per la presència de biofilms a l'inici de l'exposició (dia 9). Per altra banda, es va detectar un lleuger increment de pes dels peixos en els tractaments sense biofilm. Al final de l'experiment (després de 56 dies d'exposició), es va observar un clar increment en l'activitat de la catalasa (CAT) en el fetge dels peixos en tots els tractaments amb arsènic (desapareixent l'efecte compensatori del biofilm), ja que l'exposició prolongada del biofilm (a l'arsènic) li va fer perdre la seva capacitat d'autodepuració. Aquests resultats posen de manifest l'interès i l'aplicació d'incorporar part de la complexitat dels sistemes naturals en ecotoxicologia i donen suport a l'ús de llindars d'exposició ("criterion continuous concentration", CCC) per l'arsènic inferiors

als 150 μg/L i més propers al criteri de qualitat de l'aigua per protegir la vida aquàtica recomanat pel govern canadenc, que és de 5 μg As/L.

En el tercer article es va utilitzar la tècnica anomenada "Random Forests" per examinar els factors que millor prediuen la toxicitat en un conjunt de peixos ampli i l'anàlisi de la covariància per avaluar la importància de la sensibilitat diferencial de les espècies de peixos. Entre 13 variables, els cinc millors predictors de la toxicitat van ser, per ordre d'importància: la substància química (és a dir, número CAS), coeficient de partició octanol-aigua (log *P*), priorització de contaminants, classificació ECOSAR i espècie de peix per a la concentració letal al 50% (LC_{50}); i el número CAS, espècie de peix, log *P*, classificació ECOSAR i temperatura de l'aigua per a concentracions sense efectes observats (NOEC). L'espècie de peix va ser un predictor molt important per la tots dos criteris de valoració i amb les dues difererents tècniques estadístiques utilitzades. Diferents espècies de peixos mostren relacions molt diferents amb log *P*, sovint amb diferents pendents i amb tanta importància com aquest predictor. Per tant, s'ha de tenir precaució en extrapolar els resultats o les relacions entre espècies i cal una major investigació de les sensibilitats toxicològiques de les espècies i els mecanismes que les generen.

La protecció dels organismes aquàtics i del funcionament dels ecosistemes plantegen un gran repte ambiental. Els resultats d'aquesta tesi posen de manifest que no n'hi ha prou amb incorporar part de la complexitat dels sistemes naturals en ecotoxicologia. El monitoratge (mostreig de camp) és imprescindible per arribar a conèixer els efectes reals de la contaminació, tenint sempre en compte que aquests es poden produir a concentracions més baixes d'allò que s'esperaria, ja que, tal com es mostra en aquesta tesi, els efectes de compostos tòxics tals com l'arsènic en els peixos, el biofilm i les seves interaccions dependran d'altres factors ambientals com la disponibilitat de

nutrients. A més a més, també s'ha vist que les espècies de peixos difereixen enormement en la seva sensibilitat als tòxics i que la toxicitat es pot veure agreujada si tenim en compte les seves interaccions biològiques.

ХУРААНГУЙ

Усны экосистем нь органик бодис, хүнд металл, цацраг идэвхт бодис, нефть, нефтийн бүтээгдэхүүн, дулааны бохирдол, ахуйн хог хаягдал гэх мэт олон төрлийн бохирдуулагчдын нөлөөнд эмзэг байдалтай байна. Тухайн ямар нэгэн химийн бодисын усны организмд нөлөөлөх нөлөөллийг лабораторийн нөхцөлд ихээхэн судалж байгаа ч, байгаль дээр эдгээр бодисуудын бодит үр нөлөө нь олон хүчин зүйлүүдээс хамааран өөрчлөгдөж байдаг тул урьдчилан таамаглахад хэцүү байдаг. Энэхүү диссертацийн гол зорилго нь: i) цэнгэг усны экосистемд өөр хоорондоо харилцан үйлчлэлд оршиж байдаг гол бүрэлдэхүүн хэсэг болох биофильм болон загасанд үзүүлэх хүнцлийн хоруу чанарыг үнэлэх, байгаль дахь хүнцлийн бодит нөлөөлөл нь биологийн харилцан үйлчлэлийн үр дүнд хэрхэн өөрчлөгдөж болох талаар мэдээлэл бий болгох ii) Химийн бодисуудын загасанд үзүүлэх хоруу чанарт голлон нөлөөлдөг хүчин зүйлүүдийг холбогдлоор нь эрэмбэлэх, мөн химийн бодисын нөлөөллийг популяци болон бүлгэмдлийн түвшинд бодит байдалтай холбоход чухал шаардлагатай үзүүлэлт болох мэдрэмтгий байдлаар нь загасны төрөл зүйлүүдийг үнэлэх юм.

Эхний өгүүлэлд байгаль дахь бодит байдалтай төстэй орчинд хүнцлийн, хуримтлал болон биофильмийн өсөлт, шим тэжээлийн бодисын эргэлтэд түүний нөлөөллийг судлахын тулд загас, перифитон (хайрганы биофильм), болон ёроолын хагшаас бүхий голын хялбаршуулсан системийг байгуулан судалгаанд хэрэглэв. Нийт ууссан хүнцлийн агууламжийн ихэнх нь ёроолын хагшаасд шингэж, багахан хэсэг нь перифитонд хуримтлагдаж, туршилтын хугацаанд (60 хоног) 120 мкг/л-ээс 28.0 ± 1.5 мкг/л хүртэл эрс буурсан байна. Мөн загасны ялгаруулсан фосфор болон азотын ихэнх хэсэг нь элсний биофильм буюу эфифсаммонд шингэсэн байна. Ийм хэмжээний хүнцэлд өртөхөд, шим тэжээлийн

бодисоор ядмаг (олиготроф) нөхцөлд усан орчны хоол тэжээлийн гинжин хэлхээний анхдагч болох биофильмийн нийт биомассыг багасгаж, түүний органик фосфор ашиглах чадварыг (фосфатазын идэвх) бууруулж, замгийн өсөлтийг нэн ялангуяа цахиур замгийн өсөлтийг зогсоож, элсний биофильмийг илүү гетеротроф болгон, улмаар усны орчинг хүчилтөрөгчжүүлэх болон шим тэжээлийн бодисыг эргэлтэд оруулах зэрэг хэвийн үйл ажиллагааг нь алдагдуулж байна.

Хоёр дахь өгүүллийн зорилго нь байгаль дахь бодит байдалтай төстэй орчинд, бага хэмжээний хүнцэл нь загасанд сөргөөр нөлөөлдөг эсэхийг тодорхойлох юм. Туршилтын систем нь эхнийхтэй яг ижил бөгөөд энэ удаад дөрвөн өөр хувилбар (хяналтын (C), биофильмтэй (B), хүнцэлтэй (+As), хүнцэл болон биофильмтэй (B +As)-ыг ашиглаж хүнцлийн загасанд нөлөөлөх нөлөөг биофильм хэрхэн өөрчилж буйг судалж үзэв. Усан дахь хүнцлийн дундаж агууламж +As хувилбарт 40.5 ± 7.5 мкг/л, B+As хувилбарт 34.4 ± 1.4 мкг/л байв. Хүнцлийн энэ багахан агууламж нь загасанд шууд болон шууд бусаар нөлөөлж байгаа нь тогтоогдов. Туршилтын хугацааны эхэнд (9 дэх өдөр) загас нь хүнцлийн нөлөөнд өртөгдөн элгэн дэхь супероксиддисмутаз (СОД) болон глютатион редуктаз (ГР) энзимүүдийн идэвх өөрчлөгдөх байдал нь биофильмийн нөлөөгөөр багассан байлаа. Түүнчлэн, биофильмгүй, зөвхөн хүнцэл агуулсан хувилбарт загасны жингийн эрс өөрчлөлт ажиглагдав. Удаан хугацааны туршид (56 хоног) хүнцлийн нөлөөнд өртсөний дараа загасны элгэнд каталаз (КАТ) энзимийн идэвх эрс нэмэгдсэн бөгөөд хүнцэлд нэрвэгдсэн биофильм ус цэвэршүүлэх үүргээ алдсан учир хүнцлийн хорыг саармагжуулах эрэг нөлөө цаашид ажиглагдсангүй. Бидний судалгааны үр дүн нь экотоксикологийн судалгаанд байгалийн системийн нарийн төвөгтэй харилцан уялдааг нэгтгэн оруулж өгөхийг санал болгож байгаа бөгөөд, усан орчин дахь амьдралыг хамгаалахын тулд АНУ-д баримталж байгаа хүнцлийн

шалгуур концентраци (CCC) болох 150 мкг/л-ийг хэт их гэж үзэн, Канадын засгийн газрын тогтоосон 5 мкг/л –ийг дэмжиж байна.

Гурав дахь өгүүлэлд “Random forest” аргыг ашиглан химийн бодисуудын нөлөөллийн хэмжээг урьдчилан таамаглахад хамгийн их холбогдолтой хүчин зүйлүүдийг харьцуулан дүгнэж, мөн ковариацийн шинжилгээ хийж загасны зүйлүүдийн химийн бодисуудын нөлөөнд эмзэг байдлын ялгааг үнэлсэн. Random forest –ийн үнэлгээгээр 13 хүчин зүйлээс химийн бодисын нөлөөлөл (үхлийн дундаж концентраци (LC_{50}))- ийг илэрхийлэх хамгийн гол холбогдолтой нь химийн бодисын төрөл (CAS дугаар), тухайн бодисын спирт (октанол) болон усанд хуваарилагдалтын коэффициент (log *P*), Америкийн байгаль орчны агентлагаас жагсаасан бохирдуулагчдын эрэмбэлэлт, ECOSAR ангилал, загасны төрөл зүйл зэрэг байв. Нөлөөлөл ажиглагдаагүй концентраци (NOEC)-ийг урьдчилан таамаглахад гол холбогдолтой хүчин зүйлүүд нь CAS дугаар, загасны зүйл, log *P*, ECOSAR ангилал, усны температур зэрэг байв. Химийн бодисын загасанд үзүүлэх нөлөөг урьдчилан тааварлахад загасны төрөл зүйл нь аль аль нөлөөллийн түвшинд (LC_{50}, NOEC) ч, судалгаанд хэрэглэсэн хоёр өөр статистик тооцооллын аргаар ч маш чухал үзүүлэлт гэдэг нь тодорхой харагдаж байна. Загасны төрөл зүйлүүдийн хувьд химийн бодисын тэдэнд нөлөөлөх концентраци нь тухайн бодисын спирт (октанол) болон усанд хуваарилагдалтын коэффициентоосоо хамаарааД өөр өөр байгаа бөгөөд, регрессийн шугамын налуу нь мөн харилцан адилгүй байна. Тиймээс хор судлалын үр дүнгүүдийг загасны бүх төрөл зүйлд хамааруулж экстраполяци хийх үедээ болгоомжтой хандах, амьтан, ургамлын төрөл зүйлийн онцлог, мэдрэмтгий чанар, үүнийг бүрдүүлж байгаа механизмыг цаашид судлах шаардлагатай юм.

Усны организмууд, тэдгээрийн үйл ажиллагаа нь амьдрах орчиндоо хэвийн байгаа эсэхийг судлаж хамгаалах нь бэрхшээлтэй байдаг. Тийм учраас бидний судалгаа нь химийн бодисын нөлөөллийн судалгаа буюу экотоксикологит байгалийн системийн зарим нарийн харилцаа холбоог оролцуулан авч үзэх хэрэгтэйг харуулж байна. Химийн бодисуудын байгаль дээрх бодит нөлөөлөл нь тооцоологдож байгаагаас илүү бага концентрацид илэрч болохыг бодолцож бохирдлын бодит нөлөөллийн талаар мэдээлэл бий болгохын тулд мониторинг судалгааг явуулах шаардлагатай байна. Учир нь хүнцлийн нөлөөнд нэрвэгдсэн биофильмийн эргэж сэргэх эсэх нь усан орчны бусад үзүүлэлтүүд, жишээлбэл фосфорын агууламжаас шалтгаалдаг, мөн загасны төрөл зүйлүүд нь хоорондоо бохирдолд эмзэг байдлаараа ялгаатай учраас, тэсвэртэй зүйл нь аль хэдийн химийн бодисын нөлөөнд өртчихсөн байгаа мэдрэмтгий зүйлдээ давхар дарамтыг учруулах зэргээс үүдэн химийн бодисын дам нөлөөллийг улам илүү болгож болох юм.

INTRODUCTION

INTRODUCTION

Water pollution is one of the main issues globally threatening the enormous biodiversity of freshwater ecosystems (Dudgeon et al. 2006), which occupy less than 1% of the earth's surface, but support at least 100,000 known species, including over 10,000 freshwater fish and 90,000 invertebrates (Allan and Castillo 2007). The decline in biodiversity is far greater in freshwaters than in the most affected terrestrial ecosystems (Sala et al., 2000).

The study of streams and rivers should be addressed taking into account natural processes and human activities and directed at the urgent need for the conservation of aquatic systems (Allan and Castillo, 2007). The fluvial ecosystem integrates the biota and its interactions with all the interacting physical and chemical processes that collectively determine how it functions. Since all the processes and components of aquatic ecosystems are tightly interconnected, any disturbance can affect their structure or function. According to the Intermediate Disturbance Hypothesis (Grime, 1973; Connell 1978), the response to perturbations depends on their intensity and frequency. Although rivers can adapt to natural disturbances, chronic and persistent disturbances by humans can cause environmental alterations to which the biological system cannot respond, adapt or recover (Stevenson and Sabater, 2010).

One of the human-mediated disturbances in freshwaters and soils is caused by past and recent mining activities or industrial wastewaters, from which high concentrations of metals and metalloids drain into water and aquatic sediments (Schaller et al. 2011), which is a global problem for several types of freshwater ecosystems (Kraak et al., 1991; Biney et al., 1994; Kouba et al., 2010). For instance, in 1989 it was estimated that approximately 19,300 km of streams and rivers, and ca. 72,000 ha of lakes and wetlands worldwide were seriously damaged by mining activities (Johnson and Hallberg, 2005),

but the true scale of the environmental pollution caused by mine water discharges is difficult to assess accurately. In northern England, it was estimated that 12,000 km^2 of river catchments were directly affected by former mining activities (Macklin et al., 2002). Among all the toxic metals and metalloids, three metals (lead, mercury and cadmium) and the metalloid arsenic, have all caused major human health problems in various parts of the world (Hutton, 1987)

Arsenic in the environment

Arsenic is a major environmental pollutant (Singh,et al., 2007), as it ranks as the 20th most occurring trace element in the earth's crust (NRC, 1977).

1. Arsenic occurrence, chemistry and biotransformation

Arsenic contamination of surface waters (Table 1) and soil mostly arises when arsenic-rich geothermal fluids come into contact with surface waters and mining-related activities, because arsenic is a widely dispersed element in the Earth's crust and occurs as a constituent in more than 200 minerals. Arsenic minerals exist in the environment principally as sulphides, oxides, and phosphates (Garelick et al., 2009). There is a hazard of mobilising arsenic during gold mining activities, because gold- and arsenic-bearing minerals coexist (Garelick et al., 2009).

Baseline	Polluted European rivers	Geothermal influenced	Mining influenced	High-As ground water influenced
0.83 (0.13-2.1)	4.5-45	38 (0.2-264)	137.5 (2-7900)	235.5 (<10-21800)

Table 1. Mean arsenic concentration and ranges (µg/L) in river waters as reviewed by Smedley and Kinniburgh (2002) and Bundschuh et al. (2012).

The mechanisms by which arsenic is released from minerals are diverse and are accounted for by many (bio)-geochemical processes: oxidation of arsenic-bearing

sulphides, desorption from oxides and hydroxides, reductive dissolution, evaporative concentration, leaching from sulphides by carbonate, and microbial mobilisation (Garelick et al., 2008).

The fate of arsenic in freshwater systems is similar to other metals and metalloids. Trace elements enter the water by atmospheric precipitation, terrestrial runoff and groundwater discharge and bind to organic and inorganic particles and all these tend to sink to the bottom (Belzile and Morris, 1995). The biota in the sediment has an important role for bioaccumulation of arsenic (Fig. 1).

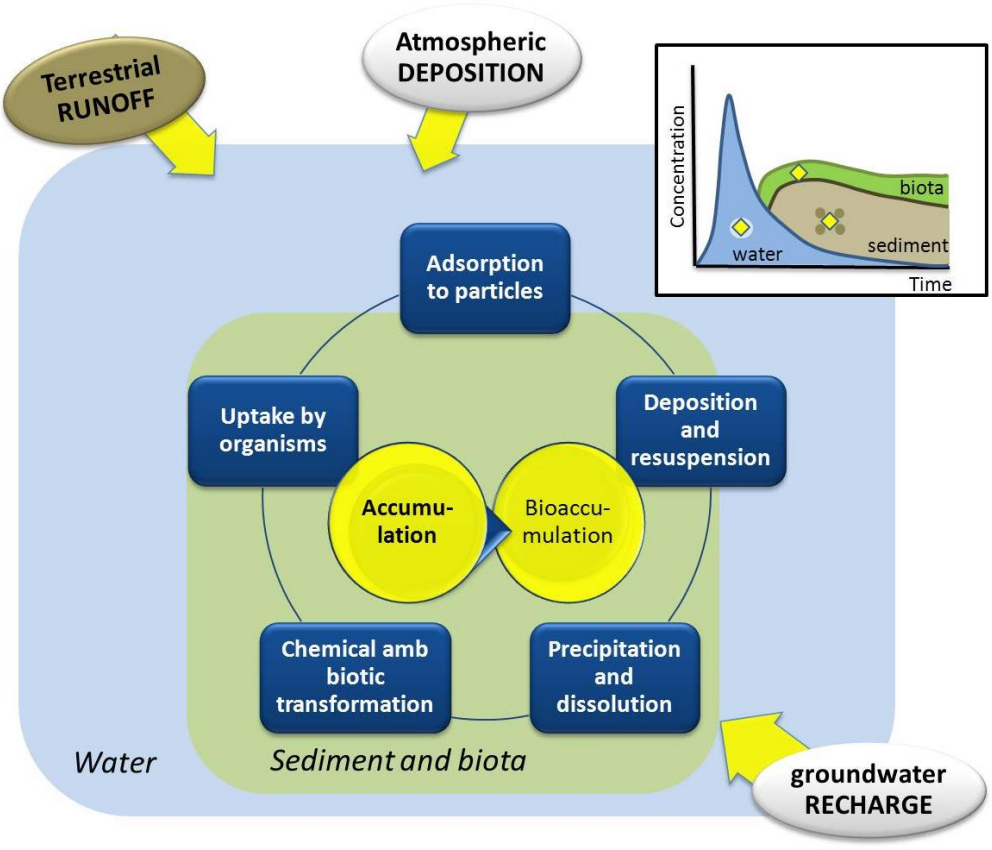

Figure 1. Sources (terrestrial, atmospheric and groundwater) and processes (dark-blue boxes) involved in the transfer of metals and metalloids (in yellow) between the different compartments: water, biota and sediments and temporal variation of concentration in each compartment after its entrance to freshwater systems (right corner graph).

In the environment, arsenic is found in inorganic and organic forms and in different valence or oxidation states. The valence states of arsenic of environmental interest are the trivalent (III) and pentavalent (V) states. Elemental arsenic has a valence state of (0), arsine and arsenides have a valence of (–III) (Hughes et al. 2011). A general scheme for the methylation of arsenic in organisms, which was proposed by Cullen et al. (1984) can be summarized as follows:

$$\mathrm{As^{V}O_4}^{3-} \xrightarrow{2e} \mathrm{As^{III}O_3}^{3-} \xrightarrow{\mathrm{Me^+}} \mathrm{MeAs^{V}O_3}^{2-} \xrightarrow{2e} \mathrm{MeAs^{III}O_2}^{2-} \xrightarrow{\mathrm{Me^+}} \mathrm{Me_2As^{V}O_2}^{-}$$

$$\xrightarrow{2e} \mathrm{Me_2As^{III}O}^{-} \xrightarrow{\mathrm{Me^+}} \mathrm{Me_3As^{V}O} \xrightarrow{2e} \mathrm{Me_3As^{III}}$$

Where $As^{V}O_4^{3-}$ - arsenate, $As^{III}O_3^{3-}$– arsenite, $MeAs^{V}O_3^{2-}$ - monomethylarsonic acid, $MeAs^{III}O_2^{2-}$ - monomethylarsonous acid, $Me_2As^{V}O_2^{-}$ - dimethylarsinic acid, $Me_2As^{III}O^{-}$ - dimethylarsinous acid, $Me_3As^{V}O$ - trimethylarsine oxide, Me_3As^{III}- trimethylarsine.

2. Arsenic toxicity

Arsenic toxicity is influenced by many chemical, environmental and biological factors. Trivalent arsenicals (e.g., arsenite, monomethylarsonous acid, dimethylarsinous acid) are considered more potent toxicants than pentavalent arsenicals (e.g., arsenate, monomethylarsonic acid, dimethylarsinic acid), and methylated and dimethylated trivalent arsenicals are more cytotoxic, more genotoxic, and more potent inhibitors of the activities of some enzymes than arsenite (Thomas et al. 2001). On the other hand, several modes of action have been described for arsenic involving different chemical species, and these may also affect different aquatic organisms differently.

2.1 Modes of action (MoA) of arsenic

A mode of action (MoA) describes a functional or anatomical change, at the cellular level, resulting from the exposure of a living organism to a substance. Toxic responses may be detected in a variety of ways in animals triggering a change in cellular functions

till death of the whole animal, but all the toxic responses may have a biochemical basis (Timbrell 1991). Several modes of action for arsenic exposure and toxicology were described in a review by Hudge et al. (2011).

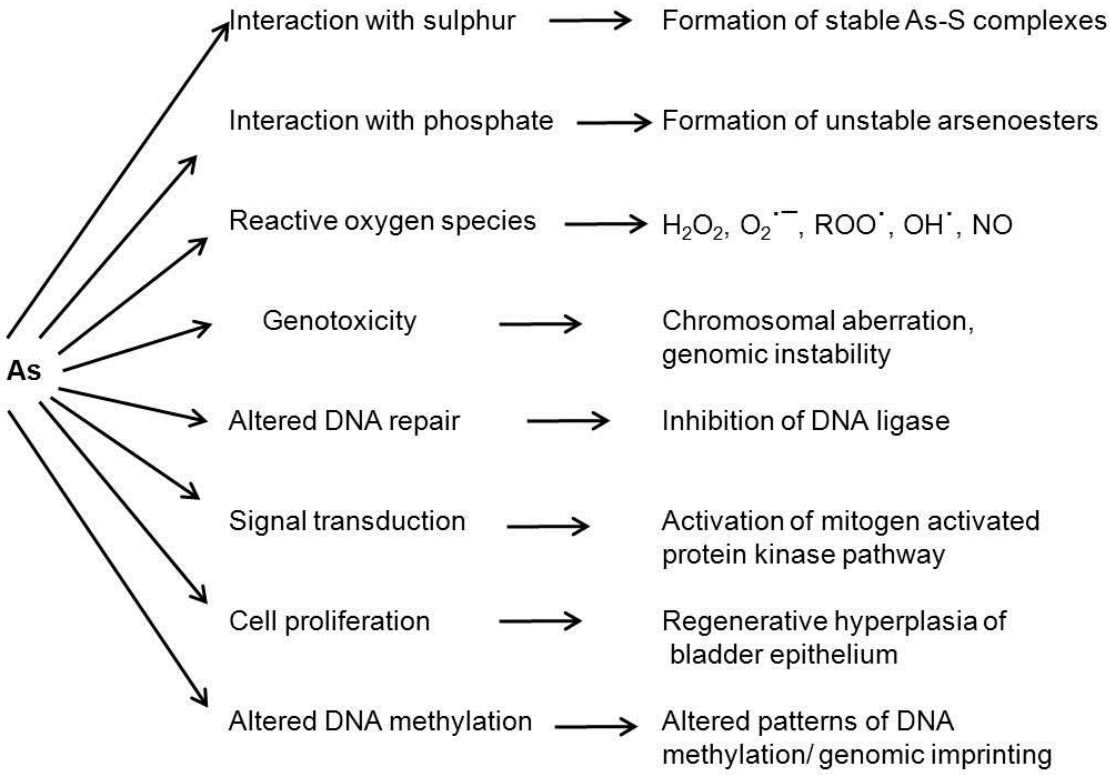

Figure 2. Modes of action for arsenic as described in Hudge et al. (2011)

Interaction with sulphur

Trivalent arsenic binds to sulfhydryl groups of tissue proteins (Hudge et al., 2011), such as the sulfhydryl group of glutathione or other cellular sulfhydryl compounds. Arsenite can inhibit pyruvate dehydrogenase (PDH) (Hughes, 2002) by binding to vicinal sulfhydryl groups within this enzyme complex. Studies by Samikkannu et al. (2003) suggest that arsenite inhibits the enzyme by generating reactive oxygen species (ROS) that inactivate the protein. This occurs at much lower concentrations of arsenite than concentrations required for inhibition by direct binding to the sulfhydryl group.

Interactions with Phosphate

Arsenic and phosphorus are in the same group and have similar physicochemical properties. Arsenate ($HAsO_4^{-2}$) exhibits very similar pK_a (acid dissociation constant) values to phosphate (HPO_4^{-2}), and forms analogous esters. However, the As–O bond is about 10% longer than the P–O bond (Dixon, 1997), rendering it less stable; the arsenate ester bond can easily dissociate. Arsenate uncouples formation of adenosine triphosphate (ATP) in vitro by a mechanism termed ‘‘arsenolysis” (Doudoroff et al. 1947). Arsenolysis can occur during glycolysis and oxidative phosphorylation in the presence of arsenate (Crane and Lipmann, 1953; Gresser, 1981). In the glycolytic pathway, arsenate can form the intermediate anhydride, 3-phosphoglyceroyl arsenate. In oxidative phosphorylation, arsenate can couple with adenosine diphosphate (Hughes et al., 2011). Both reactions form unstable arsenate anhydrides, which hydrolyse easily. The overall result is that the formation of ATP is diminished.

Reactive Oxygen Species

Arsenic generates excessive ROS by increasing the expression and activity of nicotinamide adenine dinucleotide phosphate-oxidase (NADPHO), an enzyme that produces superoxide, through several mechanisms (Hunt et al., 2014). ROS molecules attack lipids, proteins, nucleic acids and damage most cellular machinery, which often leads to alterations in cell structures and mutagenesis (Bhattacharya et al., 2007; Bonet Sánchez, 2013; Cooke, 2003). Therefore, ROS formed by arsenic are involved in several of the other MoAs including genotoxicity, signal transduction, cell proliferation, and inhibition of DNA repair (Hughes, 2011). Normally, cells defend themselves

against ROS damage with several enzymes including superoxide dismutase, catalases, and glutathione peroxidases (Bansal and Kaushal, 2014).

2.2 The potential toxicities of arsenic to different aquatic organisms

Arsenic toxicity to humans and other mammals is well documented. It causes increased mortality due to different kinds of cancer, ischemic heart disease, bronchiectasis and other diseases, such as cardiovascular and peripheral vascular disease, developmental anomalies, neurological and neuro-behavioural disorders, diabetes, hearing loss, portal fibrosis, haematological disorders (anaemia, leukopenia and eosinophilia), and carcinoma (Smith and Steinmaus, 2009; Tchounwou et al., 2012). However, the impacts on aquatic ecosystems are little known, and determination of toxicological effects of arsenic on fish and other organisms has largely been investigated in the laboratory, generally with a few surrogate species. As mentioned before, it is assumed that arsenic toxicity highly depends on its oxidation state.

Arsenic toxicity values compiled from the ECOTOX database (http://cfpub.epa.gov/ecotox/) were used to construct species sensitivity distribution curves (SSDs). SSDs allow the proportion of species affected at different levels of exposure to a specific toxicant to be calculated and plotted (Posthuma et al., 2001). SSDs curves show that arsenite (As^{III}) is more toxic than arsenate (As^{V}), especially to insects and worms. For instance, LC_{50} values (concentrations of a compound causing 50% mortality of the tested organisms) for crustaceans, rotifers, fish, algae, worms and insects are 4.6, 30.0, 40.9, 79.4, 213.0 and 604.0 mg As^{V}/L, respectively (Fig. 3A), whereas reported arsenite toxicity values for crustaceans, rotifers, Polychaeta, fish, amphibians, insects, molluscs and nematodes are 5.9, 6.0, 11.3, 24.5, 28.5, 39.3, 72.3, and 177.5 mg As^{III}/L, respectively (Fig. 3B) . When arsenite concentration reaches 36

mg/L or arsenate concentration reaches 78 mg/L it may affect 50% of aquatic organisms and 20% will be affected at 10 mg/L (Fig. 3A, B). In the ECOTOX database, no observable effect concentrations (NOEC), which is the highest concentration of toxicant in which the values of the observed responses are not statistically significantly different from controls, had more data than other sub-lethal endpoints. Based on ECOTOX, all NOEC values to aquatic organisms (except fungi) are below 10 mg/L. In contrast to $LC_{50,}$ NOEC values to fish and algae for arsenate (4.68 and 1.19 mg/L, respectively) are lower than those of arsenite (9.83 and 8.59 mg/L), indicating that sub-lethal exposure to arsenate is more influential to these organisms (Fig. 3C, D).

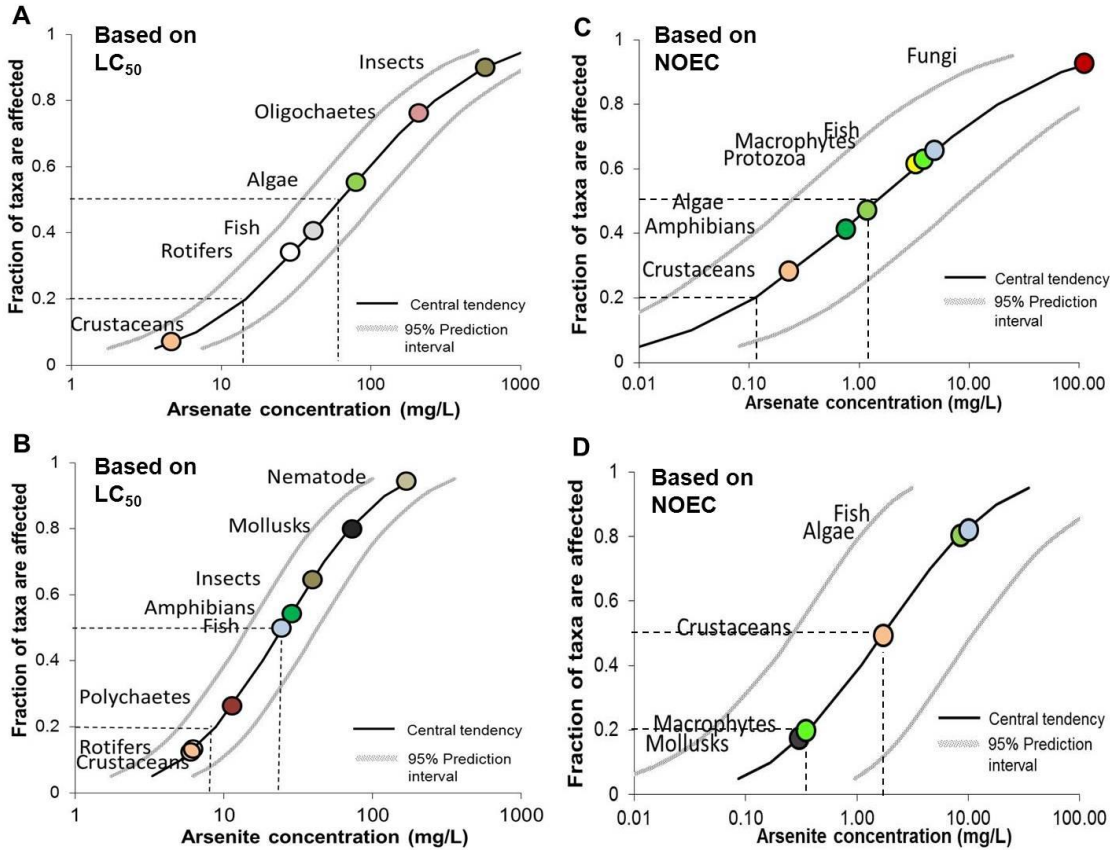

Figure 3. Species sensitivity distribution curves based on LC_{50} and NOEC values of arsenate and arsenite for aquatic organisms. Data and guides for constructing the curves were extracted from the ECOTOX database [http://cfpub.epa.gov/ecotox/.](http://cfpub.epa.gov/ecotox/)

The existing data of arsenic effects on aquatic organisms indicate low real-effects in the environment since arsenic occurrence is usually much lower than the effective

concentrations found in the ECOTOX database. ECOTOX is very limited in terms of cases (e.g. it includes no LC50 value of arsenite for algae) and has low ecological realism because it is mainly based on acute toxicity to a single species. This drawback highlights the interest in investigating arsenic toxicity after longer exposure and at a larger scale of biological organization, in order to account for the effects that exposure may cause on the structure and function of aquatic communities and ecosystems.

Biofilms

Freshwater biofilms are communities mainly composed of diatoms and green algae as well as cyanobacteria, bacteria, protozoa and fungi, all embedded together in a polymeric matrix (Sabater and Admiraal, 2005). The polymeric matrix is mostly constituted by water and polysaccharides and other compounds are found in small amounts, such as extracellular DNA, proteins and lipids, particulate materials and detritus (Mora-Gómez et al., 2016). Aquatic biofilms are classified by the substratum and environmental conditions where they develop (Mora-Gómez et al., 2016). For instance, biofilms attached to submerged rocks are called epilithon or periphyton, but those found in sediments are called epipsammon, epipelon or hyporheic biofilms (Romaní et al., 2010; Mora-Gomez et al., 2016). In this thesis, I will use the term periphyton or periphytic biofilm for biofilm growing on glass tiles (originally collected by scraping stream cobbles), and epipsammon or epipsammic biofilm for those developed in the sediment. The functioning of freshwater biofilms is vital in the environment both in maintaining and improving ecological health and in degrading and transforming pollutants, creating primary production, oxygenating the environment, and in carbon and nutrient cycling (Mora-Gómez et al., 2016). Since biofilms are communities, there are several interactions within biofilm components, such as

mutualism, commensalism, predation, grazing, parasitism or competition (Sathe and Dobretsov, 2016), and also between microorganisms in biofilms and higher (macro) organisms, providing food, and vitamins for grazing macro-organisms, and receiving organic nutrients from them (Thompson et al. 2004).

The use of biofilm to assess chemical toxicity in aquatic environments has been increasing (Guasch et al., 2012, 2016). Heavy metal effects are cumulative and increase their toxicity after repetitive pulses (Guasch et al., 2010) leading to various effects on biofilm, such as changing pigment, biomass, physiological activities, and algal species composition (Sabater et al., 2007). Long-term exposure changes the species composition of a community and has a selective influence for tolerant species (Clements et al. 2000; Guasch et al., 2003). Moreover, biologically-caused changes in stream ecosystems influence the kinetics of biogeochemical reactions, such as solubility of minerals, sorption of metals onto particle surfaces, transformations between oxidised and reduced species, and metabolism of aquatic biota (Balistrieri et al., 2012).

1. What is known about arsenic toxicity to biofilms?

From studies that proposed to use biofilm for bioremediation of arsenic and other pollutants (e.g Battaglia-Brunet et al., 2002; Belval et al., 2009; Dictor et al., 2012), it is known that arsenic oxidising and reducing bacteria in biofilms may contribute to arsenic biotransformation in the aquatic environment. In river systems, it has been shown that epipsammic biofilms play an important role in water quality, increasing arsenic retention in the sediment (Prieto et al., 2013).

While biofilm is one important component of aquatic ecosystems and is commonly used to assess chemical toxicity in the aquatic environment (Guasch et al., 2016), little information exists about arsenic effects on biofilms. A microcosm experiment

performed by Barral-Fraga et al. (2016), demonstrated that biofilm exposure to arsenic at environmentally realistic concentration (130 μg/L, during 13 days) may be sufficient to inhibit algal growth and productivity, also causing selection for tolerant small-sized species. The effects of arsenic on these parameters, such as inhibiting photosynthetic capacity, algal growth, changing community composition and reducing the P uptake ability of the community were only detected in P-starved communities (Rodriguez-Castro et al., 2015). Arsenate uptake by algal cells was generally higher, thus As^{V} was more toxic than As^{III}, especially at the near neutral pH 6.8. (Pawlik-Skowrońska et al., 2004). Effective arsenic concentrations in biofilms (<0.13 mg/L) contrast with those reported in the ECOTOX database (one order of magnitude higher). It is also important to highlight that arsenate toxicity is inversely related to phosphate concentration (Levy et al., 2015; Rodriguez-Castro et al., 2015). In this thesis, we will evaluate the effects of low arsenic concentration on structural and functional attributes of fluvial biofilms in the presence of freshwater fish since microbial communities are expected to drive the biotransformation of arsenic and modify the bioavailability of both arsenic and nutrients to other freshwater organisms.

Fish

Fish are not only important for the tens of millions of people who rely on this resource for income and food but also for ecosystem health as they play an enormous role in structuring the food web and driving nutrient cycling. Focusing on this second aspect, fish may be a mobile source of nutrients. For instance, spawning runs of migrating fish may import substantial amounts of marine-derived nutrients or nutrients from downstream to upstream river reaches and lakes through excretion, release of gametes, and their own mortality, especially if many or all die after reproducing, such as

salmon (Naiman et al., 2002). In an experiment in tropical streams, grazing fish had a significant impact on nitrogen demand and the response to nitrogen enrichment was significantly greater on substrates accessible to natural fish assemblages compared to substrates where grazing fish were excluded, indicating that fish had a dual effect of consuming algae and regenerating nutrients (Flecker et al., 2002). Bioturbation by fish may also influence carbon flow and ecosystem metabolism. In the experiments carried out by Taylor et al. (2006) in open and fish-entrance restricted stream sections, it was observed that, in the absence of fish, the amount of the particulate organic carbon (POC) on the streambed was higher and its downstream flux declined due to reduced bioturbation and consumption; heterotrophic respiration increased due to greater biofilm growth; primary production doubled, and because respiration increased more than primary production, net ecosystem metabolism showed a greater deficit.

1. What is known about the effects of chemicals on freshwater fish?

Ecotoxicologists generally assess the impacts of pollutants on freshwater fish using standard toxicity tests. However, toxicity tests are limited to a few surrogate species (Escher and Hermens, 2002). Methods have been developed to extrapolate data for a few species to many species based on interspecies correlation (e.g. Dyer et al., 2008; Wu et al., 2016) and from few substances to many substances based on their structural similarities or physicochemical properties, e.g. octanol-water partition coefficient (log *P*) (Netzeva et al. 2008). This simplified approach contrasts with real aquatic systems, characterised by having a mixture of chemicals, many fish species and different environmental conditions. Therefore, all the above possible predictors should be taken into account when predicting the toxicant effect on fish, and they need to be ranked. In this thesis, we will examine the most important predictors and their possible interactive effect on toxicity to fish by accessing widely-used toxicological endpoints, 50% lethal

concentrations (LC_{50}) and no observed effect concentrations (NOEC) using meta-analyses.

2. Arsenic toxicity to fish

Compared to other toxic metals and metalloids, arsenic toxicity has been poorly addressed. Based on ecotoxicology data for the Spanish fish species extracted from the ECOTOX database (http://cfpub.epa.gov/ecotox/), arsenic was the least tested contaminant among the toxic metals and metalloids that are in the toxic pollutants list (http://www2.epa.gov/eg/toxic-and-priority-pollutants-under-clean-water-act) (Fig. 4).

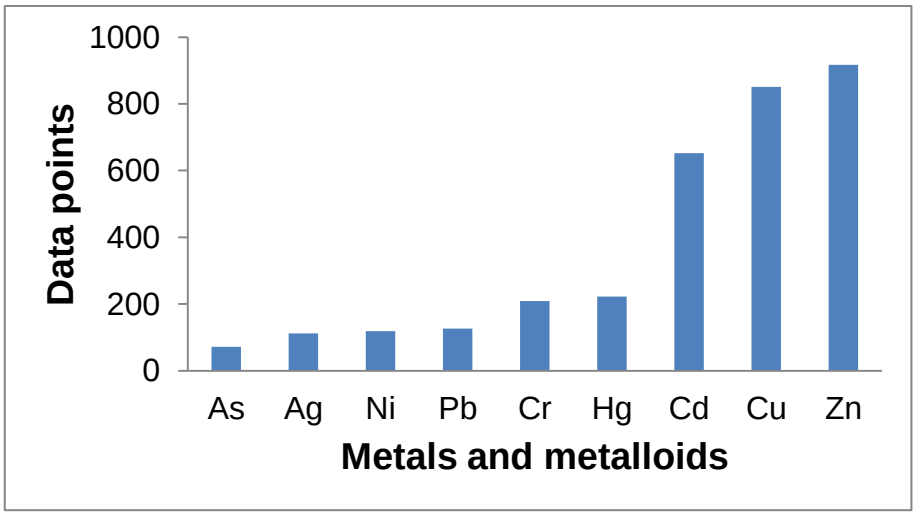

Figure 4. Number of cases of LC_{50} per compound (metals and metalloids) for Spanish freshwater fish listed in the ECOTOX database.

Since arsenic toxicity data was scarce, a literature search in the Web of Knowledge (http://webofknowledge.com) was conducted to gather more information about the arsenic effects on fish (further methods are given in the Supplementary Information).

The meta-analysis results show that fish mortality, growth and arsenic accumulation effects were clear both in acute and chronic exposure to arsenic. Nevertheless, mortality and growth effect tests were conducted with a very high concentration of arsenic (on average around 100 mg/L), whereas arsenic accumulation after chronic exposure was detected at a much lower concentration (Fig. 5).

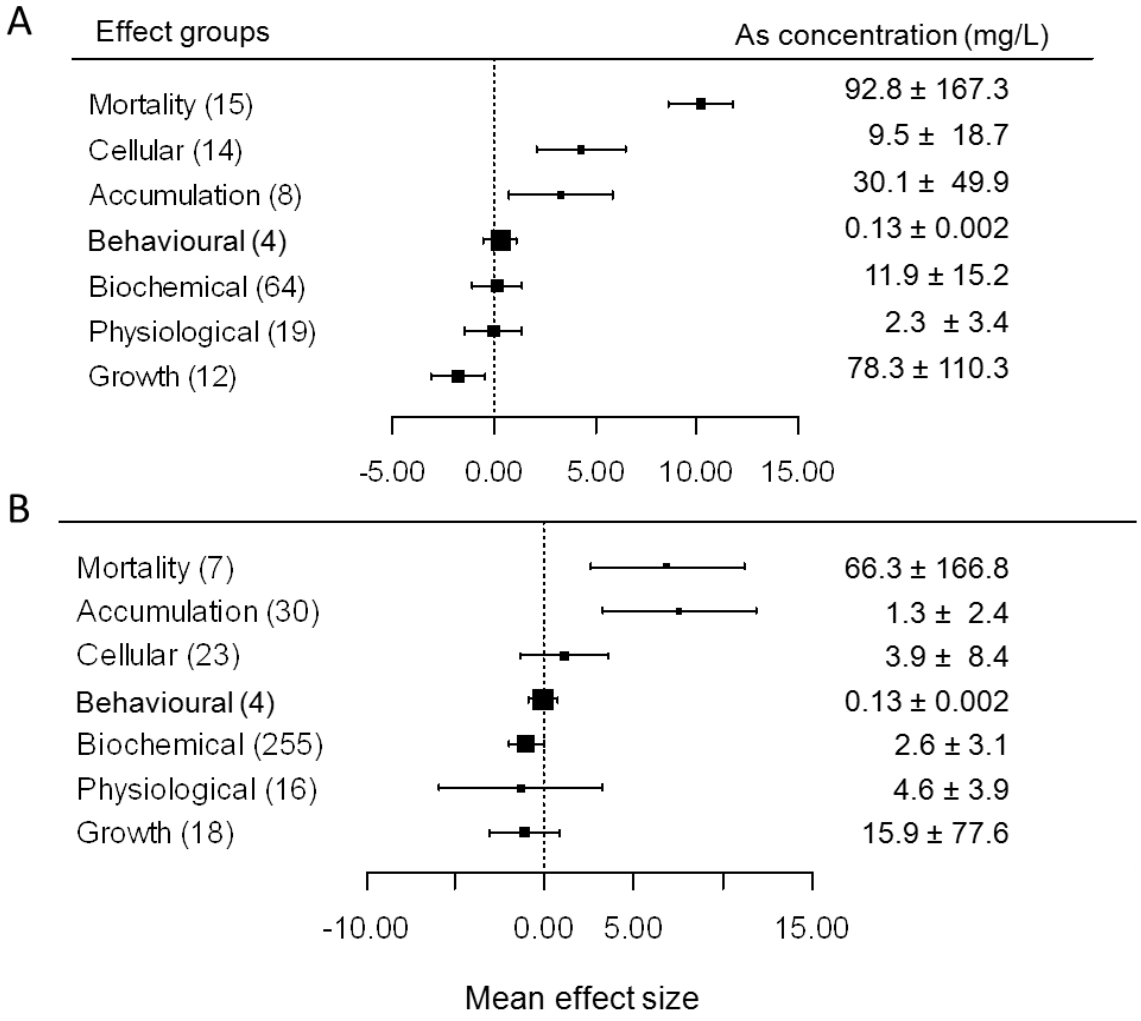

Figure 5. Arsenic effects on main groups of responses of fish during acute (A) and chronic (B) exposure. Sample size for each group is given in parentheses. The bars around the mean denote 95% confidence intervals based on random-effect model. Numbers (right) are mean and standard deviations of tested arsenic concentration (mg/L)

Behavioural, biochemical and physiological effects were evaluated at lower concentrations compared to growth and mortality, but they were not significantly different from control and the effects were heterogeneous. The largest number of data correspond to biochemical responses both in acute and chronic exposures, whereas studies reporting effects on fish behaviour are very scarce.

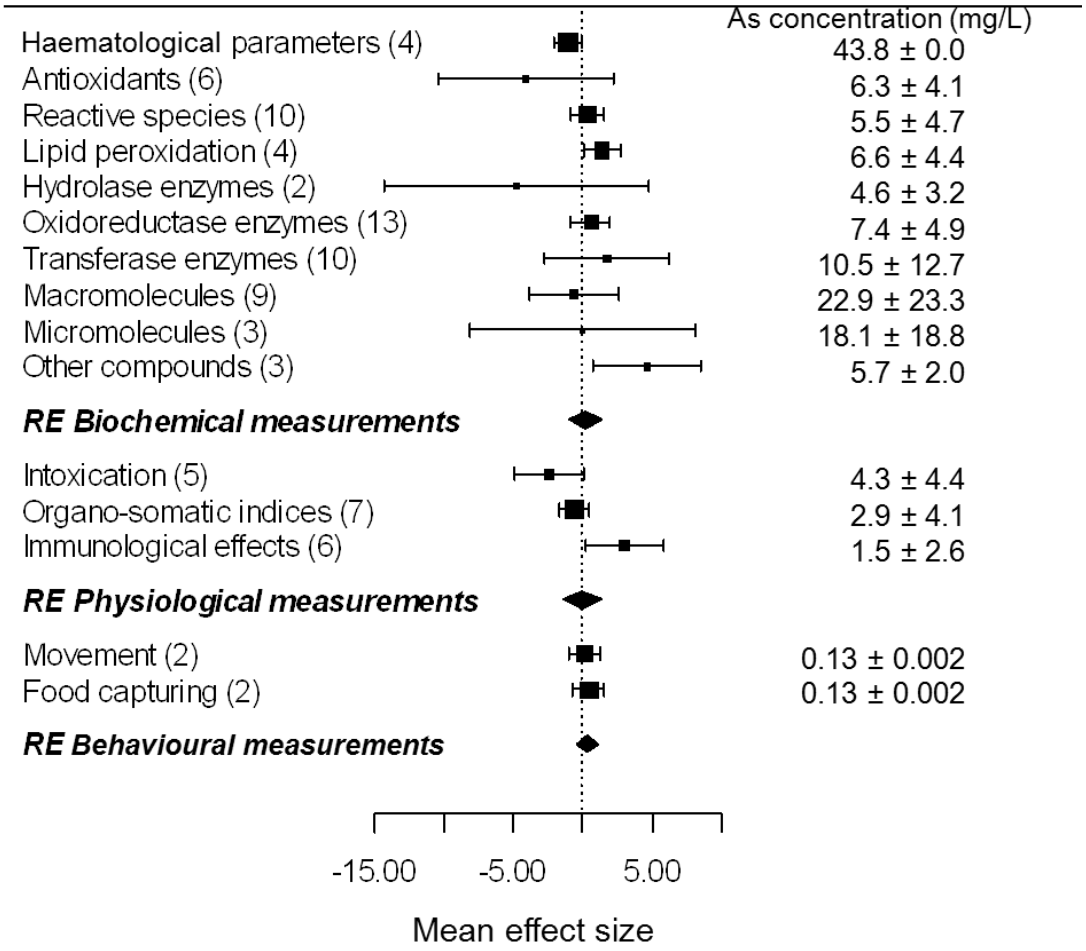

Figure 6. Arsenic acute exposure effects on diverse biological measurements of fish. Sample size for each effect is given in parentheses. The bars around the mean denote 95% confidence intervals based on random-effect model. Numbers (right) are mean ± SD of tested arsenic concentration (mg/L)

Among biochemical variables some responses were negative and some had positive changes under arsenic exposure. For instance, haematological parameters decreased, while lipid peroxidation and some breakdown compounds increased (Fig 6). Oxidoreductase enzyme activities tend to have increased during acute exposures. Other biochemical measurements had heterogeneous responses during acute arsenic exposures (Fig. 7). Among the physiological measurements, intoxication ability always decreased and immunological effects increased. There were only two behavioural measurements; each had two sub-variables and neither of them were significantly different from

controls. In fact, these behavioural measurements were tested with the lowest average concentration compared to the other effects.

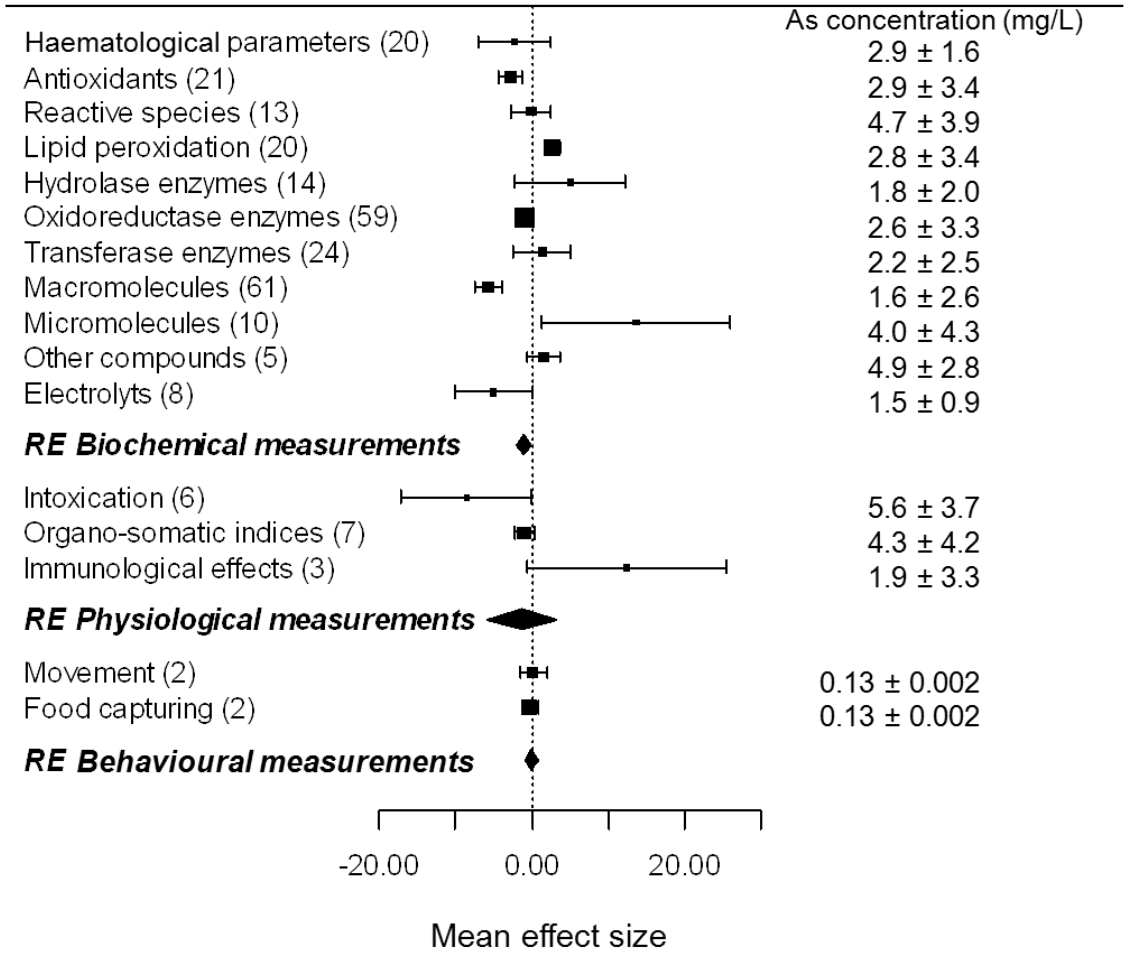

Figure 7. Arsenic chronic exposure effects on several biological measurements of fish. Sample size for each effect are given in parentheses. The bars around the mean denote 95% confidence intervals based on random-effect (RE) model. Numbers (right) are mean ± SD of tested arsenic concentration (mg/L)

During chronic exposures, it seems that antioxidants and oxidoreductase enzyme activities tend to decrease, and lipid peroxidation increases at lower concentrations (on average 6.4 mg/L). A decrease in macromolecules and an increase in micromolecules were detected during long term arsenic exposure, but the tested concentrations were higher (20 mg/L on average) (Fig. 7). Physiological and behavioural measurements have the same response as in acute exposures (Fig. 6 and 7). Although the results of

experimental studies, which have been conducted to evaluate arsenic effect on fish, show that these organisms are affected by arsenic and show several different responses, the average concentrations of arsenic used in the experiments were 1-2 fold higher than environmental contamination of arsenic measured in natural aquatic systems (Rosso et al. 2011; Alonso et al. 2014). Despite being useful for generating guidelines to protect against the mortality of aquatic animals, acute lethality tests ignore impairments in the functioning of the organisms that may occur after much lower toxicant exposures (Scott and Sloman, 2004). Even if animals are not overtly harmed by a contaminant, they may be unable to function in an ecological context if their body cells have a biochemical alteration.

Biotic interactions

Biofilms play a key role in the functioning of aquatic ecosystems. Biofilms can actively influence the sorption, desorption, and decomposition of pollutants. Biologically-caused changes in stream ecosystems influence the kinetics of biogeochemical reactions, such as solubility of minerals, sorption of metals onto particle surfaces, transformations between oxidised and reduced species, and metabolism of aquatic biota (Bourg and Bertin, 1996; Fuller and Davis, 1989). During exposure to toxicants, these are uptaken by the biofilm and microbiological processes are affected, the algae often losing their ability to perform photosynthesis. Since biofilms are the base primary production in rivers, their quality and quantity influences the ecosystem health and fitness of higher trophic level organisms (Figure 8). Finally, biofilms affect bioavailability and hence toxicity to fish, so in fish toxicology it is also important to provide as much ecological realism as possible.

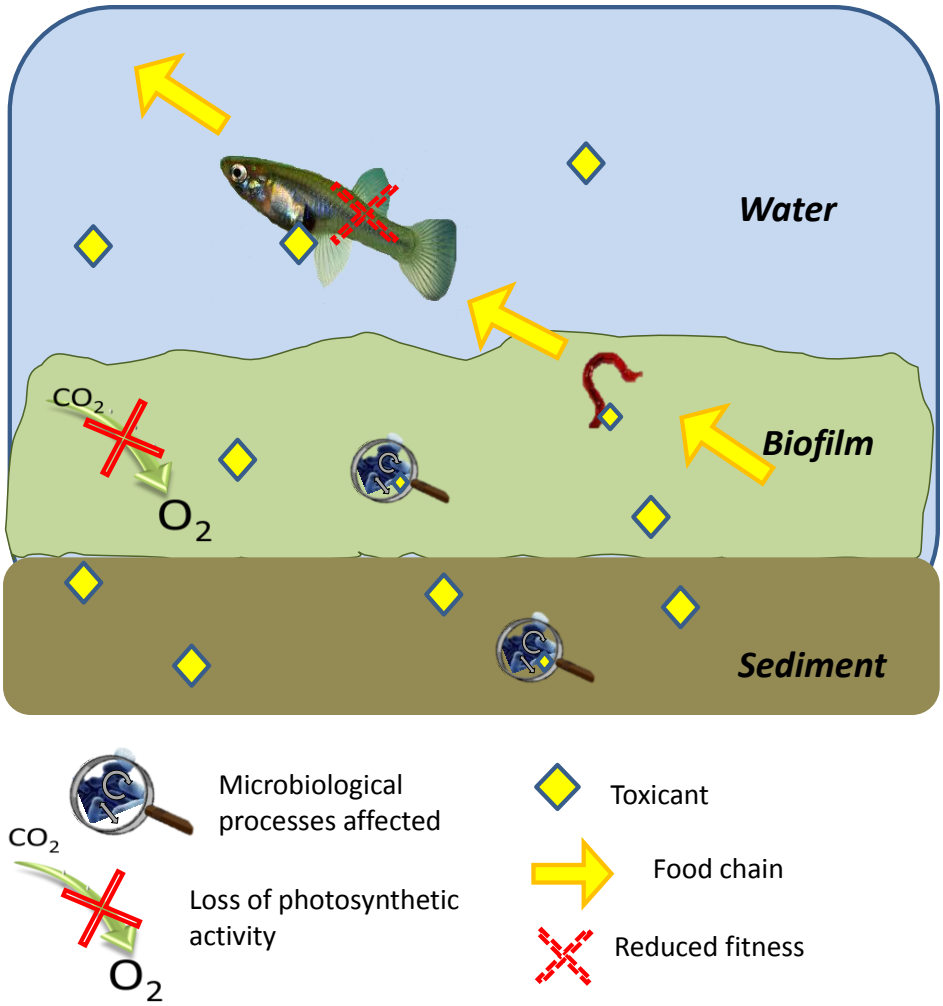

Figure 8. Biological impacts of metals and metalloids (arsenic) on microbial communities and fish.

OBJECTIVES

As mentioned above, a general drawback concerning the risk assessment of chemicals in the environment is the main difficulty for linking toxicity data obtained in the laboratory to the real effects in the ecosystem. Thus, it is very important to provide as much ecological realism as possible in toxicity testing to better understand real toxicity. It is not possible to determine effects of toxicants on a given organism by separating it from its community and ecosystem.

The main aims of this PhD thesis are: i) to evaluate arsenic toxicity in two major, interacting components of the freshwater ecosystem: biofilm and fish; to provide information on environmentally realistic exposures and on biotic interactions that modulate toxicity, and ii) to rank predictors of toxicity to fish and quantify the differences in sensitivity among fish species - a key aspect needed to link chemical pollution data with their real effects on wild fish populations and communities.

HYPOTHESES

We hypothesised:

i) Concerning the fate and toxicity of arsenic to fish: a close interaction between fish and fluvial microbial communities can have a particular influence on toxicity, influencing the biotransformation and bioavailability of both arsenic and nutrients, thus providing clues to better understand arsenic toxicity in fluvial ecosystems.

ii) Concerning toxicant influences on fish: toxic potentials (range of toxicity) of different chemicals for fish should vary not only in relation to their structural classification but also markedly among fish species.

A simplified fluvial system with microbial communities and fish was used to address the interaction between these two ecosystem components for nutrient bioavailability when exposed to arsenic. Finally, we conducted random forest analyses to examine the factors that best predict toxicity in a set of widespread fish, and analyses of covariance to further assess the importance of differential sensitivity among fish species.

RESULTS

Article 1

Arsenic toxicity effects on microbial communities and nutrient cycling in indoor experimental channels mimicking a fluvial system

Aquatic Toxicology (2015) 166: 72-82

Aquatic Toxicology 166 (2015) 72–82

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Arsenic toxicity effects on microbial communities and nutrient cycling in indoor experimental channels mimicking a fluvial system

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ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 18 June 2015
Accepted 8 July 2015
Available online 17 July 2015

Keywords:
biofilm
ecosystem functioning
arsenic
nutrient
fish

ABSTRACT

The toxicity of chemicals in the environment is influenced by many factors, such as the adsorption to mineral particles, active biological surfaces, biotransformation and/or nutrient concentration. In the present study, a simplified fluvial system including fish, periphyton and sediment was used to investigate the fate and effects of environmentally realistic concentration of arsenic (As) on biofilm growth and nutrient cycling. Total dissolved arsenic concentration decreased exponentially from 120 μg/L to 28.0 ± 1.5 μg/L during the experiment (60 days), mostly sinking to the sediment and a smaller percentage accumulated in the periphytic biofilm. Most P and N, which was provided by fish, was also retained in the epipsammic biofilm (growing on sediment grains). We conclude that exposure to this concentration of arsenic under oligotrophic conditions is changing the quality and quantity of the base of the aquatic food chain and its respective contribution to nutrient cycling, and normal functioning of the ecosystem. The effects include lowering the total biomass of biofilm and its potential ability to use organic P (i.e., phosphatase activity), inhibiting algal growth, especially that of diatoms, decreasing nitrogen content, and making the epipsammic biofilm more heterotrophic, thus reducing its ability to oxygenate the aquatic environment.
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1. Introduction

Arsenic (As) in drinking water is a serious public health problem affecting many countries, with millions of people throughout the world being exposed (Mandal and Suzuki, 2002). In addition to naturally occurring high concentrations of As in freshwater (Safiuddin and Karim, 2001; Rodríguez-Lado et al., 2013; Bundschuh et al., 2012; Alonso et al., 2014), several parts of the world have been affected by As. Since it has poisoned soils, sediments and water as a result of past and recent mining activities (Smedley and Kinniburgh, 2002; Wang and Mulligan, 2006; Inam et al., 2011; Battogtokh et al., 2013). While the effect of arsenic on human health has been studied in relation to contaminated groundwater problems, the impacts of this substance on the aquatic ecosystem are little known.

The toxicological effects of arsenic on aquatic organisms have been investigated in the laboratory. However, predicting real effects in the environment is difficult, since toxicity is influenced by many factors such as the adsorption to mineral particles, active biological surfaces, biotransformation and/or nutrient availability (Levy et al., 2005; Rodriguez Castro et al., 2014; Wang et al., 2013). In the present study, a simplified fluvial system, including periphytic biofilm, sediment and fish, was used to investigate the fate and effects of environmentally realistic concentration of arsenic on the functional and structural attributes of biofilms.

Biofilms are communities mainly composed of diatoms and green algae as well as cyanobacteria, bacteria, protozoa and fungi, all embedded in an extracellular matrix (Sabater and Admiraal, 2005). These communities play a pivotal role in the functioning of aquatic ecosystems. They are major sources of primary production, being crucial in the cycling of key nutrients such as phosphorus and nitrogen within freshwaters (McNeely et al., 2007; Romaní et al., 2004; Lear et al., 2012). Biofilms can be used as warning systems for the detection of the effects of toxicants on aquatic systems due to the sensitivity and integration of a large diversity of physiological responses of the species constituting the biofilm (Sabater et al., 2007; Lear et al., 2012; Burns and Ryder, 2001). Several studies have highlighted biofilm sensitivity to a large panel of toxicants, such as metals (e.g., Serra et al., 2010; Corcoll et al., 2011; Bonet et al., 2012), herbicides (Guasch et al., 2003; Pesce et al., 2008), and pharmaceuticals (Proia et al., 2011, 2013; Corcoll et al., 2014). The effects of arsenic on biofilm communities and the role of biofilm in the adsorption, uptake and/or transformation of arsenic have been recently investigated (Rodriguez Castro et al., 2014).

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http://dx.doi.org/10.1016/j.aquatox.2015.07.005
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In fluvial ecosystems, biofilm communities developing on sediments may also be affected by As, but this has been much less studied than periphyton. The biofilm developing on sediment plays a major role in organic matter degradation, the biofilm structure being less complex and its bacteria being more exposed to changes in the flowing water than in the usually thicker periphytic biofilm (Romaní and Sabater, 2001). At the same time, sediments are expected to play an important role in arsenic fate. Sediments quickly remove As from streams containing high As inputs (Prieto et al., 2013; Woolson, 1977). Adsorption of dissolved arsenic onto particulate phases has been actively studied and reviewed (Sharma and Sohn, 2009). The process mostly explained relating solid (intrinsic) sediment surface area and concentration of the solution (Goldberg et al., 2001) and bonding on mineral surfaces leading to a quick removal of As from streams (Woolson, 1977). Furthermore, in aquatic systems trace elements may bind to dead plankton, particles of decaying plant material or faecal pellets of animals and humic particles, and all these tend to sink to the bottom, and this mechanism helps to remove trace elements from the water column (Belzile and Morris, 1995). Aquatic sediments are also a crucial site for phosphorus retention and cycling, which might also affect As toxicity.

Nutrient availability has a strong influence on arsenic toxicity to freshwater algae. For example, Levy et al., (2005) showed that a 10-fold increase in phosphate concentration (0.15-1.5 mg) caused an 18 times decrease in As toxicity (i.e 72-h IC50 value for *Monoraphidium arcuatum* increased from 0.25 mg As(V)/L to 4.5 mg As(V)/L). Similarly, in the experiments performed by Wang et al., (2013), As toxicity to algae was reduced about 2000 times with phosphorus (EC50s were 33,502.7 μgAs/L and 14.1 μgAs/L for +P and −P, respectively). Results from Rodriguez Castro et al. (2014) showed that chronic exposure to 130 μgAs/L inhibited algal growth up to 61% in P-starved conditions, but not when P-availability was higher, whereas P-uptake capacity was already affected in P-starved communities at the lowest tested concentration (15 μgAs/L).

Since the effects of As on organisms increase under low phosphorus availability, oligotrophic streams might be highly sensitive to As. In these systems, nutrient content fluctuations are highly affected by the presence of fish which are expected to drive the biogeochemical cycles (McIntyre et al., 2008), thereby possibly affecting arsenic toxicity to freshwater algae indirectly. The importance of fish in nutrient cycling was recently reviewed, highlighting the ability of fish to recycle nutrients within a habitat, or translocate nutrients across habitats or zones (McIntyre et al., 2008; Vanni, 2002). The presence of fish can contribute to inorganic phosphorus availability in water as observed in a previous experiment where phosphate concentration significantly increased (from 3 to 15 μgP/l) after the addition of fish (Magellan et al., 2014; Barral-Fraga et al., 2015).

This study aimed to evaluate the fate and effects of a 60-day exposure to environmentally realistic concentration of arsenic on functional and structural attributes of biofilm in a simplified fluvial system including the interaction between fish, periphytic and epipsammic biofilm and sediments. Effects of arsenic on fish were also evaluated and will be detailed in another paper.

The fate of arsenic in the three different compartments (periphytic and epipsammic biofilm and fish) was quantified and a set of biofilm endpoints related to their structural and functional attributes (community composition, biomass growth and microbial extracellular enzyme activity) in control and arsenic treatments were examined in biofilm growing on sediments (epipsammon) and those growing on top of the illuminated substrata of the channel (periphyton). We hypothesised that the interaction between fish, sediment and fluvial microbial communities in terms of toxicant and nutrient inputs and retention can modify As toxicity in each compartment.

2. Materials and methods

2.1. Experimental setup

Six experimental units (eu), three as controls and three for arsenic exposure were used. Each one consisted of a one-metre long channel for biofilm growth (mimicking a flowing system), and a ninety-litre aquarium (mimicking a pool) for sediment and fish exposure with a pH control system (JBL Proflora m603). Each channel and aquarium were connected for water circulation with an eight-litre aquarium between them (Fig. 1). The channel surfaces were covered with small (1.4 cm^2) and larger (14 cm^2) sand-blasted glass substrata placed at the bottom of each channel (covering the whole bottom) to allow the growth of periphytic biofilm, and the bottom of the big aquarium was filled with 10 cm of coarse grain sediment (gravel) to allow the growth of epipsammic biofilm. The sediment was purchased from the "Center Verd" gardening center in Girona, Spain, and its grain size composition was 15% of 4.5–9.5 mm, and 85% of 2–4.5 mm in diameter size. Water flowed constantly at a controlled flow rate (2.9 ± 0.2 l/min) and was recirculated using a hose and a submersible pump (EDEN Typ: T0; series 107; 400 l/h; 230 V approx.; 50 Hz; 5 W; 0.8H max m.) from the big aquarium. Water in the big aquarium was also circulated through the sediment by another submergible pump (EHEIM Typ: 1048 21 9; series 12,084A; 10 l/min.; 230 V approx.; 50 Hz; 10 W; 1.5H max m.) placed below the sediment to simulate hyporheic water fluxes.

All experimental units were placed in a room under controlled temperature (20 °C), light irradiance from LEDs Grow Light (Voltage: 220Vac/50 Hz; power: 120 W; Ip50) with a darkness/light cycle of 12 h/12 h. Light measurements were done in three points (both ends and middle) above each channel and adjusted to 140 ± 9.8 μmol photons m^{-2} s^{-1}. Every pH control system had an automatically established pH range (7.6–7.65). Control of pH is crucial not only for biofilm and fish life but also for the solubility and speciation of metals.

Colonisation of periphytic and epipsammic biofilm lasted for 21 days without arsenic. Colonisation inocula were collected by scraping the surface of randomly chosen rocks from a pristine stream (Llémena River, NE Spain), and the scraped biofilm added to each experimental unit twice a week during three weeks as described in Serra (2009). Phosphate solution (10 μg/L) was also added twice a week as a nutrient supply for algal growth.

Arsenic was added on day 22 and the fish were placed in the aquaria 3 days later (colonisation day 25). Arsenic (Sodium(meta) arsenate or sodium dioxoarsenate – $NaAsO_2$ – molecular weight: 129.91 g/mol) solution was added to the big aquarium to reach 120 μg/L, within the range recently noted in a large set of streams and rivers (Rosso et al., 2011; Alonso et al., 2014).

The fish used in this experiment were eastern mosquitofish (*Gambusia holbrooki*). Eight fish (29.4 ± 7.6 mm) were added to each aquarium (total weight per aquarium 2.52 ± 0.33 g) and they were fed every day with commercial, frozen bloodworms (*Chironomus* spp.) as described by Magellan et al. (2014). The experiment was ended after 82 days of biofilm growth so biofilms were exposed to As(V) for 60 days, and fish exposure lasted for 56 days. Water was completely renewed the day before arsenic addition in order to have exactly the same nutrient conditions for all experimental units. After arsenic addition, 25% of water was renewed at days 33 and 49 and their corresponding concentration of arsenic added. Water lost due to evaporation was refilled whenever necessary.

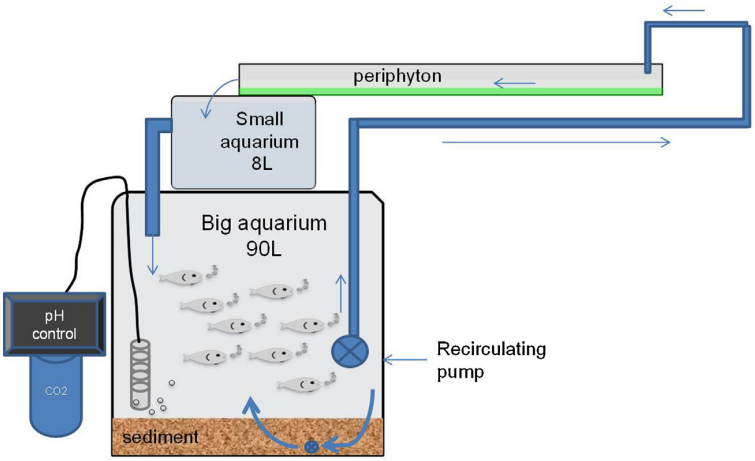

Fig. 1. Schematic diagram of the experimental settings (see main text for details).

2.2. Physicochemical parameter measurements

Physical and chemical parameters (water temperature, dissolved oxygen, pH and conductivity) were measured with appropriate probes (HQPortable Meters, HQ40d18, HACH Company) each week during the whole experimental period (82 days). Phosphorus (soluble reactive) [P(i)] was determined by a modified molybdenum blue method (Carvalho et al., 1998). Ammonium was measured following Reardon et al., (1966). Nitrate analysis was done with a minispectrometer measuring formation of a redviolet azo dye with N-(1-naphthyl) ethylene diammonium dichloride (AQUANAL@-plus Nitrate kit).

2.3. Arsenic analyses

Water samples for total As analyses were taken every week and were prepared using 5 mL of unfiltered water and acidified immediately with 1% of HNO_3 (from 65% suprapure, Merck). Total As analyses were done with inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies) in the Technical Research Services (STR) of the University of Girona (http://www.udg.edu/serveis/STR).

At the end of the experiment, As in periphytic and epipsammic biofilms and fish was also measured. Three replicates of periphytic biofilm samples from each channel were collected scraping larger (14 cm^2) sand-blasted glass substrata. For epipsammic biofilms, sediment (sand) was collected in triplicate from the big aquarium by an uncapped syringe from the first cm in depth (final sediment volume of 1 cm^3). The samples were then stored in glass vials. Fish were sampled from each aquarium five times at fixed days: two fish at day 4, 25 and 57 and one at day 9 and 41. The samples were lyophilised, homogenised, weighed and digested with 4 mL of HNO_3 (from 65% suprapure, Merck) and 1 mL of H_2O_2 (33% suprapure Merck) in high performance microwave (Milestone, Ethos Sel). After digestion, samples were diluted to 10 mL with Milli-Q water and weighed. Total As analyses were done with inductively coupled plasma mass spectroscopy following the same procedure used for water samples.

2.4. Nutrient cycling and stoichiometry

Nutrient cycling (P and N) from fish and nutrient stoichiometry (C, N, P content and molar ratios) for periphyton, sediment, fish and water were measured. N and P input into the experimental units from the fish (excreted plus egested) was calculated based on the following mass balance equation, developed by Schindler and Eby (1997). An example for phosphorus is given:

$$P_{ex} + P_{eg} = P_{cons} - P_{gr} \quad (1)$$

where

P_{ex}—excreted phosphorus
P_{eg}—egested phosphorus
P_{cons}—consumed phosphorus with food
P_{gr}—phosphorus allocated to fish growth

Nutrient allocation to fish growth was calculated from fish growth. We measured every fish weight 2 times, when placed in the system and when taken for As accumulation analysis, and fish growth was calculated as the mass difference between those 2 measurements. The weight gained by each specific fish per day was calculated by dividing total weight growth by the number of days that the fish was in the experiment. Nutrient consumption with food was calculated by knowing the amount of eaten food and food composition. The fish were fed every day with bloodworms, added to the aquaria one by one (after the first one was consumed, the next one was given) and the total weights of given food were measured daily.

The sediment samples were taken using the method described in the arsenic analysis section. Samples were stored in glass vials and were sonicated for 15 min in 10 mL Milli-Q-filtered water to achieve complete detachment of the epipsammic biofilm. Sediment extract suspensions, periphytic biofilm extracts, fish and fish food were dried (80 °C) and weighed for the elemental analysis. Carbon and nitrogen was measured using an elemental analyser (PerkinElmer 2400) in the Technical Research Services of the University of Girona (http://www.udg.edu/serveis/STR). Total phosphorus was measured after digestion using oxidation reagent at high pressure. After digestion, total dissolved phosphorus was quantified using the method detailed in the protocol of soluble reactive phosphorus. Quantification of the C, N and P contents of the different compartments (periphytic biofilm, epipsammic biofilm, fish and water) were calculated based on their dry mass and elemental content.

2.5. Response of microbial communities to As exposure

2.5.1. Chlorophyll-a fluorescence measurements

Photosynthetic efficiency, photosynthetic capacity and minimum fluorescence yield were measured with the PhytoPAM (Pulse Amplitude Modulated) fluorimeter (HEINZ WALZ, Effeltrich,

Germany), using "PhytoWin EDF" software. Measurements were done on periphyton by collecting five small glass substrata from each channel every 3 days during the whole experimental period. The PHYTO-PAM employs light-emitting-diodes (LED) to excite chlorophyll fluorescence light pulses at four different wavelengths (470, 535, 620 and 650 nm), which can be used, respectively, to gain information on the relative abundance of differently pigmented organisms, such as, for example, green algae (green), diatoms (brown) and cyanobacteria (blue) (Kuhl and Jorgensen, 1992).

Fluorescence measurements under light conditions were carried out with actinic light provided by the instrument. One saturation pulse was applied and the effective PSII quantum yield (Photosynthetic efficiency, Y_{eff}) was obtained. Effective PSII quantum yield is defined as a measure of the photosynthetic efficiency of the community (Schreiber, 2004). Glass substrata were then placed in the dark for one hour. A saturation pulse was applied and the minimal fluorescence yield was obtained. The minimal fluorescence yield of a dark-adapted cell (F_0) is proportional to its chlorophyll a concentration (Rysgaard et al., 2001). The maximal PSII quantum yield (Y_{max}) was also obtained in the saturation pulse performed under dark conditions. This parameter is defined as a measure of the photosynthetic capacity of the community (Corcoll, 2012).

2.5.2. Total biomass, chlorophyll-a, live dead bacteria and autotrophic index

Biofilm biomass was measured both for periphyton and epipsammon at the end of the experiment, on triplicate samples taken following the same procedure used for As accumulation measurements. Total biomass content was measured by calculating the ash-free dry weight (AFDW). Biofilm samples were dried at 105 °C for 24 h in order to calculate dry matter. Afterwards the samples were combusted in an oven at 450 °C (Obersal MOD, MF12-124, Spain) for 4 h and were then weighed again to calculate the mineral matter content. The AFDW was calculated subtracting the mineral matter from the total dry matter. Results were expressed in mg/cm^2 for periphytic biofilm and in mg/cm^3 for epipsammic biofilm samples.

Chlorophyll-a was measured using the Jeffrey and Humphrey (1975) spectrophotometric method. In brief, 5 small glasses were taken from each channel, placed into glass vials separately and 5 mL 90% of acetone were added to each. After 2 min of sonication, samples were left for 24 h in refrigeration in darkness for chlorophyll extraction. Before filtering, samples were sonicated again for 2 min. The absorbance of filtrated liquid was read at 430 nm (peak of carotenoids and degraded chlorophyll), 665 nm (peak of chl-*a*) and 750 nm (turbidity) using 1 cm cuvette. The autotrophic index (AI) was calculated as the ratio of AFDW to chlorophyll-a (Grossey and La Point, 1988).

Bacteria were counted separately as active and dead cells in the biofilm community using the SYTO 9 dye and the propidium iodide stain. These stains differ in their ability to penetrate the intact membrane of bacterial cells. When used alone, the Syto-9 stain labels both alive and dead bacteria. In contrast, propidium iodide only penetrates bacteria with damaged membranes, reducing Syto-9 fluorescence when both dyes are present (Berney et al., 2007). Ten mL of autoclaved Milli-Q water were added to triplicate samples of both periphytic and epipsammic biofilms and sonicated for 2 min to detach bacteria from the substrates. The suspension was diluted 10 times (0.2 mL sample 1.8 mL water) and 3 μL of the staining solution were added. After incubation for 30 min, the stained samples were filtered through black polycarbonate filters (0.2 μm, Whatman). Filters were mounted with one drop of immersion oil on grease-free slides, covered with cover slips, and bacterial cells were counted using a fluorescence microscope (Eclipse E600, Nikon, Japan). Twenty fields were counted for each filter.

2.5.3. Extracellular enzyme activities: β-glucosidase and phosphatase

The extracellular enzyme activities of β-D-1,4-glucosidase (EC 3.2.1.21) and alkaline phosphatase (EC 3.1.3.1) in the biofilm were determined spectrofluorometrically using fluorescent methylumbelliferone (MUF; from Sigma–Aldrich). Three small glasses colonised with periphyton and 1 cm depth of triplicated epipsammon samples were collected from each experimental unit and 4 mL of filter-sterilised water from each experimental unit was added to the respective samples. Samples were incubated with MUF-β-D-glucoside for β-glucosidase and with MUF-phosphate for phosphatase activity at a final concentration of 0.3 mM, during one hour in the dark, and in a shaker at 20 °C.

At the end of the incubation, glycine buffer (1/2, v/v, buffer/sample) (pH = 10.4) was added to the samples, blanks and standards of MUF. Measurement of the fluorescence in a fluorimeter at 365/455 nm excitation/emission was carried out (Tecan, infinite M200 Pro 4). The results were expressed in nmol MUF released from each cm^2 biofilm and/or sediment surface area and also in nmol MUF released per mg ash-free dry weight (nmol MUF g AFDW^{-1} h^{-1}).

2.6. Statistical analyses

The physical and chemical parameters (temperature, pH, dissolved oxygen, conductivity, ammonium, phosphate and nitrate) were analysed using one-way repeated measures ANOVA to test for differences between control and As treatments during the experiment. Total As exposure was determined integrating the curve of As concentration measured weekly and divided by the exposure time to calculate average exposure concentration per day (μg As L^{-1} day^{-1}). Photosynthetic fluorescence parameters were compared using linear models with R environment (2008) considering treatments as a factorial variable and exposure time as a numerical variable, with curve fitting. The linear models were carried out with the data from datasets that followed significant trends. Non-repeated measurements (which were measured at the end of the experiments) in control and As treatments were compared by *T* test (independent samples). ANOVA and *T* test performed with SPSS software (IBM SPSS statistic 20).

3. Results

3.1. Physicochemical parameters

Physicochemical parameters in water were similar in the six experimental units at the beginning of the experiment and changed during biofilm colonisation. Water temperature (20.9 ± 0.2 °C in average) increased slightly at the end of the experiment but was not significantly different between treatments. Water pH was not significantly different between treatments, but there were marginal interactive effects of As × day. Even though we tried to maintain the pH during the experiment by adding CO_2, it was slightly higher in the control than in As treatment (8.0 ± 0.1 for control and 7.9 ± 0.1 for As treatment). After adding the fish, dissolved oxygen decreased in As treatment and remained slightly lower than in controls until the end of the experiment. Conductivity was also higher in the As treatment at the end of the experiment (Supplementary (S) Table 1). Phosphate concentration in water was always low. There was a marked reduction of nitrate concentration over time, which was more pronounced with As, while ammonium concentration was not significantly different among treatments (S. Table 1).

Table 1
Arsenic fate in the compartments of the experimental system. Arsenic acumulation per dry and wet weight (in μg/kg DW and μg/kg WW, respectively) and total accumulation in each substrate (in μg and %) in an experimental unit.

Compartment	As per DW (μg/kg)	As per WW (μg/kg)	Total As (μg)	Total As (%)
Water		28.0 ± 0.5	2286 ± 96	19.5 ± 0.8
Periphyton	15964 ± 4837	1756 ± 532	37.3 ± 22.0	0.32 ± 0.19
Epipsammon	626.8 ± 150.7	539.1 ± 129.0	9328 ± 2300	79.7 ± 19.5
Fish	46.5 ± 45.5	10.6 ± 10.3	0.12 ± 0.06	0.0009
Total			11700	100

Total dissolved arsenic concentration decreased exponentially, from 120 μg/L (initial concentration added to the aquaria), down to 28.6 ± 1.19 μg/L after 60 days (S. Fig. 1). The As mostly sank to the sediment due to its large total volume (8727 ± 570 cm^3), a lower fraction was taken up by and/or adsorbed to the periphytic biofilm (with surface 648 cm^2), and a very small proportion was found in fish tissues (Table 1). On the other hand, As concentration was higher in the periphytic biofilms, followed by sediment and fish (Table 1).

3.2. Nutrient cycling and stoichiometry in control and arsenic treatments

P content (% of total dry weight) of eastern mosquitofish and their food (bloodworms) were 1.18 ± 0.07% and 0.83 ± 0.07% (respectively) while N content was 8.81 ± 0.29%, and 9.64 ± 0.01%, respectively. Using the mass equation for calculation, it resulted in 8.97 ± 0.9 mg of P and 111.2 ± 10.9 mg of N added per fish to the 90 L aquarium after 56 days, or 12.5 μgP/L and 154.4 μgN/L per week. Excreted + egested P was slightly different between treatments; 1.86 ± 0.08 μgP/hour and 1.62 ± 0.08 μg P/h for control and As treatment, respectively. Thus, the overall P contribution of fish to the experiment was lowered with As (Table 2). Even though fish made a significant contribution to the phosphorus input to the aquaria, dissolved phosphate concentration remained low, indicating that most phosphorus input was taken up and retained as biofilm growth. Total P content in both periphytic and epipsammic biofilm was also smaller in the As than in the control treatment (Table 2).

While there was no significant difference for fish N excretion between the control and As treatments (Table 2), total N in periphytic biofilm was significantly decreased with As, indicating that arsenic had also influenced biofilm N content. Total carbon, nitrogen and phosphorus contents and the elemental ratios were similar in biofilms exposed to As and those non-exposed (Table 3), but C, N, P contents were up to 30% lower with As both in periphyton and epipsammon. In periphyton, C:P was 3.5 times and N:P was 1.9 times higher than in epipsammon, showing that the former had been more P-starved.

3.3. Effects of arsenic on periphyton growth and photosynthesis

Periphytic biofilm successfully colonised on illuminated glass substrata. Just before adding As, the minimal fluorescence (F_0) for each treatment was 48 ± 15.4 and 41.3 ± 9.1 for control and As treatment channels respectively, and photosynthetic activity was slightly low (Y_{eff} 0.26 ± 0.03 and Y_{max} 0.47 ± 4.5), without significant difference between treatments. F_0 of diatoms was 76–77% and the fluorescence of blue green and green algae had a smaller percentage.

The minimal fluorescence yield (F_0) increased with time, and overall tendency was a bit lower in the presence of As (S. Fig. 2A, $p = 0.09$). The maximal PSII quantum yield (Y_{max}) and the effective PSII quantum yield (Y_{eff}) increased with time until the saturation points and gradually decreased with no differences between treatments (S. Fig. 2B and C) indicating that temporal patterns of photosynthetic activity for overall community were not affected by arsenic.

The minimal fluorescence yield of blue greens(Fig. 2A) had a similar tendency as overall F_0, whereas that of green algae (Fig. 2B) was higher in As treatment after its exposure and tends to have linear increase. For diatoms (Fig. 2C), the minimal fluorescence was significantly lower in As treatment than in controls ($p < 0.001$) during the whole experimental period without interaction effect As and time ($p = 0.995$, S. Table 2). The photosynthetic efficiency of cyanobacteria (Y_{eff} blue, Fig. 2D) and diatoms (Y_{eff} brown, Fig. 2F) rapidly decreased after adding As to the treatments and then increased fast, indicating acute toxicity effect of As on biofilm (Fig. 2D and F), but not showing common tendency through time. The photosynthetic efficiency was similar between the treatments for cyanobacteria ($p = 0.835$), and green algae ($p = 0.361$), but the efficiency of diatoms was significantly higher in As treatments than in control (Fig. 2F, $p < 0.001$).

Considering the relative fluorescence of these groups of algae and cyanobacteria, the proportion assigned to diatoms decreased significantly over time (Fig. 2I), whereas the percentage assigned to green algae increased with time (S.Table 2, Fig. 2H). The decline of diatoms and increase in green algae were marked in As treatments. The blue–green fraction increased significantly with time in both treatments (Fig. 2G).

Chlorophyll-a of periphyton was 1.77 ± 0.72 μg/cm^2 for controls, and 1.45 ± 0.48 μg/cm^2 for As treatments. Biofilms also colonised the surface of the sediment grains. Therefore, epipsammon developed in the sediment placed in the bottom of the big aquarium. The chlorophyll-a of the epipsammon was significantly lower in the presence of As than in controls (Fig. 3, A); 3.3 ± 0.45 μg/cm^3 and 1.1 ± 0.57 μg/cm^3 for control and As treatments, respectively. Ash free dry weight (AFDW), used as a surrogate of total biomass, was lower in periphytic biofilm under As influence than in controls ($p = 0.007$), while this parameter in epipsammic biofilm was not much affected ($p = 0.63$). It was 48% lower in periphytic biofilm and 8% lower in the epipsammic biofilms with As, compared to controls (Fig. 3B). In contrast, the autotrophic index in periphytic biofilm was not different between treatments ($p = 0.56$, 1330 ± 368 in control, 1062 ± 654 in As), but in the epipsammic biofilm the index was significantly higher in As treatment ($p = 0.007$, 1300 ± 168 in control, 3606 ± 761 in As) (Fig. 3C), showing that As was making this biofilm more heterotrophic.

3.4. Bacterial abundance and L/D ratio

Total bacterial density was higher in epipsammic (37.6 ± 24.2 × 10^6 cell/cm^2 in control, 65.6 ± 32 × 10^6 cell/cm^2 in As), than in periphytic biofilm (15.2 ± 2.9 × 10^6 cell/cm^2 in control, 12.6 ± 6.1 × 10^6 cell/cm^2 in As), but the presence of As had no effect on total number of bacteria. Even though the ratio of live to dead bacteria (L/D) in periphyton was not affected by As, effects of arsenic were much higher in the epipsammon, causing a high bacterial mortality (L/D 0.05 ± 0.02 compared to 0.16 ± 0.05 in the controls, Fig. 3D).

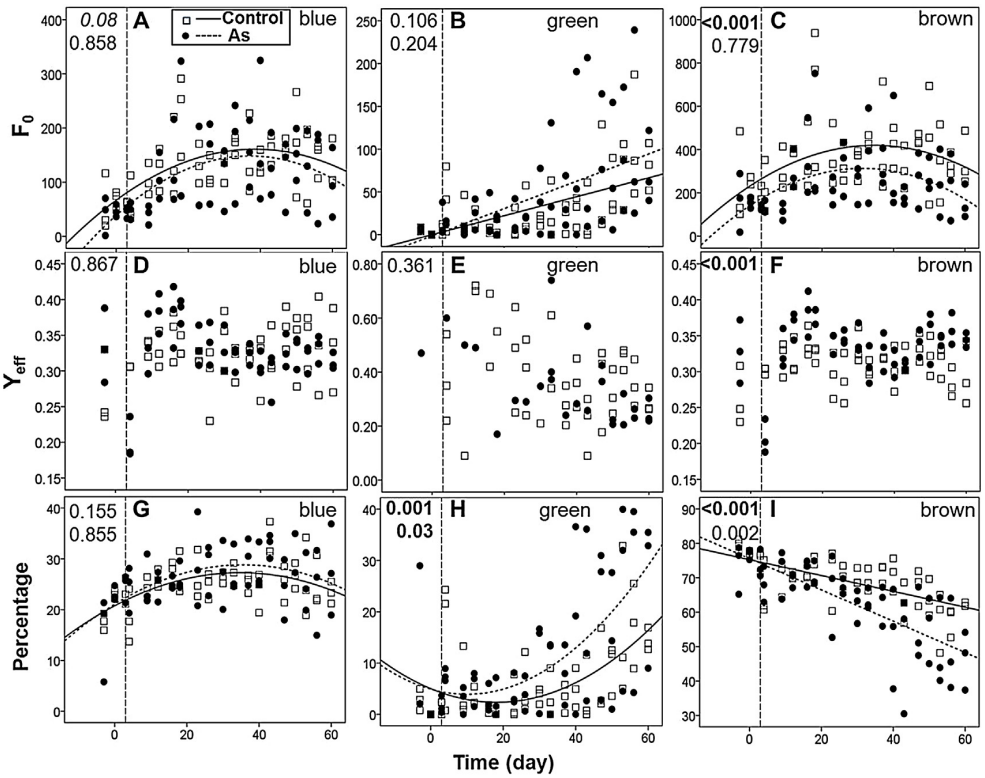

Fig. 2. The minimal fluorescence yield (F_0, A–C), the effective PSII quantum yield (Y_{eff}, D–F), and percentage of the algal groups (G–I) in control and arsenic treatments over time. The open squares and solid line stand for control, black circles and pecked lines for arsenic treatment and the lines correspond to linear and quadratic regressions. The vertical line indicates the day when fish were added to the treatments. The values at the top left denote the *p* values based on general linear model tests between control and arsenic treatments: As as factor (top) and arsenic × day as factor (bottom). For Y_{eff} of all groups, the number indicates the main effect of Arsenic.

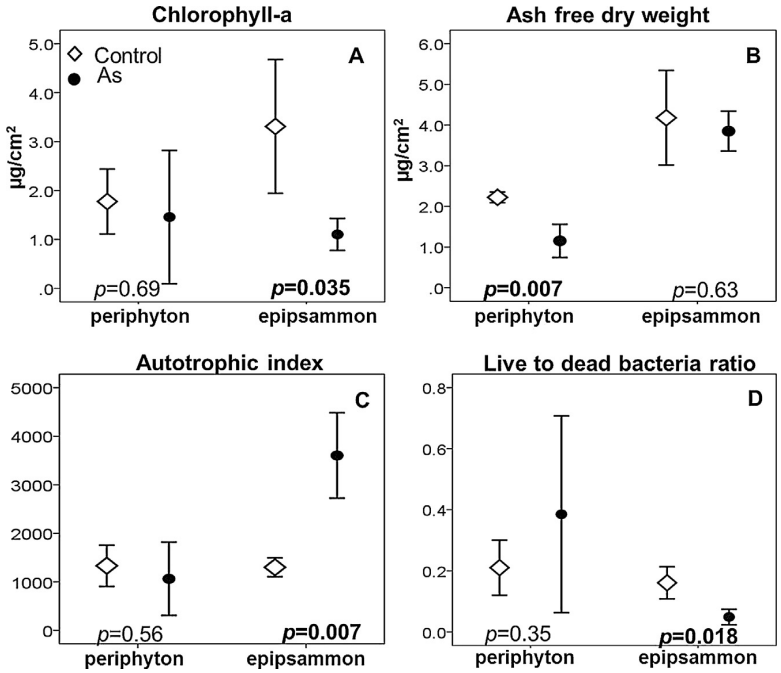

Fig. 3. Average values and standard errors of Chlorophyll a (A), ash-free dry weight (B), autotrophic index (C) and ratio of live/dead bacteria (D) in periphytic and epipsammic biofilm, as measured at the end of the experiment. Statistical significance (*p* values) of *T* test are in the bottom of each compared pair (control and As treatment). When $p < 0.05$ was highlighted in bold.

Table 2
Excreted or adsorbed N and P in the system compartments calculated at the end of the experiment. Numbers in italics when significance value $p < 0.1$, and in bold when $p < 0.05$ based on T test (4 degrees of freedom).

Calculated total amount (in an experimental unit)	C	As	p
Excreted + Egested P by fish (mg in 56 days per aquarium)	9.57 ± 0.29	8.36 ± 0.87	*0.079*
P in periphyton (mg)	1.78 ± 0.9	0. 61 ± 0.19	*0.092*
P in epipsammon (mg)	3.7 ± 1.1	2.3 ± 0.26	*0.094*
Total P in water (90L)	0.23 ± 0.05	0.23 ± 0.05	1.000
Excreted + Egested N by fish (mg in 56 days per aquarium)	118.5 ± 4.9	103.9 ± 10.8	0.1
N in whole periphyton (mg)	31.3 ± 8.2	13.6 ± 4.0	**0.028**
N in epipsammon (mg)	50.9 ± 24.6	31.0 ± 9.3	0.262
Total N in water (90L)	8.37 ± 2.5	4.0 ± 4.0	0.191

Table 3
The average values and standard deviations for carbon, nitrogen and phosphorus contents and their molar ratios in periphytic and epipsammic biofilms at the end of the experiment.

Parameters	Periphytic biofilm			Epipsammic biofilm		
	Control mean ± SD	As mean ± SD	p	Control mean ± SD	As mean ± SD	p
Carbon (%)	23.4 ± 2.6	19.7 ± 2.5	0.15	15.2 ± 5.5	11.6 ± 1.8	0.33
Nitrogen (%)	0.72 ± 0.16	0.64 ± 0.15	0.58	0.99 ± 0.35	0.68 ± 0.17	0.24
Phosphorus (%)	0.04 ± 0.02	0.028 ± 0.01	0.32	0.07 ± 0.02	0.06 ± 0.01	0.45
C:N	39.0 ± 5.3	36.6 ± 4.3	0.57	17.8 ± 0.7	20.0 ± 2.1	0.16
C:P	1650 ± 501	1812 ± 99	0.61	558 ± 47	523 ± 44	0.40
N:P	41.8 ± 9.3	49.7 ± 3.4	0.24	31.4 ± 3.3	26.2 ± 2.7	0.10

3.5. Extracellular enzyme activities

Phosphatase activity per surface area was significantly lower in As treatments than in controls both in periphyton and epipsammon (Fig. 4A), following a similar pattern to that observed for biofilm biomass (Fig. 3B), but with less activity in the epipsammic biofilm. Phosphatase activity per unit of biomass (AFDW) was similar between treatments, but slightly lower in the epipsammon with As (Fig. 4B), indicating that the remaining biomass was slightly less efficient. Arsenic did not affect the β-glucosidase activity per area (Fig. 4C) but it caused a clear increase in the activity per unit of biomass in periphyton (Fig. 4D).

4. Discussion

Recently it has been shown that environmentally realistic concentrations of arsenic may have toxic effects on biofilms (Rodriguez Castro et al., 2014). Our study system not only shows toxicity on autotrophic and heterotrophic components of aquatic biofilms but further indicates a differential effect on periphyton and on epipsammon. The latter is modulated by nutrient cycling in fish and the accumulating arsenic and nutrients in sediments.

Our study system simulated a nutrient-poor fluvial system where nutrient cycling and arsenic fate are mainly driven by fish and sediments. The mass balance equation reveals that fish egestion and excretion provide inorganic N and P to the system. The calculated nitrogen input from fish (62.4 ± 9.5 μgN/h g) was 2.8 times higher than the nitrogen excretion by mosquito fish measured in a lab experiment (Uliano et al., 2010), but similar and in the lowest range of values obtained by McIntyre et al. (2008) in field experiments. Phosphorus input (0.055 ± 0.01 μmol P/h fish) was also in the lower range of values of phosphorus excreted by a fish per hour reported by McIntyre et al. (2008), and it was comparable for the small-bodied (average 0.31 ± 0.04 g) fish. In our nutrient-poor system, fish biomass (27.5 ± 3.7 g/m^3) was similar to the one measured in nature (Gardner et al., 2013) and thus may act as a nutrient subsidy (Ruegg, 2011). In contrast to our hypothesis, P and N inputs from fish were not high enough to overcome nutrient limitation. Measured phosphate and nitrate concentration in water were low, and their incorporation to periphytic algal growth was also low. Most inputs of P and N were retained in the sediment revealing a key role of this compartment and the associated epipsammic biofilm in nutrient removal, leading to nutrient-limiting conditions, mainly for periphytic biofilm growth. Our systems showed low N and P contents in water, below the range found in oligotrophic rivers (Mas-Martí et al., 2015; Sabater et al., 2011), as well as low periphytic chl-a concentration (2.82 μg/cm^2 on average), also characteristic of nutrient-poor systems, confirming that periphyton was nutrient-limited. Moreover, phosphatase activity was in the upper range of those measured in an oligotrophic stream, thus suggesting phosphorus limitation for these biofilm communities (Romaní and Sabater, 2001).

Besides the total amount of N and P absorbed, it is interesting to highlight the difference in stoichiometry between periphytic and epipsammic biofilm. P and N contents and the C:N ratio were higher in the epipsammon, and closer to fish and food ratios, thus indicating higher nutrient availability in this compartment, probably due to the fact that most particulate organic matter (detached algae or fish faeces) was deposited on the sediment placed in the bottom of the aquaria. On the other hand, extracellular enzyme activities were higher in periphyton than in epipsammon, either per surface area or per biomass unit (Fig. 4). Since total biomass and the chl-a were similar, the low activity in the epipsammon in our system might be related to the higher availability of organic matter leading to lower efficiencies (Romani et al., 1998).

Following the same pattern as nutrient balance results, arsenic retention was mainly attributed to the sediment with the least contribution of periphyton (Table 1). These results are in agreement with those reported in the literature highlighting the role of sediments on arsenic removal by adsorption (Borgnino et al., 2012; Mandal et al., 2012) Recently it was reported that epipsammic biofilms on the sediment play a key role in the fate and mobility of As in riverine environments and particularly in the transference of As from the water column to the sediment (Prieto et al., 2013). Our results and those from the literature agree with the role that sediments play as arsenic sinks in natural systems, thus moving exposure from the water column to the benthic compartment. The release of As from sediments is driven by redox chemistry (Ferguson and Gavis, 1972) in which iron oxide binding phases are reduced (Bennett et al., 2012). A study focused on the mobilization of arsenic in freshwater and estuarine sediments (Bennett et al., 2012) indicated that As(III) was the primary species mobilized from the solid phase to the water column in anoxic conditions and

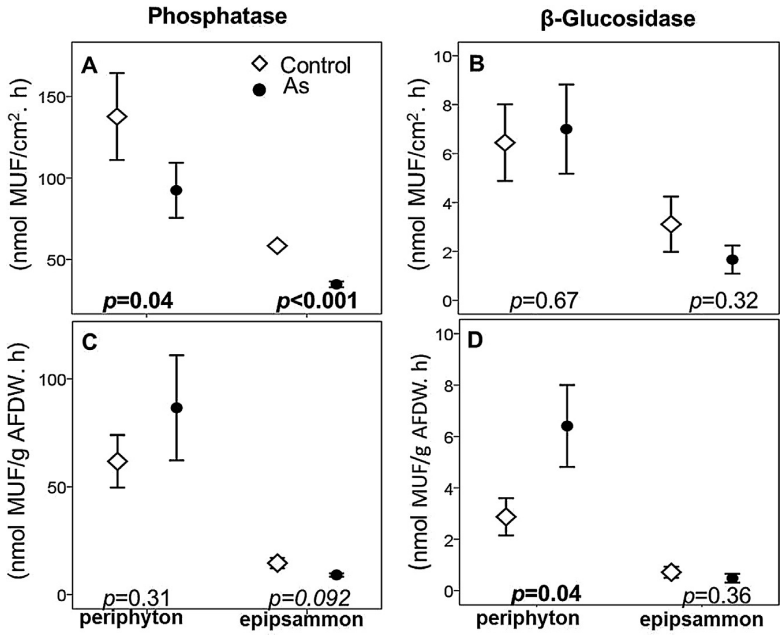

Fig. 4. Average values and standard errors of phosphatase (A and C), β-glucosidase (B and D) in periphytic and epipsammic biofilms at the end of the experiment: The results were expressed in nmol MUF released from each cm^2 biofilm or sediment surface (A and C) and expressed in nmol MUF released per mg ash-free dry weight (B and D). Statistical significance (p values) of the T test are in the bottom of each compared pairs (control and As treatment). When $p < 0.1$ was highlighted as italics and $p < 0.05$ was highlighted as bold.

As(V) mobilized substantially during reoxygenation. Since our system is oxic, we expected having mostly As(V). However in nature, with large diurnal fluctuations of oxygen or lake stratification, As adsorbed to sediment can be released to water (Ferguson and Gavis, 1972; Moriarty et al., 2014; Rieuwerts et al., 2014), affecting again the organisms inhabiting this compartment.

Total As accumulation in periphyton was lower than in the sediment but also remarkable, supporting its use as a biomarker of As exposure in natural water. Overall, As concentration in water had already lessened more than 4 times than at initial exposure (S. Fig. 1), whereas biofilm As contents reached up to 16 mg As/kg DW. Algae in nature accumulate up to 382 mg/kg (DW) of arsenic depending on the conditions (Neff, 1997). For example, periphyton exposed to 130 μg/L of arsenic for 4 weeks accumulated 3750 μgAs/g DW (Rodriguez Castro et al., 2014), while background arsenic concentrations in freshwater biota are less than 1 μg/g (Rahman et al., 2012).

In our experiment, arsenic exposure changed over time simulating a pulse of 120 μg/L and a further 60-day exposure to the remaining concentration of 28 ± 0.5 μg/L. The average exposure concentration (37.3 μg/L) was between four and five times lower than the criterion continuous concentration (CCC) (150 μg/L), which is an estimate of the highest concentration of a substance in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (USEPA, 2014). However, arsenic exposure affected the activity and biomass accrual of periphytic and epipsammic biofilm. Arsenic affected algal growth, more specifically diatom growth (Fig. 2). Overall, As-exposed periphytic biofilm accumulated 48% less biomass than controls with a reduction of diatom growth by 32.1%. Several studies showed that toxicants inhibit algal photosynthesis (Barranguet et al., 2003; Pesce et al., 2010; Corcoll et al., 2011) and that metals may selectively affect diatoms (Bonet Sánchez, 2013; Corcoll et al., 2011; Serra et al., 2010). Clearly acute toxicity effects of As were observed on the photosynthetic efficiency of diatoms and cyanobacteria (Fig. 2D and E), suggesting this parameter may be useful for detecting recent contamination. The lower photosynthetic performance of the community could also be attributed to the energetic cost of detoxification (Lelong et al., 2012). Arsenic selectively affected diatoms, in agreement with the results obtained by Rodriguez Castro et al. (2014) at 130 μg/L of As under P-limiting conditions, but not at the lowest tested concentration (15 μg/L).

In addition to the photosynthetic performance and growth of periphytic algae, arsenic exposure affected the bacterial component of biofilm, the biofilm's capacity to use organic matter and the heterotrophy of the whole community. Our results showed the total phosphatase activity (expressed per unit of surface) decreased in both the periphytic and epipsammic biofilms (Fig. 4A), while phosphatase activity per biofilm biomass was not affected (Fig. 4B), suggesting that the observed decrease was mainly due to the decrease in biomass. Both algae and bacteria are responsible for phosphatase activity and thus this significant decrease could be either due to a decrease in biomass (i.e., decrease in chlorophyll or AFDW) or accumulation of non-active biomass (as shown by the increase in dead bacteria in the epipsammon or decreasing photosynthetic efficiency of periphyton). In contrast to phosphatase activity, β-glucosidase activity (expressed per unit of biofilm biomass) significantly increased in periphytic biofilm exposed to As (Fig. 4D), indicating that bacteria increased their efficiency in the use of available polysaccharides probably released from decaying algae. β-glucosidase activity is involved in the decomposition of simple polysaccharides and, in contrast to phosphatase, mainly microbial heterotrophs are responsible for this activity. Haack and McFeters (1982), demonstrated that algae may supply bacteria with DOC resulting from excretion processes during photosynthesis (exudates) and after algal cell death in biofilm. Also, Middelboe et al. (1995) suggested the increasing bacterial use of polysaccharides from decaying algae by the increase in β-glucosidase activity at the end of an algal bloom. In a previous study focused on the effects of the herbicide diuron on biofilms, such a

cascade effect on bacteria after algal cell death was also found to show an increase in peptidase activity (Ricart et al., 2009). This response, however, was not shown for the epipsammic biofilm, probably due to the accumulation of dead bacteria in the sediment and reduced autotrophy (measured as the AI or AFDW/chl-*a* ratio) with As. In this case, arsenic exposure prevented the colonisation of sediment grains by algae, compared to the non-exposed sediment that accumulated similar chl-*a* to that of the periphytic biofilm.

These direct effects of exposure had several indirect effects on water chemistry and nutrient contents. The reduction in algal growth and activity influenced water conductivity by reducing the role that photosynthesis plays on the precipitation of salts (Wetzel, 2001). Moreover, in the arsenic treatments, dissolved oxygen and pH were slightly lower resulting also from photosynthesis inhibition and enhanced heterotrophy (lower CO_2 uptake and lower O_2 production).

Slight effects on fish were also observed, influencing their nutrient inputs. The whole fish input of N and P decreased slightly with arsenic (around 12%). This difference was related to the daily food consumption of the fish in As treatment, which was slightly lower leading to lower nitrate concentration in water and lower total N accumulated in periphytic biofilm. Biofilm P contents were also lower, but differences were marginally significant (Table 1). Biofilms are at the base of the food chain of the stream, fuelling energy to the upper trophic level (Power et al., 2013) and driving carbon and nutrient cycles (Vadeboncoeur and Steinman, 2002). Our results showed that the whole biomass (AFDW) decreased, algal growth decreased and that epipsammic biofilms became more heterotrophic due to Arsenic, thus reducing their ability to oxygenate and purify water. On top of that, the total amount of N in periphytic biofilm dropped, changing food quality and quantity to the higher trophic levels.

5. Conclusion

In contrast to our hypothesis, fish and sediment could not ameliorate As exposure effects on biofilm. In spite of the role that fish played as a resource subsidy, nutrient as well as As retention in sediments were high keeping phosphate similar to background ambient P concentration found in Mediterranean oligotrophic streams (Sabater et al., 2011) and similar to the P-limiting conditions established in the experiment conducted by Rodriguez Castro et al. (2014). We can conclude that sediment exerted a double and antagonistic role reducing As concentration but enhancing As toxicity by reducing nutrient concentration. On the other hand, the negative effects of arsenic on the role that fish played as a resource subsidy, exacerbated nutrient limitation, thus enhancing As effects on the growth and contribution to the nutrient cycling of periphytic and epipsammic biofilm. The maximum exposure (120 μg/L) and the remaining As concentration in water (28 ± 0.5 μg/L) are similar to values measured in naturally As polluted fresh waters and near mining areas (Inam et al., 2011; Battogtokh et al., 2013; Rosso et al., 2011; Alonso et al., 2014), where processes similar to those described in this experiment may drive the fate of arsenic. The remaining As concentration is five times lower than the criterion continuous concentration (150 μg/L) demonstrating that this criterion will not preserve the ecological integrity of fluvial systems.

We can conclude that exposure to arsenic at environmentally realistic concentrations will directly and indirectly affect the base (primary producers) of the aquatic food chain and its respective contribution to nutrient cycling impairing the normal functioning of the ecosystem.

Contributors

Experimental design: Helena Guasch (HG); the experiment, biochemical analyses and data analyses: Baigal-Amar Tuulaikhuu (BT) and HG; extracellular enzyme activities: Anna Romani (AR) and BT; writing: BT and HG; editing, revising: HG, AR and BT.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

Financial support was provided by the Spanish Science and Education Ministry (Project CTM2009-14111-CO2-01 and the Spanish Economy and Competitiveness Ministry (Project CGL2013-43822-R). Baigal-Amar Tuulaikhuu benefitted for a doctoral fellowship from the Techno 2 Program of the European Union Erasmus Mundus partnership. Thanks to Kit Magellan for providing the fish and giving recommendations for maintaining and measuring them. Thanks also to Laura Barral for helping with the lab work, and Luisa Matas and Imma Arrom from the Technical Research Services of the University of Girona for guiding arsenic and elemental analyses.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.07.005

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Supplementary tables and figures of the article 1

S. Table 1. Average values and standard deviations of physicochemical parameters in the water during the experiment. The symbols (˟) attached when $p<0.1$, and (*) when $p<0.05$ based on the results of repeated measures ANOVA comparing control and As treatments

Parameters	Treat		Day 0	Day 3	Day 6	Day 11	Day 18	Day 25	Day 32	Day 38	Day 46	Day 52	Day 59	P values		
														Day	As	As× day
Temp (^{O}C)	C	Mean	19.6	20.9	21.2	20.6	20.7	20.8	20.1	20.7	21.4	21.4	23.6	**<0.001**	0.25	0.88
		SD	0.1	0.3	0.1	0.1	0.6	0.2	0.3	0.3	0.2	0.3	0.1			
	As	Mean	19.6	20.8	21.1	20.4	20.3	20.5	20.0	20.4	21.4	21.2	23.4			
		SD	0.1	0.2	0.1	0.2	0.6	0.3	0.3	0.1	0.2	0.1	0.1			
pH	C	Mean	7.73	7.79	8.16	8.03	7.98	8.03	8.08	8.05	8.10	8.12	8.00	**<0.001**	0.15	*0.09*
		SD	0.09	0.09	0.11	0.06	0.12	0.05	0.07	0.11	0.06	0.09	0.10			
	As	Mean	7.68	7.65	7.87	7.96	7.97	8.00	8.07	8.02	8.08	8.02	7.90			
		SD	0.12	0.10	0.22	0.07	0.09	0.06	0.05	0.03	0.03	0.05	0.04			
Con-duc-tivity	C	Mean	381	386	388	395	396	401	401	404	**403˟**	**401***	**402***	**<0.001**	0.16	0.25
		SD	1.7	2.1	2.1	4.2	1.5	1.5	4.0	5.0	**4.5**	**3.8**	**3.8**			
	As	Mean	381	387	392.3	403.7	406.7	410.7	407.0	411.7	**413˟**	**413***	**414***			
		SD	4.6	5.3	4.9	7.2	9.8	16.7	13.1	10.8	**5.9**	**2.1**	**2.9**			
DO (mg/l)	C	Mean	8.72	8.25	**8.14***	**8.09˟**	8.10	8.06	8.30	8.12	7.87	7.81	6.87	**<0.001**	0.21	0.22
		SD	0.04	0.02	**0.05**	**0.16**	0.06	0.05	0.16	0.08	0.15	0.18	0.39			
	As	Mean	8.64	8.06	**7.92***	**7.73˟**	7.90	8.06	8.27	7..93	7.73	7.74	6.38			
		SD	0.08	0.19	**0.02**	**0.22**	0.30	0.22	0.17	0.29	0.15	0.26	0.48			
PO4-P (µg/L)	C	Mean		5.0	6.4	12.0	4.2		5.6	4.7	bdl	bdl	2.9	**<0.001**	0.9	0.87
		SD		1.0	2.8	3.3	3.0		3.1	1.1			0.6			
	As	Mean		5.0	5.2	12.5	7.1		4.6	3.9	bdl	bdl	2.8			
		SD		4.1	1.6	2.6	3.4		1.5	1.2			0.6			
NH_4-N (µg/L)	C	Mean	17.3	28.5	30.8	bdl	bdl	29.8	bdl	bdl	15.3	bdl	5.2	**0.003**	0.56	0.99
		SD	13.6	17.3	15.0			24.4			11.0		0.3			
	As	Mean	20.7	32.1	23.9	bdl	bdl	29.4	bdl	bdl	24.0	15.6	14.7			
		SD	17.0	18.2	4.9			28.3			27.2	18.4	14.5			
NO_3-N (µg/l)	C	Mean		571			**67˟**			**90˟**	38	47	**88˟**	**<0.001**	0.45	*0.06*
		SD		52			**18**			**24**	28	34	**28**			
	As	Mean		656			**41˟**			**40˟**	63	8	**29˟**			
		SD		105			**8**			**27**	40	3	**31**			

S. Table 2. Curve fitting (the best fit for each parameter) for the data of minimum fluorescence yield **(F_0)**, the maximal PSII quantum yield **(Y_{max})** and Photosynthetic efficiency (**Y_{eff}**) measured each three day in periphytic biofilms and these values separated by algal groups.

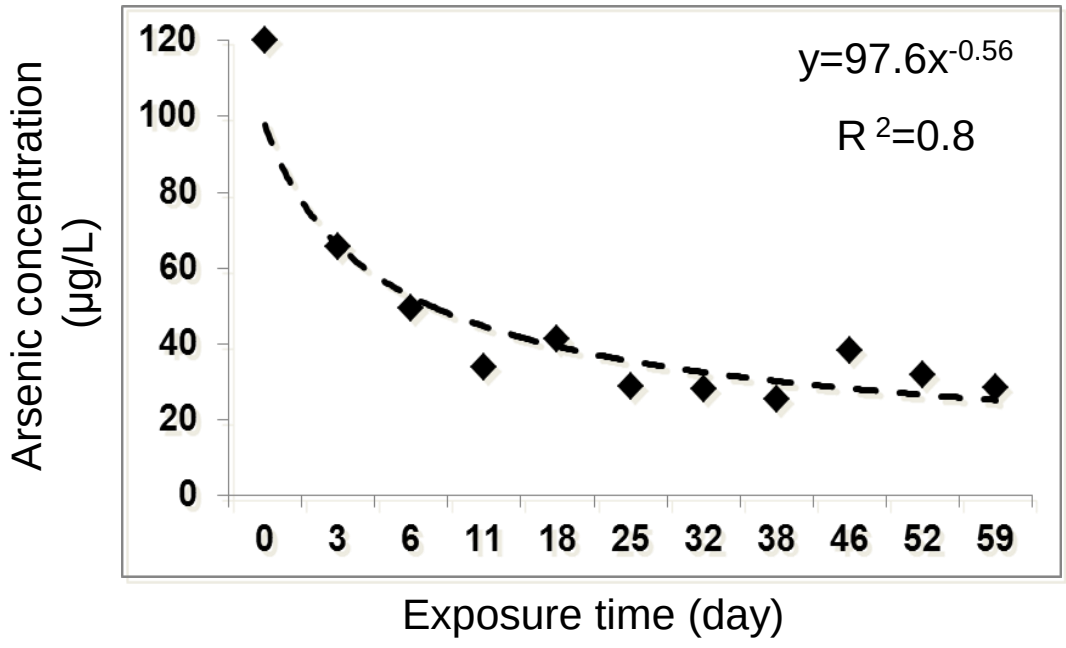
Parameter	Treat-ment	Model equation	Model Summary				
			R^2	F	df1	df2	Sig.
F_0	C	quadratic	0.28	11.01	2	57	**<0.0001**
	As	quadratic	0.28	11.05	2	57	**<0.0001**
Y_{max}	C	quadratic	0.26	9.75	2	57	**<0.0001**
	As	quadratic	0.31	12.54	2	57	**<0.0001**
Y_{eff}	C	quadratic	0.17	5.90	2	57	**0.005**
	As	quadratic	0.14	4.72	2	57	**0.013**
F_0 blue	C	quadratic	0.33	13.88	2	57	**<0.0001**
	As	quadratic	0.27	10.59	2	57	**<0.0001**
F_0 green	C	linear	0.33	28.23	1	58	**<0.0001**
	As	linear	0.30	24.92	1	58	**<0.0001**
F_0 brown	C	quadratic	0.16	5.47	2	57	**0.007**
	As	quadratic	0.18	6.21	2	57	**0.004**
Y blue	C	linear	0.07	4.18	1	52	**0.046**
	As	linear	0.01	0.13	1	52	0.722
Y green	C	linear	0.08	3.66	1	40	0.063
	As	linear	0.27	9.42	1	25	**0.005**
Y brown	C	cubic	0.15	3.00	3	50	**0.039**
	As	cubic	0.14	2.73	3	50	0.053
% blue	C	quadratic	0.28	11.17	2	57	**<0.0001**
	As	quadratic	0.22	8.18	2	57	**0.001**
% green	C	quadratic	0.30	12.35	2	57	**<0.0001**
	As	quadratic	0.39	18.56	2	57	**<0.0001**
% brown	C	linear	0.43	44.33	1	58	**<0.0001**
	As	linear	0.57	75.54	1	58	**<0.0001**

S. Table 3. Results of the general linear model considering the effects of As (factorial) and exposure time (continuous) variables on biofilm chlorophyll fluorescence parameters. **F_0**- minimum fluorescence yield, **Y_{max}** - the maximal PSII quantum yield, **Y_{eff}** – Photo-synthetic efficiency as the former 2 parameters separated by algal groups: blue, green and brown.

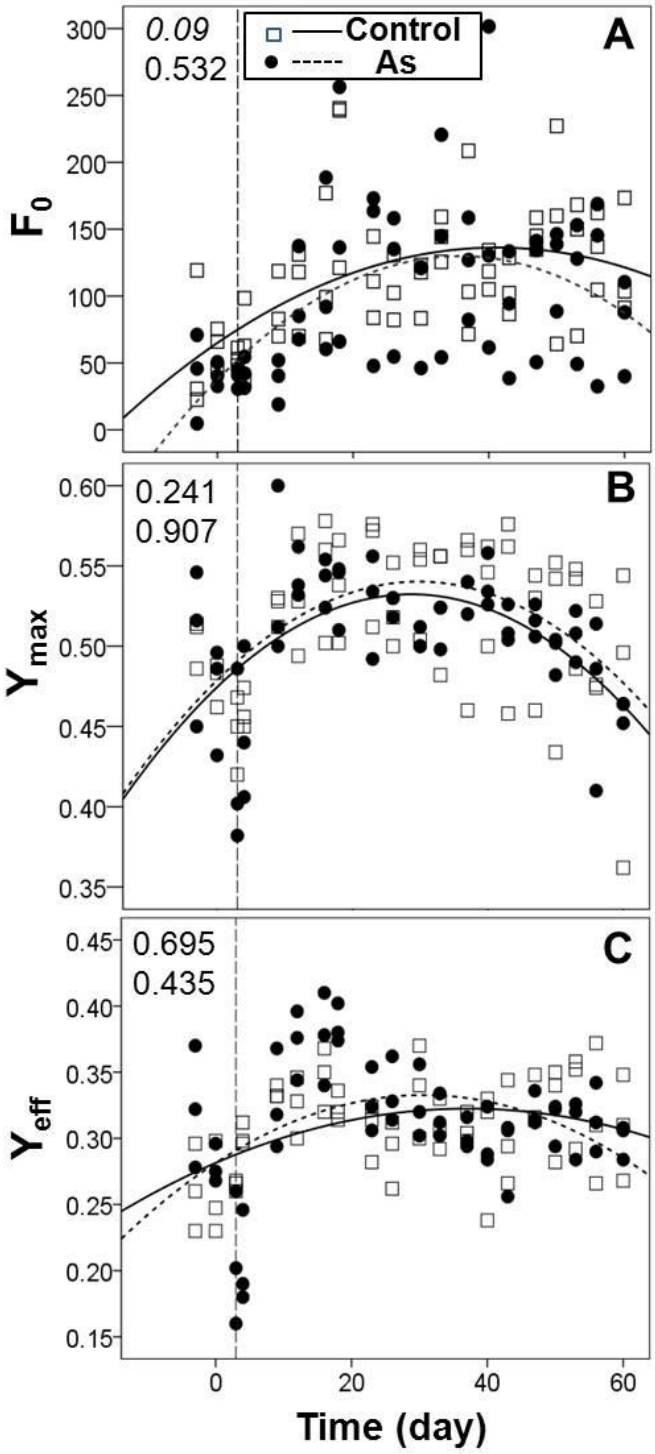
Parameter	**Source**	**Intercept**	**As**	**Day**	**As × Day**	**Error**
F_0	SS	97.9	6678	97754	2898	47.75
	d.f.	1	1	2	2	114
	P	<**0.001**	*0.09*	<**0.001**	0.532	
Y_{max}	SS	0.51	0.001	0.063	0.0003	0.04
	d.f.	1	1	2	19	114
	P	<**0.001**	0.241	<**0.001**	0.907	
Y_{eff}	SS	0.311	0.0003	0.02	0.003	0.04
	d.f.	1	1	2	2	114
	P	<**0.001**	0.695	<**0.001**	0.435	
F_0 blue	SS	111.4	10015	153241	997	57.21
	d.f.	1	1	2	2	114
	P	<**0.001**	*0.08*	**0.001**	0.858	
F_0 green	SS	-1.53	4433	83719	2722	40.84
	d.f.	1	1	1	1	116
	P	0.868	0.106	**0.001**	0.204	
F_0 brown	SS	245.0	351524	458715	10121	142.2
	d.f.	1	1	2	2	114
	P	<**0.001**	<**0.001**	<**0.001**	0.779	
% blue	SS	26.2	40.9	729	6.27	4.47
	d.f.	1	1	2	2	114
	P	<**0.001**	0.155	**0.001**	0.855	
% green	SS	10.9	685	4102	488	8.27
	d.f.	1	1	2	2	114
	P	<**0.001**	**0.001**	<**0.001**	**0.03**	
% brown	SS	62.9	1061	5478	549	4015.0
	d.f.	1	1	2	2	80
	P	<**0.001**	<**0.001**	<**0.001**	**0.002**	

S.Table 4. The average values and standard deviations of periphytic and epipsammic biofilm parameters measured at the end of the experiment. Statistically significant values (p<0.05) are in bold based on T test values considering Arsenic as factor.

Parameters	Periphytic biofilm			Epipsammic biofilm		
	Control mean±SD	**As mean±SD**	***P***	**Control mean±SD**	**As mean±SD**	***P***
Chlorophyll a (µg/cm^2)	1.77±0.7	1.45±0.5	0.69	3.3±0.4	1.1±0.5	**0.035**
AFDW (mg/cm^2)	2.22±0.1	1.15±0.3	**0.007**	4.17±1.0	3.84±0.4	0.63
β glucosidase (nmol MUF/cm^2·h)	6.38±1.2	7.11±2.5	0.67	2.99±1.8	1.82±0.3	0.32
β glucosidase (nmol MUF/g AFDW·h)	2.9±0.5	6.4±2.1	**0.04**	0.71±0.36	0.48±0.13	0.36
Phosphatase (nmol MUF/cm^2·h)	137.5±13.2	92.5±23.9	**0.04**	58.3±1.4	34.6±1.7	**0.001**
Phosphatase (nmol MUF/g AFDW·h)	61.8±3.08	86.6±37.5	0.31	14.6±4.2	9.1±1.1	*0.092*
Total bacteria (×10^6/cm^2)	15.2±2.9	12.8±6.1	0.56	37.6±24.2	65.6±32.0	0.29
Live to dead bacteria ratio	0.21±0.08	0.39±0.05	0.35	0.16±0.05	0.05±0.02	**0.018**



S. Figure 1. Arsenic concentration in the water as weekly measured values



S. Figure 2. The minimal fluorescence yield (F_0), the maximal PSII quantum yield ($\boldsymbol{Y}_{max}$), and the effective PSII quantum yield (F_{eff}) in control and arsenic treatments over time. The open squares and solid line stand for control; black circles and pecked lines for arsenic treatment, and the lines correspond to linear and quadratic regressions. The vertical line indicates the day when fish were added to the treatments. The values at the top left denote the *p* values based on linear model (with line fitting) tests between control and arsenic treatments: As as factor (top) and arsenic×day as factor (bottom).

Article 2

Effects of low arsenic concentration exposure on freshwater fish in presence of fluvial biofilms

Science of The Total Environment (2016) 544:467 - 475

Science of the Total Environment 544 (2016) 467–475

Contents lists available at ScienceDirect

Science of the Total Environment

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

Effects of low arsenic concentration exposure on freshwater fish in the presence of fluvial biofilms

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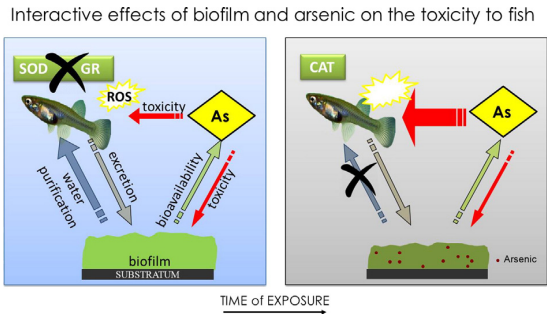
HIGHLIGHTS

- Biofilm activity may counterbalance arsenic toxicity in the short term
- Biofilm protection will disappear when it loses their normal functioning
- Two-month exposure to low arsenic affects biofilm, fish and their interactions
- Chronic arsenic concentration criterion may not protect aquatic life.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 September 2015
Received in revised form 20 November 2015
Accepted 24 November 2015
Available online xxxx

Editor: D. Barcelo

Keywords:
Arsenic
Antioxidant enzyme activity
Biofilm
Ecological realism
Mosquitofish

ABSTRACT

Arsenic (As) is a highly toxic element and its carcinogenic effect on living organisms is well known. However, predicting real effects in the environment requires an ecological approach since toxicity is influenced by many environmental and biological factors. The purpose of this paper was to evaluate if environmentally-realistic arsenic exposure causes toxicity to fish. An experiment with four different treatments (control (C), biofilm (B), arsenic (+As) and biofilm with arsenic (B + As)) was conducted and each one included sediment to enhance environmental realism, allowing the testing of the interactive effects of biofilm and arsenic on the toxicity to fish. Average arsenic exposure to Eastern mosquitofish (*Gambusia holbrooki*) was 40.5 ± 7.5 μg/L for +As treatment and 34.4 ± 1.4 μg/L for B + As treatment for 56 days. Fish were affected directly and indirectly by this low arsenic concentration since exposure did not only affect fish but also the function of periphytic biofilms. Arsenic effects on the superoxide dismutase (SOD) and glutathione reductase (GR) activities in the liver of mosquitofish were ameliorated in the presence of biofilms at the beginning of exposure (day 9). Moreover, fish weight gaining was only affected in the treatment without biofilm. After longer exposure (56 days), effects of exposure were clearly seen. Fish showed a marked increase in the catalase (CAT) activity in the liver but the interactive influence of biofilms was not further observed since the arsenic-affected biofilm had lost its role in water purification. Our results highlight the interest and application of incorporating some of the complexity of natural systems in ecotoxicology and support the use of criterion continuous concentration (CCC) for arsenic lower than 150 μg/L and closer to the water quality criteria to protect aquatic life recommended by the Canadian government which is 5 μg As/L.

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http://dx.doi.org/10.1016/j.scitotenv.2015.11.126
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1. Introduction

Arsenic (As) is a highly toxic element and its carcinogenic effect on living organisms is well known (Ng et al., 2003). Elevated concentrations of metals and metalloids in water and aquatic sediments deposited from mining or industrial waste waters are a global problem (Schaller et al., 2011). There are many examples of rivers and lakes being contaminated with arsenicals and other polluters from current and old mining activities (Casiot et al., 2005; Inam et al., 2011; Wong et al., 1999). Predicting real effects in the environment requires an ecological approach since toxicity is influenced by many environmental factors (e.g. substrate type, nutrient contents, redox status) and biological ones (e.g. biotransformation) and differs among aquatic organisms. Contaminants are accumulated on organic and inorganic sediment particles and their associated microorganism communities (Schaller et al., 2011). Microorganisms play a key role in the biogeochemical cycle of arsenic. They bioaccumulate inorganic arsenicals (iAs), biotransform to methylarsenicals and complex organoarsenicals inside their cells, and then release back to the water and mineralize methyl and organoarsenicals producing iAs species (Rahman et al., 2012). In natural waters, arsenic is mostly found in inorganic form as oxyanions of trivalent arsenite (As(III)) or pentavalent arsenate (As(V)) (Hasegawa et al., 2010; Smedley and Kinniburgh, 2002). Arsenite is assumed to be the most toxic form to most organisms, including humans, whereas As(V) is more toxic than As(III) to algae (Knauer et al., 1999). In well oxygenated aquatic systems iAs should be mostly as As(V).

While arsenic toxicity to invertebrates and algae has been described at low concentration: 20 μg/L (LOEC for *Daphnia magna* growth in a 21-day flow-through chronic bioassay) and 50 μg/L (14-day EC50 of growth inhibition for the green alga *Scenedesmus obliquus*), respectively, freshwater fish has lower sensitivity. The lowest chronic LC50 for fish reported in the literature was 550 μg/L for rainbow trout, *Oncorhynchus mykiss*, after 28 days' exposure (CCME, 2001). Numerous data are available on the effect of arsenic on fish reproduction (Boyle et al., 2008), growth, development and survival (D'Amico et al., 2014; Erickson et al., 2011; Gonzalez et al., 2010, 2006; Li et al., 2009).

Existing data regarding the biochemical basis and mechanisms of arsenic toxicity support the use of antioxidant enzymes as early warning signallers of arsenic toxicity in freshwater fish. One of the earliest responses to arsenic toxicity is an increase in reactive oxygen species (ROS) (Flora, 2011), during their redox cycling and metabolic activation processes that cause lipid peroxidation and DNA damage (Ratnaike, 2003). Normally, cells defend themselves against ROS damage with several enzymes including superoxide dismutase, catalases, and glutathione peroxidases (Bansal and Kaushal, 2014). The tripeptide glutathione (GSH) directly or indirectly regulates the scavenging of ROS both as an important component of antioxidant defence system in fish and also as a molecule containing a thiol group, in which arsenic has affinity. So glutathione and its dependent enzymes such as glutathione reductase and glutathione S transferase are expected to respond to arsenic exposure in fish (Srikanth et al., 2013). There are few studies measuring antioxidant enzyme activities of freshwater fish exposed to low concentrations of arsenic. Kim and Kang (2015) observed increases in SOD and GST activities in liver and gill of juvenile rockfish (*Sebastes schlegelii*) exposed to 200 μg/L sodium arsenite for 20 days, whereas Sarkar et al. (2014) found a triphasic alteration in CAT activities in the brain of zebrafish exposed to 50 μg/L arsenic trioxide for 90 days.

Given the intricacies of the feedback and cycling interactions contributing to arsenic toxicity in fish, in previous studies a simplified fluvial system was used to examine the interacting effects of naturally occurring periphytic biofilms, thus incorporating some of the complexity of natural systems in a laboratory experiment and allowing some specific processes from whole-ecosystem effects to be disentangled (Barral-Fraga et al., 2015; Magellan et al., 2014). Using this experimental setup, Magellan et al. (2014) found that mosquitofish exposed to arsenate (As(V), 130 μg/L over 13 days) experienced an increase in the amount of weight gained and a higher level of aggressive behaviour; effects which were aggravated by the presence of periphytic biofilms, while periphytic biofilms suffered a reduction in algal species richness, a marked inhibition of algal growth and a strong reduction in diatom cell biovolume and these effects were reported by Barral-Fraga et al. (2015).

In this study, we investigated the effects of a longer exposure (56 days) of arsenic on mosquitofish (*Gambusia holbrooki*) under the influence of periphytic biofilms (growing on illuminated glass substrata) and epipsammic biofilms (growing on sediment grains), thus increasing the complexity, and hence realism of experimental conditions, by including sediments. By including periphytic and epipsammic biofilms, the influence that adsorption and/or biotic activity may have on arsenic toxicity was also evaluated.

Biofilms — communities embedded within a polysaccharide matrix — play a key role in the functioning of aquatic ecosystems. Biologically, biofilm changes chemical exposure in stream ecosystems by influencing solubility of minerals, sorption of metals onto particle surfaces, transformations between oxidized and reduced species, and metabolism of aquatic biota (Balistrieri et al. 2012). Eventually, these changes will affect bioavailability and hence toxicity in fish.

The purpose of this paper was to evaluate the effects of environmentally-realistic arsenic exposure on fish under the interactive influence active biofilm communities. Effects caused to periphytic and epipsammic biofilms of this experiment were previously evaluated and reported in detail in Tuulaikhuu et al. (2015). Periphytic and epipsammic biofilms were grown under conditions of phosphorus limitation since effects of arsenic on organisms increase under lower phosphorus availability (Rodriguez Castro et al., 2014; Wang et al., 2013) and under a well-oxygenated environment to ensure that AsV was the dominant arsenic species. Arsenic exposure influenced the quality and quantity of the biofilm and its ability to purify and oxygenate the aquatic environment. Sediments played a double and antagonistic role in arsenic toxicity to biofilm by removing arsenic from the water column but enhancing its toxicity by retaining nutrients (Tuulaikhuu et al., 2015). Arsenate (As(V)) toxicity to periphyton photosynthesis and phosphate uptake under P-limiting conditions has been demonstrated at low concentration (15 μg As/L), highlighting the role of phosphate on As(V) toxicity to these aquatic communities (Rodriguez Castro et al., 2014). In this periphytic community, P-uptake capacity was already affected and algal growth was inhibited up to 61% in P-starved conditions, but not when P-availability was higher.

Generally short-term exposure or low arsenic concentrations results in an increase of the activity of these enzymes, while higher exposure may lead to a reduction of these activities if the antioxidant defences are overwhelmed (Flora, 2011). Given the low arsenic concentration used, exposure effects were expected to be chronic, thus exposure lasted for eight weeks and the effects based on antioxidant enzyme activities (AEAs) which are expected to respond to low-dose exposure. We evaluated five different AEAs (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST), and glutathione reductase (GR)), in liver and gill proteins of eastern mosquitofish (*Gambusia holbrooki*) to assess the arsenic effect on this fish. Although enzymes are early warning signallers of toxicity, they are highly sensitive to changes in environmental conditions. Thus, it is essential to provide the greatest possible ecological realism in toxicity testing to better understand real toxicity. Since sediments removed a large part of arsenic from the water column (Tuulaikhuu et al., 2015), we also expected that the remaining concentration may not affect the AEAs of the fish, but the already stressed biofilms might have some effects.

2. Methods

2.1. Experimental set up

Twelve independent experimental units with three replicates of 4 different treatments each were: control (C) (with neither As (V) nor biofilm), biofilm (B), arsenic (+As) and biofilm with arsenic (B + As). Each experimental unit had three main components: a large tank (90 L), in which fish were not in contact with the periphytic biofilm, a channel (90 × 8.5 × 7.5 cm) containing sand blasted glass tiles to provide substrate for the periphytic biofilms and a smaller tank (8 L) (S. Fig. 1). We filled the bottom of the large tanks (10 cm depth) with coarse grain sediment (grain size composition was 15% of 4.5–9.5 mm, and 85% of 2–4.5 mm in diameter size) to allow the growth of epipsammic biofilm. The large tanks filled with 90 L dechlorinated tap water, and the water circulated through the system. The experimental settings are well described in Magellan et al. (2014) and in the previous part of our research (Tuulaikhuu et al., 2015).

Before adding arsenic, biofilm colonisation took place in the B and B + As treatments. Colonisation inocula were collected by scraping the surface of randomly chosen rocks from a pristine stream (Llémena River, NE Spain), and the scraped periphytic biofilm added to each experimental unit twice a week during three weeks as described in Serra (2009). Phosphate solution (10 μg/L) was also added twice a week as a nutrient supply for algal growth.

2.2. Exposure

Arsenic (sodium(meta)arsenite — $NaAsO_2$ — molecular weight: 129.91 g/mol CAS 7784-46-5) solution was added to the big aquaria to reach 120 μgAs/L on day 22 and fish were placed in the aquaria 3 days later (at colonisation day 25) as described in Tuulaikhuu et al. (2015). The experiment was completed at day 82, thus biofilms were exposed to arsenic for 60 days, and fish exposure lasted for 56 days (the last fish were sampled one day before the experiment ended). Water was completely renewed the day before an arsenic addition, in order to have the similar nutrient conditions for all experimental units. Twenty five percent of the water was renewed at days 33 and 49, to avoid nutrient increase in the system and further water was not changed because there were only 2 fish left in each aquarium. When the water was changed, arsenic was added to the aquarium (+As and B + As treatments) in order to maintain the exposure level, but water lost due to evaporation was refilled whenever it was necessary without adding arsenic.

2.3. Physicochemical parameter measurements

Physical and chemical parameters (water temperature, dissolved oxygen, pH and conductivity) were measured with an appropriate probe (HQPortable Meters, HQ40d18, HACH Company) each week during the whole experimental period (81 days). Phosphorus (soluble reactive) [P(i)] was determined by a modified molybdeum blue method (Carvalho et al., 1998). Ammonium was measured following Reardon et al. (1966). Nitrate analysis was done with a minispectrometer measuring the formation of a red violet azo dye with N-(1-naphthyl) ethylene diammonium dichloride (AQUANAL®-plus Nitrate kit). Total arsenic analyses were done with inductively coupled plasma mass spectroscopy (ICP-MS 7500c Agilent Technologies) in the Technical Research Services (STR) of the University of Girona (http://www.udg.edu/serveis/STR). Water samples for total arsenic analyses were taken every week and were prepared using 5 mL of unfiltered water and acidified immediately with 1% of HNO_3 (from 65% suprapure, Merck). At the end of the experiment, arsenic in biofilms (in triplicate samples) and in fish was also measured.

2.4. Experimental fish

The fish used in this experiment were Eastern mosquitofish (*G. holbrooki*), provided by fish researchers of the Institute of Aquatic Ecology, University of Girona, Spain (IEA, UdG). Magellan et al. (2014) described that the fish were collected from the Ter (42.0451°N, 3.1960°E) and Muga (42.2527°N, 3.0756°E) rivers and had been kept for several months in big tanks (4 × 4 × 2 m) outside the faculty for experimental purposes. Eight fish (29.4 ± 7.6 mm) were added to each aquarium (total weight per aquarium 2.52 ± 0.33 g) and they were fed every day with commercial, frozen bloodworms (*Chironomus* spp.). The bloodworms were added to the aquaria one by one (after the first one was consumed, the next one was given until they stop eating) and the total weights of given food were measured daily. The daily average fish food consumption was 16.1 ± 2.8%, 18.1 ± 6.0%, 15.5 ± 5.1% and 13.5 ± 2.6% of fish weight for the treatments C, +As, B and B + As respectively. There was no statistically significant difference between treatments ($p = 0.65$).

We weighed each fish twice (when they were placed in the system and when they were taken for further analysis), and fish growth was calculated as the mass difference between these 2 measurements. Fish sampling was performed 5 times (we took 2 fish at day 4, 1 at day 9, another 2 at day 25, another 1 at day 41 and the last 2 fish at day 57). The gained weight of each fish per day was calculated dividing the total weight growth by the number of days that the fish were in the aquarium. Fish were always sampled at the same time of the day, to have no daily differences between the treatments. Once collected, the fish were placed on ice (without using an anaesthetic) and their liver and gills were dissected and put in different eppendorfs for enzyme analyses. Other parts of the fish body were kept in the freezer at −65 °C for arsenic bioaccumulation analyses. To do so, fish samples were lyophilized, homogenized, weighed and digested with 4 mL of HNO_3 (65% suprapure, Merck) and 1 mL of H_2O_2 (33% suprapure, Merck) in high performance microwave digestion (Milestone, Ethos Sel). After digestion, samples were diluted to 10 mL with milli-Q water and weighed. Finally, total arsenic concentration was analysed by ICP-MS, following the same procedure used for water samples.

2.5. Protein extraction and quantification

Protein quantification and AEA analyses were done 4 times throughout the 56 days. In the days following dissection, the organs were thawed gradually in fresh ice powder and were homogenized with 2 pulses of 30 s with a ceramic mortar. Samples were kept on ice during all the processes. Proteins were extracted with a sodium and potassium phosphate buffer (100 mM Na_2HPO_4/KH_2PO_4, 100 mM KCl, 1 mM EDTA and pH = 7.4) and centrifuged at 15,000 *g*, for 30 min, at 4 °C. The quantification was done following the Bradford (1976) method using a Coomassie Brilliant G-250 dye reagent (Bio-Rad, Laboratories GmbH, Munich, Germany) and bovine serum albumin as a standard. The absorbance was measured using a microtiter plate reader (Infinite® M200 PRO).

2.6. Antioxidant enzyme activity (AEA) analysis

AEA measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®), and changes in absorbance were followed using a microtiter plate reader (Infinite® M200 PRO). The antioxidant enzyme activities, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD), were measured following the protocols used in the same laboratory (Bonet et al., 2012; Bonnineau et al., 2013). Optimization of AEA substrate was performed in triplicate samples and with 5 different concentrations for each activity. The optimum concentrations were 30 mM of H_2O_2 for CAT, 3 mM of H_2O_2 for APX, 0.15 mM of

NADPH for GR, 5 mM of GSH for GST and 75 μM of WST-1 for SOD. AEA assays were done according to Bonet Sánchez (2013).

2.7. Statistical analyses

The physical and chemical parameters and arsenic concentration in water were analysed using repeated measure ANOVA for the exposure period (from day 22), considering biofilm as a factor when comparing control (C) and biofilm (B) treatments and arsenic as a factor when comparing biofilms (B) and biofilms plus arsenic (B + As) treatments. Repeated measures ANOVA with two between-subjects factors (arsenic and biofilm) was conducted to know if there is effect of arsenic and/or biofilm and interaction of those two factors on the AEAs and to assess changes over time. Normality of the data was checked with Shapiro–Wilk method and $\log_{10}$ transformation was used when needed. Dependent variable's (AEA's) equality of the error variance across was tested with Levene's method with SPSS.

The differences in gained weight of fish per day among the treatments were analysed using analysis of covariance (ANCOVA). A treatment was considered a factor with 4 levels (C, +As, B and B + As), and fish length was considered a covariate.

To understand the response of the set of endpoints measured in each experimental unit (AEA, arsenic bioaccumulation in fish and changes in fish weight) to the different treatments over time, within principal component analyses (PCA) were performed. In this particular case of PCA, the mean of the samples in a same group, i.e. collected at the same time is subtracted from each sample of this group for each variable. All the group centres are, therefore, at the origin of the factorial map and samples are represented with maximum variance around this origin. Therefore, the patterns of variation obtained at each sampling time can be compared one to the other (Bonnineau, 2011). Within PCAs were carried out using the "ade4" package (Dray et al., 2007) in the R software, developed by R Core Team (2015).

3. Results

3.1. Physical and chemical conditions

Physicochemical parameters in water were similar among the treatments at the beginning of the experiment and changed during biofilm colonisation. Water temperature was 20.9 ± 1.0 °C on average and slightly increased at the end of the experiment but was similar between treatments. Water pH was higher in the B treatment than in C at most times, except the first day when it was lower than C (Table 1). Comparing the +As and B + As treatments, pH was higher with the presence of periphytic biofilm in the latter half of the experiment period (Table 1). Even though we tried to maintain the pH during the experiment by adding CO_2, it was slightly higher in the aquaria with biofilm (B and B + As). Conductivity increased from day 18 onwards and was higher (+8.2%) in the +As than in the B + As treatment and was also slightly higher (+7.8%) in the C than in the B treatments, especially at the end of the experiment (Table 1). Dissolved oxygen (DO) was usually higher in the B treatment (+3.8%) than in C. Comparing the B + As and +As treatments, midway through the experiment (at days 25 and 32), dissolved oxygen was also higher (+5.6%) in B + As than in As, whereas the overall difference between treatments was not statistically significant. Phosphate (PO_4–P) and ammonium (NH_4–N) concentrations in water were low all over the experiments with no clear differences between treatments. The variations in nitrate concentrations were high and no clear pattern was observed between treatments through the experiment (Table 1).

3.2. Arsenic concentration

When fish were added to the aquaria the initially added 120 μg/L As had lessened to 65.7 ± 8.0 μg/L for the B + As treatment and to 80.1 ± 9.4 μg/L for the As treatment, respectively, and further decreased down

Table 1
Average of physicochemical parameters in the water during the experiment (60 days). Symbols (** when $p < 0.01$, * when $p < 0.05$, and ˇwhen $p < 0.1$) attached when they are significantly different based on the results of repeated measure ANOVAs, which compared control vs. biofilm (C vs. B) and arsenic vs. biofilm plus As (As vs. B + As) treatment. *bdl* means below detection limit. Numbers in italic when significance value $p < 0.1$, and in bold when $p < 0.05$ as well.

Para meters	Treat	Day 0	Day 3	Day 6	Day 11	Day 18	Day 25	Day 32	Day 38	Day 46	Day 52	Day 59	*p* values		
													Day	B	B × day
Temp (°C)	C	19.6	21.1	21.2	20.5	20.3	20.7	20.3	20.9	21.6	21.3	23.5	**0.001**	0.96	0.74
	B	19.6	20.9	21.2	20.5	20.6	20.8	20.1	20.7	21.4	21.4	23.6			
	As	19.6	20.9	21.2	20.3	20.3	20.3	19.9	20.4	21.2	21.3	23.4	**0.001**	0.85	0.89
	B + As	19.6	20.8	21.1	20.4	20.3	20.4	20.0	20.4	21.4	21.2	23.4			
pH	C	**7.9***	7.7	**7.9***	**7.7****	8.1	**7.8****	**7.9****	**7.9**	**7.9****	*7.9ˇ*	*7.8ˇ*	**0.006**	0.11	**0.03**
	B	**7.7***	7.8	**8.2***	**8.0****	8.0	**8.0****	**8.0****	**8.0**	**8.1****	*8.1ˇ*	*7.9ˇ*			
	As	7.7	7.6	8.0	7.8	7.9	7.9	**7.8****	**7.8***	**7.9****	**7.8***	**7.7***	**0.001**	0.19	0.10
	B + As	7.7	7.7	7.9	7.9	8.0	8.0	**8.1****	**8.0***	**8.1****	**8.0***	**7.9***			
Conductivity (μS/cm)	C	**376****	385	391	403	**408***	**424****	**424***	**437***	**442***	**443***	**449***	**0.001**	**0.02**	**0.001**
	B	**381****	386	388	495	**396***	**401****	**401***	**404***	**403***	**401***	**402***			
	As	378	387	393	407	413	430	*432ˇ*	**447***	**465***	**459***	**464****	**0.001**	**0.04**	**0.001**
	B + As	381	387	392	403	406	410	*407ˇ*	**411***	**413***	**413***	**414****			
DO (mg/L)	C	8.7	**8.0****	8.0	**7.7***	**7.7***	**7.8***	7.9	**7.9***	*7.6ˇ*	7.3	6.6	**0.001**	*0.07*	0.25
	B	8.7	**8.2****	8.1	**8.1***	**8.1***	**8.1***	8.3	**8.1***	*7.8ˇ*	7.8	6.9			
	As	8.7	7.8	*7.9ˇ*	7.4	7.5	**7.5***	**7.8***	7.7	7.5	7.3	6.1	**0.001**	0.16	0.18
	B + As	8.6	8.0	*7.9ˇ*	7.7	7.9	**8.0***	**8.2***	7.9	7.7	7.7	6.4			
PO_4-P (μg/L)	C		5.0	6.4	12.0	4.2		5.6	4.7	bdl	bdl	2.8	**0.001**	0.29	0.55
	B		4.5	5.2	8.4	7.3		5.0	3.7	bdl	bdl	2.8			
	As		6.0	4.5	8.0*	3.3		4.2	3.3	bdl	bdl	2.6	**0.001**	**0.05**	0.19
	B + As		5.0	5.2	12.5*	7.1		4.6	3.8	bdl	bdl	2.8			
NH_4–N (μg/L)	C	8.3	38.6	19.9	5.9	bdl	19.8	bdl	bdl		9.2	6.6	**0.004**	0.59	0.71
NO_3–N (μg/L)	B	17.3	28.5	30.8	bdl	bdl	29.8	bdl	bdl		bdl	5.3			
	As	Bdl	31.6	23.8	bdl	bdl	18.1	bdl	bdl		bdl	25.1	**0.001**	0.55	0.78
	B + As	17.0	32.1	23.9	bdl	bdl	29.4	bdl	bdl		15.0	14.7			
	C		617			47			**24***	*91ˇ*	53	*31ˇ*	**0.001**	0.75	0.19
	B		571			67			**90***	*37ˇ*	46	*88ˇ*			
	As		445			56			60	72	24	42	**0.001**	0.28	**0.02**
	B + As		656			41			39	63	8	49			
As (μg/L)	As	120	80.1	*61.6ˇ*	42.0	40.2	37.5	**33.9***	31.7	47.9	35.8	27.8	**0.001**	0.21	0.18
	B + As	120	65.7	*49.4ˇ*	33.7	41.4	28.9	**28.0***	25.3	38.4	31.8	28.6			

to 27.8 ± 13.3 μg/L and 28.6 ± 1.19 μg/L in As and B + As treatments respectively after 60 days (Table 1). So the daily average As exposure to fish was 40.5 ± 7.5 μg/L for the As treatment and 34.4 ± 1.4 μg/L for the B + As treatment for 56 days. The repeated measure ANOVA shows that the biofilm in the channels did not make a significant contribution to arsenic concentration reduction, if the whole exposure period is considered ($p = 0.21$). However, there were slight differences at the beginning (at day 6) when As concentration was slightly higher with biofilm ($p = 0.06$), and in the middle of the experiment (at day 32) when it was significantly higher in the As treatment than in the B + As one ($p = 0.038$). Arsenic concentrations in the periphitic and epipsammic biofilms were 15.9 ± 4.8 mg/kg and 0.6 ± 0.1 mg/kg dry weight respectively.

3.3. Fish growth and antioxidant enzyme activities

Overall, arsenic did not affect fish weight growth. However, regarding fish size, smaller fish gained less weight and bigger fish gained more weight in the As treatment compared with the fish in other treatments (Fig. 1 and Table 2).

Arsenic exposure mostly affected AEAs in the liver (Fig. 2) and seldom in the gills of eastern mosquitofish (S. Table 1). At the shorter time of exposure (day 9) arsenic has a significant effect on SOD and GR activities and the interaction was statistically significant, indicating and these effects were influenced by the presence of periphytic biofilm (Fig. 2A and C). Further these activities did not change much, except that there was a slight decrease of GR activities in the B and B + As treatments at day 25 (Fig. 2C). GST activity was significantly higher in +As treatments, lower in B treatments but the interaction was not significant. Catalase activity (Fig. 2B) increased significantly with arsenic (in both As and B + As treatments) at the end of the study (day 57).

Results of within PCA (Fig. 3) show the response of the set of endpoints measured: gained weight of fish per day, arsenic in fish body (except gill and liver) and the AEAs both in liver and gills. At the beginning of the experiment (day 9), treatments were not isolated from each other, showing a large variability. At day 25, there was an obvious separation between the B treatment and the rest of the treatments and this separation are mostly correlated positively with high APX and CAT activities in the liver and also APX activity in the gill, and negatively with fish weight, arsenic concentration in fish and GST activity in liver. At day 41, there was still a separation of B treatments. At day 56, arsenic

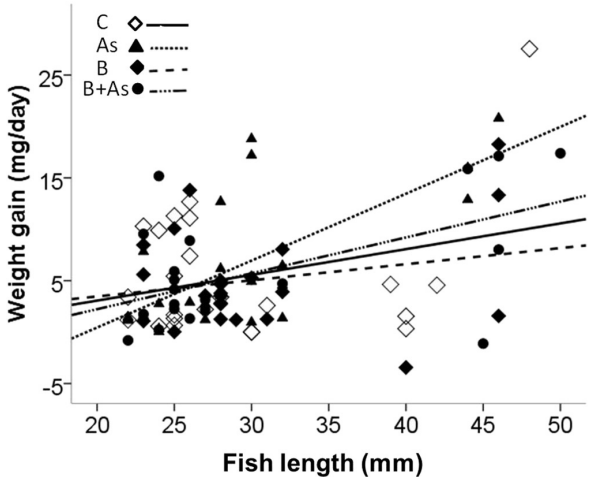

Fig. 1. Gained weight of fish in the treatments a day linked to their body length. Control treatment (C) in open diamond and black line; arsenic treatment (As) in triangle and dotted line; biofilm treatment (B) in black diamond and dashed line; biofilm plus As treatment (B + As) in circle and dotted–dashed line. The lines show a linear regression of the gained weight and fish length.

Table 2
Analyses of covariance for the variable "Gained weight a day", with arsenic and biofilm as a categorical factor and fish length as a covariate. SS — sum of squares, d.f — degrees of freedom, and *p* probability of showing an effect. Numbers in italic when significance value is $p < 0.1$.

	Gained weight a day (mg)		
	SS	d.f.	*p*
Fish length	608.07	1	< 0.0001
Arsenic (As)	18.42	1	0.413
Biofilm (B)	14.98	1	0.463
Fish length: As	88.37	1	*0.075*
Fish length: B	51.78	1	0.174
Error	5.25	84	

effects were clearly shown since all treatments with arsenic (+As and B + As) were separated (left side of the graph) from those having no As (C and B) (right side of the graph) and this separation is mostly correlated with CAT activities in the liver. B + As and +As could not be separated from each other, B + As being less variable than +As. C was more variable than the B treatment but both treatments overlapped and were also closely located (Fig. 3).

4. Discussion

In our experiment, arsenic exposure changed over time. It was mainly retained in the sediment with less contribution of periphyton as described in a previous publication focused on the fate and effects of arsenic on periphytic and epipsammic biofilms that included only the B and B + As treatments, the two treatments that had periphytic biofilms (Tuulaikhuu et al., 2015). The average exposure concentrations to arsenic were relatively low: 34.4 ± 1.4 μg/L for B + As and 40.5 ± 7.5 μg/L for +As treatment, within the range found in polluted surface waters. This low concentration affected both, periphytic communities and fish.

4.1. Effects of arsenic on fish growth

In contrast to our expectations, arsenic effects on fish growth were biomass dependent. Some studies show fish growth decreases during arsenic exposure; for example, Lima et al. (1984) exposed fathead minnow (*Pimephales promelas*) and flagfish (*Jordanella floridae*) to arsenite (4.3 mg/L and 4.12 mg/L for the two species) in 29-day and 31-day tests and found that growth was significantly reduced. However, in our experiment (2 orders of magnitude lower concentration) the growth of small-bodied fish was significantly lower and larger females gained more weight in the +As treatment compared with the control. In a similarly designed study (Magellan et al., 2014), larger individuals of this species also gained more weight with arsenic, and it was attributed to the induced aggressive behaviour of bigger fish leading to the capture of more food than smaller fish. Toxicity data from 173 tests including chronic exposure to metals, pesticides, unclassified organics, inorganic compounds, detergent chemicals and complex effluents were examined and, whereas larval growth was reduced by 36%, adult growth was seldom (5%) reduced at the lowest effect concentrations (Woltering, 1984). Our results indicate that environmentally realistic concentrations of arsenic may influence growth. However, gaining fish weight and/or growth reduction cannot be good indicators of low dose toxicity, since fish growth may depend on other factors such as behaviour, physiology and the growth stage of the exposed fish.

4.2. Antioxidant enzyme activities

Arsenic exposure mostly affected AEAs in the liver and seldom in the gills of eastern mosquitofish. Arsenic effects were detected at day 9 (SOD and GR activities in the liver were slightly higher in +As than in C treatment), later at day 25 (GST in the liver was higher in both arsenic

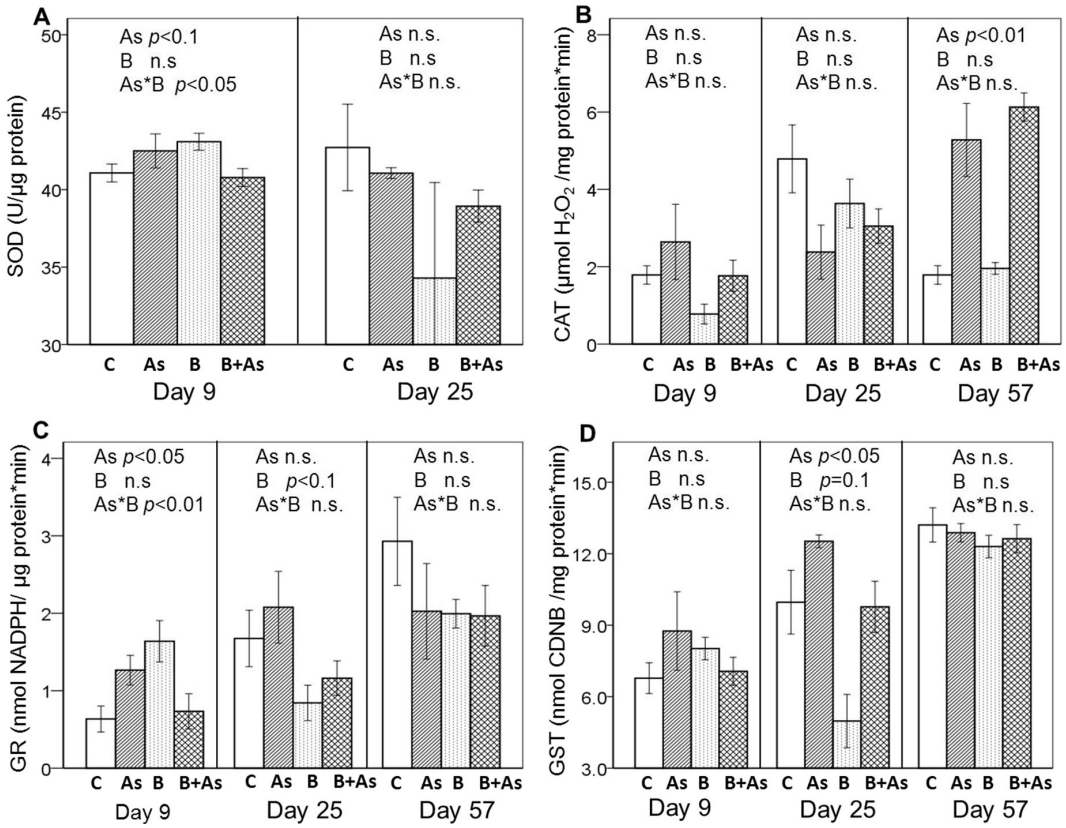

Fig. 2. Antioxidant enzyme activities (AEA): A superoxide dismutase (SOD), B catalase (CAT), C glutathione reductase (GR) and D glutathione-S-transferase (GST) in liver of fish in different treatments. The letters on the bottom of the bars represent type of the treatments: control (C), with arsenic (As), with biofilm (B) and both with arsenic and biofilm (As + B). In the upper part of each group of bars, the results of repeated measure ANOVA with two between subject-factors (arsenic (As) and biofilm (B)) are shown. The bars represent mean of three replicates of the treatments and ± standard errors.

treatments compared with their respective controls) and at the end of the experiment, after 56 days of exposure when CAT in the liver of exposed fish was clearly enhanced (more than two times higher than their respective controls). It is interesting to highlight that SOD and GR activities at day 9, showed an opposite response to the presence of biofilm (when we compare control and biofilm treatments) than to arsenic (when we compare biofilm and biofilm + As treatments), leading to a significant interaction in the two way ANOVA. The effects of arsenic seem to be lower than expected in the B + As treatment. In fact SOD and GR activities were higher in the + As than in the B + As treatments, and the latter was similar to controls indicating an ameliorating effect of periphytic biofilms on arsenic toxicity.

After 24 days of exposure, GST activity in the liver was affected (positively) by arsenic and negatively by the presence of periphytic biofilms. The elevated levels of GST could be indicating a shift toward a detoxification mechanism under exposure to longer periods to arsenic. The liver of fish is the main organ for xenobiotic metabolism (Lushchak, 2011) and this enzyme represents 10% of total cytosolic liver proteins (Leblanc, 1994). GST activity has been studied in different fish species in the tissues of liver, kidney and gill in both laboratory and under field conditions (Srikanth et al., 2013). In a chronic exposure (90 days) to As_2O_3 (1 mg/L), GST levels in fish liver first increased at day 7, then decreased after 14–30 days when compared with control. However, this activity increased during prolonged exposures (Allen and Rana, 2004). GST levels in captured fish from heavy metal contaminated areas have shown significant increases (Fonseca et al., 2011; Oliva et al., 2012). CAT, GST activities and levels of GSH were induced in the organs of fish exposed to coal ash, which contains numerous organic and inorganic toxic compounds including more than a dozen heavy metals and dioxins (Ali et al., 2004). Allen and Rana (2004) showed that arsenic manifests its toxicity by inducing oxidative stress and antioxidant enzymes, especially the glutathione-dependent enzymes, which play a protective role in arsenic toxicity as shown in the present investigation.

Lower activities in treatments with periphytic biofilms support our hypothesis about the positive effect of periphyton on the prevailing environmental conditions. In fact, GST was on average lower in the B control than in the C treatment, and this can be attributed to environmental factors (Winston and Giulioz, 1991). Based on this observation, we can also suggest that the effects of arsenic in the treatment with biofilm (B + As) could also be indirect and attributed to environmental changes, stemming from the effects that arsenic caused on the structure and function of periphytic biofilms, losing its water purification capacity approaching the environmental conditions prevailing in the control (treatment without periphytic biofilms). Periphyton facilitates fish habitat by improving water quality by taking up ammonia and nitrate, trapping suspended solids, producing oxygen, breaking down organic matter and increasing nitrification producing oxygen, helping also to decompose fish wastes (Keshavanath, 2014). In our experiment, the role that periphytic biofilms play on water purification was clearly demonstrated as having higher oxygen and lower conductivity in the presence of biofilm (Table 1).

The most significant increase occurred in the CAT activity in the liver at the end of the experiment in + As and B + As treatments but the interactive influence of biofilms was not further observed. Water dissolved oxygen was similar between the + As and B + As at that time,

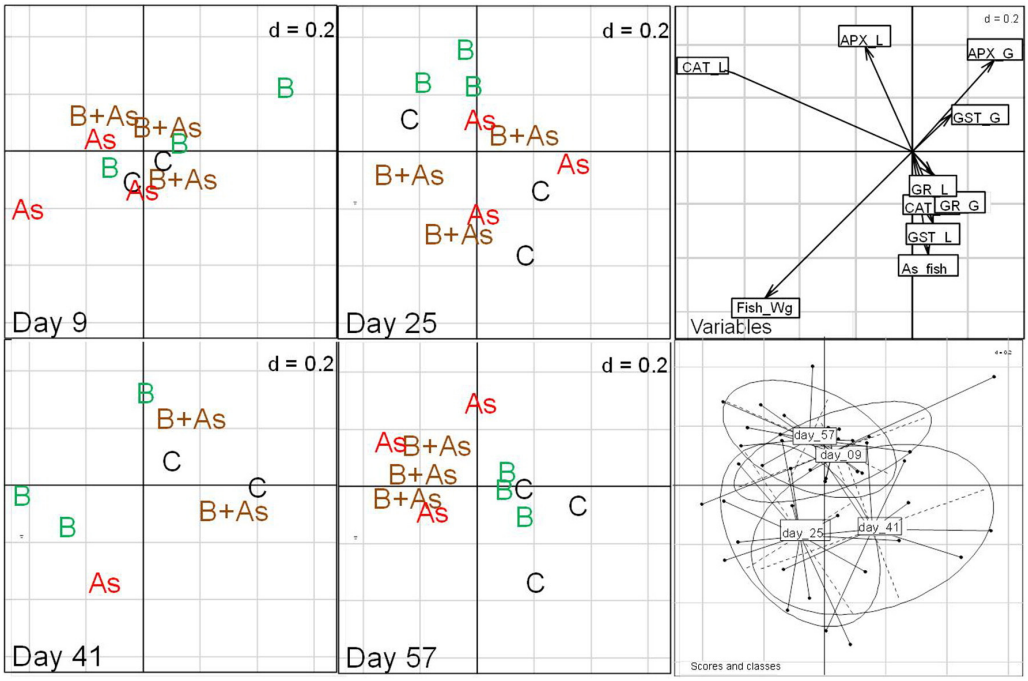

Fig. 3. Temporal variations and dispersion between replicates based on within PCA analyses on data of AEA, As contents in fish and changes in fish weight in each treatment. Projected inertia was 21.0% for axis 1 and 17.1% for axis 2. Treatment: C-control (black), B-biofilm (green), As-arsenic (red), B + As-biofilm + arsenic (brown). Variables (top right) represents the covariances between the 10 variables and the two first axes of the within PCA. Scores and classes show the projections of the individuals onto the plane defined by the axes of the within PCA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

showing that the arsenic-affected periphytic biofilm has lost its role in water purification having lower biomass and alkaline phosphatase activity (Tuulaikhuu et al., 2015).

The increase in CAT activity was very marked and related with arsenic toxicity. In comparison with our results, Atli et al. (2006) reported that the response of CAT activity in different tissues of the freshwater fish *Oreochromis niloticus* exposed to different metals was variable depending on tissues, metals and their concentrations. An inhibition of the CAT activity is commonly reported in experiments exposing fish to high arsenate or arsenite concentrations. For instance Altikat et al. (2013) reported that CAT activity decreased in *Cyprinus carpio* after 30 days of exposure to 0.5-1 mg/L, in *Clarias batrachus* exposed to 1 mg/L sodium arsenite for 60 days (Kumar and Banerjee, 2014), and also in *O. niloticus* exposed to 7 mg/L sodium arsenite for 24 days (Zahran and Risha, 2014). On the other hand, an increase in the CAT activity is commonly found in metal polluted environments. For instance, when indigenous mussels, *Mytilus galloprovincialis* of the Saronikos Gulf of Greece, were used for monitoring heavy metal pollution, CAT activities increased 2–3 times at polluted sites when they were compared with the control site (Filho et al., 2001; Vlahogianni et al., 2007). Viana et al. (2013) showed higher CAT activity in the liver of fish and histopathological changes in samples from high and medium contaminated rivers. In our experiment, we tried to mimic low but extended arsenic exposure, thus it is not surprising that our results agree with those obtained in the field as a response to chronic metal exposure.

While arsenic toxicity to fish has largely been investigated (e.g. Altikat et al., 2014; Bagnyukova et al., 2005; Bhattacharya et al., 2007), very few studies show toxicity at low concentrations (5 out of 145 papers from Web of Science, which show results of experiments of fish exposure to arsenic), and our results agree with these few studies. For instance, Ventura-Lima et al. (2009) showed an increase in glutathione levels in the gills of zebrafish (*Danio rerio*) after exposure to 10 μg/L sodium arsenite for 48 h; de Castro et al. (2009) presented impaired long-term memory and increased protein oxidation for zebrafish exposed to 10 and 100 μg/L potassium dihydrogen arsenate for 96 h, and Kumar et al. (2014), found marked genotoxicity effects for goldfish (*Carassius auratus*) exposed to arsenic trioxide at 10 μg/L (15 days).

Weight gain effects were mainly seen in treatments with no periphyton, supporting the hypothesis that periphyton may play a protective role. However, it was probably lost as periphytic biofilm was being affected by arsenic and losing this protective role. It is represented in the within PCA results showing no interaction between treatments and a clear separation between all treatments with arsenic from those without arsenic at the end of exposure.

Our results highlight again the interest and application of our experimental setup specifically designed to examine the interacting effects of naturally occurring periphytic biofilms, thus incorporating some of the complexity of natural systems in ecotoxicology.

5. Conclusions

We can therefore conclude that chronic exposure of mosquitofish to 34–40 μg As/L will cause effects, but the presence of periphytic biofilm may influence the response. The average exposure concentrations to arsenic for fish were between 3.75 and 4.5 times lower than the criterion continuous concentration (CCC) (150 μg/L), which is an estimate of the highest concentration of a substance in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect (USEPA, 2014). This low concentration of arsenic not only affected biofilm, as detailed in our previous publication (Tuulaikhuu et al., 2015), but also affected fish as shown by the effects observed in different AEAs and fish growth. AEA induction seems to be a good mechanism to cope with oxidative stress, producing a transitory and/or defensive response, whereas changes in biomass are expected to affect more the population persistence. These results support the use of lower CCC, such as the water quality criteria to protect aquatic

life recommended by the Canadian government which is 5 μg As/L (CCME, 2001).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Financial support was provided by the Spanish Science and Education Ministry (Project CTM2009-14111-CO2-01) and the Spanish Economy and Competitiveness Ministry (Project CGL2013-43822-R). Baigal-Amar Tuulaikhuu benefited from a doctoral fellowship from the Techno 2 Program of the European Union Erasmus Mundus partnership. Thanks to Kit Magellan for providing the fish and giving recommendations for maintaining and measuring them. Thanks also to Laura Barral for helping with the lab work, and Luisa Matas and Imma Arrom from the Technical Research Services of the University of Girona for guiding arsenic analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.11.126.

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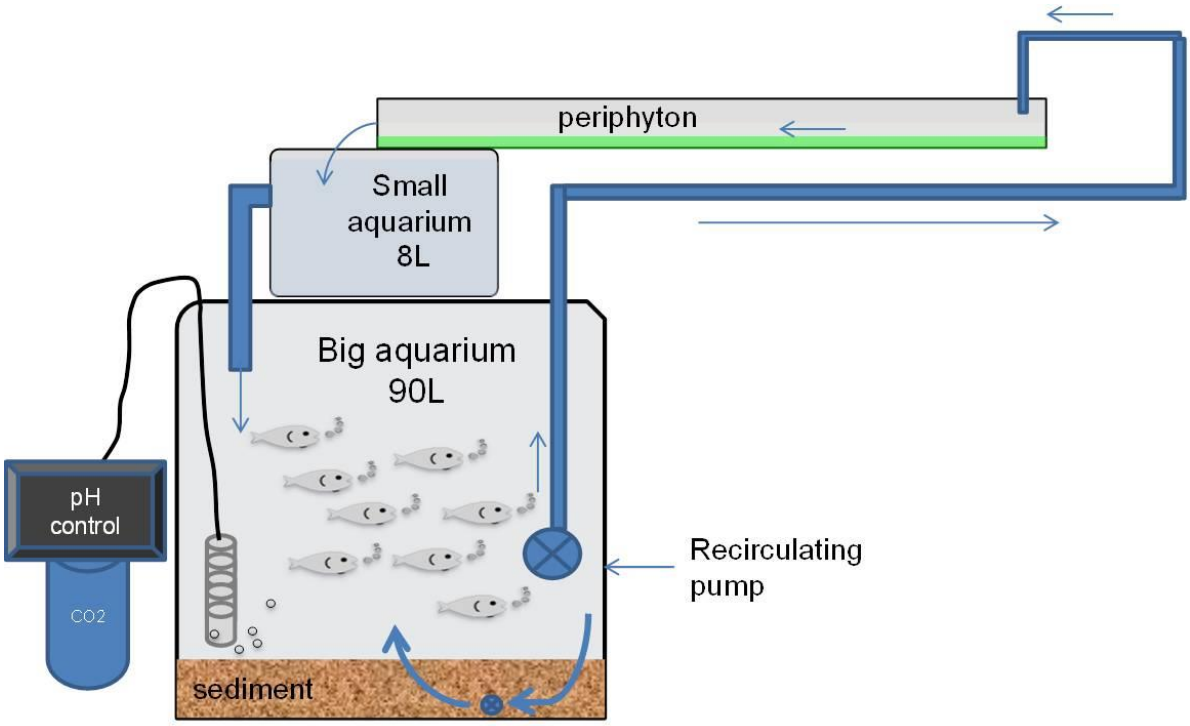
Supplementary table and figure of the article 2

S.Table Measured values of antioxidant enzyme activities in liver and gills of mosquito fish exposed to sodium arsenite for 56 days. Antioxidant enzyme activities (AEA): Ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutathione-S-transferase (GST), superoxide dismutase (SOD),

Enzyme activity	Day	Average	In liver				In gill			
			C	As	B	B+As	C	As	B	B+As
APX (nmol ascorbate/ mg protein*min)	9	mean	2.24	3.65	3.88	3.93	3.58	2.11	3.99	4.30
		SD	1.59	0.69	0.92	1.19	1.38	1.43	1.19	0.95
	25	mean	0.50	0.57	1.00	0.60	2.43	2.33	2.87	2.33
		SD	0.78	0.55	0.10	0.30	1.38	0.74	1.19	1.40
	41	mean	0.15	0.01	0.54	0.43	5.00	2.00	1.50	3.50
		SD	0.07	0.00	0.09	0.33	1.41	0.00	0.71	0.71
	57	mean	6.40	6.97	4.73	6.07	5.60	7.53	7.63	7.60
		SD	0.62	1.33	0.61	0.55	1.47	1.56	2.65	0.82
CAT (μmol H_2O_2 mg / protein*min)	9	mean	1.79	2.64	0.78	1.77	0.56	0.36	0.81	0.61
		SD	0.41	1.69	0.44	0.70	0.42	0.18	0.21	0.56
	25	mean	3.46	2.38	3.64	3.05	2.75	1.95	1.88	1.58
		SD	2.18	1.21	1.09	0.77	0.67	0.72	0.70	0.44
	41	mean	1.38	2.29	3.44	2.22	1.15	1.05	0.96	1.31
		SD	0.64	0.00	0.48	2.01	0.02	0.00	0.05	0.25
	57	mean	1.69	5.28	1.96	6.13	2.02	1.91	1.60	1.42
		SD	0.69	1.64	0.26	0.63	0.38	0.89	0.51	0.28
GR (nmol NADPH/ min /μg of protein)	9	mean	0.64	1.27	1.64	0.73	1.09	0.89	0.75	1.18
		SD	0.29	0.33	0.46	0.39	0.44	0.22	0.30	0.26
	25	mean	1.67	2.08	0.84	1.16	1.54	1.11	0.93	1.25
		SD	0.63	0.80	0.40	0.38	1.28	0.13	0.45	0.28
	41	mean	2.50	3.00	2.50	2.50	2.50	2.00	1.50	2.50
		SD	0.71	0.00	0.71	0.71	0.71	0.00	0.71	0.71
	57	mean	2.93	2.03	1.99	1.97	1.87	1.68	1.63	1.74
		SD	0.99	1.07	0.32	0.68	0.32	0.48	0.52	0.36
GST (nmol CDNB conjugated/mg protein*min)	9	mean	6.78	8.75	8.02	7.06	3.66	3.27	4.19	4.28
		SD	1.12	2.86	0.81	1.02	0.40	0.47	1.64	0.77
	25	mean	9.96	12.52	4.98	9.77	3.71	4.48	3.76	3.61
		SD	2.32	0.46	1.94	1.86	0.51	0.24	0.79	0.20
	41	mean	17.50	16.50	15.50	18.25	7.75	4.50	4.25	8.50
		SD	2.83	0.00	0.71	3.18	1.06	0.00	0.35	1.41
	57	mean	13.21	12.88	12.30	12.63	4.27	3.83	4.23	4.57
		SD	1.24	0.66	0.82	1.02	0.26	1.28	1.15	0.40
SOD (U/μg of protein)	9	mean	41.08	42.50	43.10	40.78	33.41	38.64	37.82	35.86
		SD	0.99	1.90	0.94	0.99	4.70	2.51	5.83	1.89
	25	mean	42.72	41.06	34.29	38.93	27.55	31.79	24.52	19.62
		SD	4.84	0.60	10.69	1.80	22.53	17.63	18.91	11.03
	41	mean	44.42	44.03	44.26	43.55	38.05	35.69	33.65	40.25
		SD	0.11	0.00	1.00	0.89	2.67	0.00	3.56	0.67

S. Figure. Schematic diagram of the experimental settings



Article 3

Examining predictors of chemical toxicity in freshwater fish using the Random Forest technique

Submitted

Examining Predictors of Chemical Toxicity in Freshwater Fish using the Random Forest Technique

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Abstract. Chemical pollution is one of the main issues globally threatening the enormous biodiversity of freshwater ecosystems. The toxicity of substances depends on many factors such as the chemical itself, the species affected, environmental conditions, exposure duration, and concentration. We used the random forest technique to examine the factors that best predict toxicity in a set of widespread fishes and analyses of covariance to further assess the importance of differential sensitivity among fish species. Among 13 variables the five best predictors of toxicity with random forests were, by order of importance, the chemical substance (i.e. CAS number), octanol-water partition coefficient (log *P*), pollutant prioritization, ECOSAR classification and fish species for 50% lethal concentrations (LC_{50}) and the CAS number, fish species, log *P*, ECOSAR classification and water temperature for no observed effect concentrations (NOEC). Fish species was a very important predictor for both endpoints and with the two contrasting statistical techniques used. Different fish species displayed very different relationships with log *P*, often with different slopes and with as much importance as the latter predictor. Therefore, caution should be exercised when extrapolating toxicological results or relationships among species and further research of species-specific sensitivities and the mechanisms that cause them is needed.

Key words: toxicology, log P, species-specific, sensitivity

GENERAL DISCUSSION

GENERAL DISCUSSION

Effects of chemicals on microbial communities (biofilms) and fish

This thesis aimed to evaluate the effects of a toxic compound in two major, interacting components of the freshwater ecosystem, biofilm and fish. In agreement with our hypotheses, it was shown that not only microbial communities but also sediment particles reduced arsenic exposure and nutrient availability. In this case, a clear sink of the toxic compound from the water phase to the sediment was responsible for a reduced exposure of fish to arsenic. In spite of the low concentration of arsenic (34.4 ± 1.4 (mean ± SD) µg/L for B+As and 40.5 ± 7.5 µg/L for As treatment) antioxidant enzyme activities (AEAs) underwent several changes with an increase of GR (day 9), GST (day 25) and CAT (day 57) activities in the liver of the exposed mosquitofish compared to those non-exposed. These results are in agreement with the findings of Ventura-Lima et al. (2009) and de Castro et al. (2009), who showed biochemical effects on zebrafish after exposure to 10 µg/L sodium arsenite for 48 h and to 10 and 100 µg/L potassium dihydrogen arsenate for 96 h (respectively) and Kumar et al. (2014) that found genotoxicity effects for goldfish exposed to arsenic trioxide at 10 µg/L (15 days). We can therefore conclude that either arsenite or arsenate cause toxicity to different fish species at concentrations between 10 and 100 µg/L **(article 2**).

It was also shown that arsenic effects were influenced by the presence of biofilm (significant interaction) at the beginning of the exposure (article 2). Biofilm not only contributed to reduce arsenic concentration in water (lower bioavailability) but also to improve water quality (due to its role in water purification). We hypothesise that when arsenic exposure was conducted without biofilm, chemical stress (arsenic toxicity) was added to environmental stress (poor water quality, Figure 1).

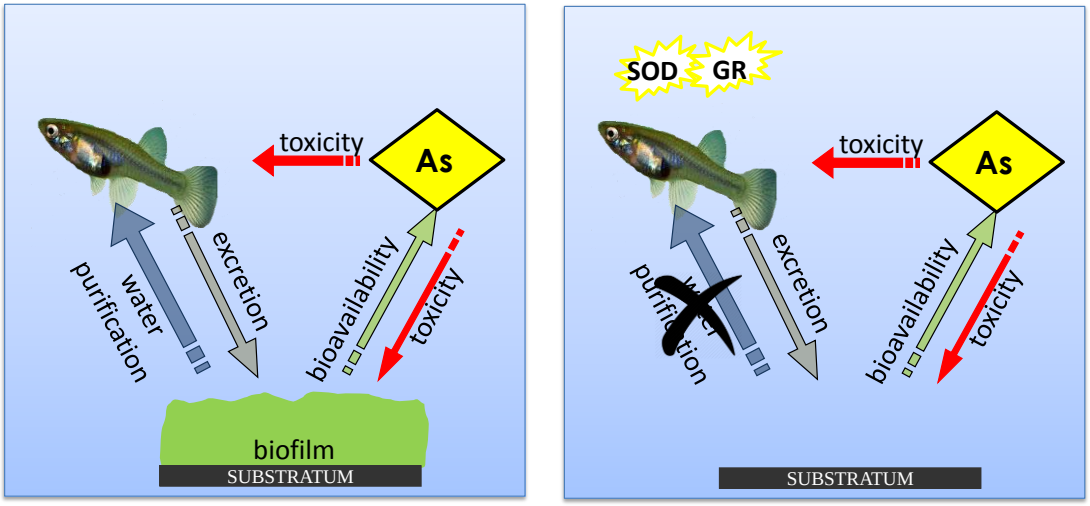

Figure 1. Influence of biofilm on the effects of arsenic on fish. Arsenic effects were not significant with the presence of biofilm whereas antioxidant enzyme activities (superoxide dismutase, SOD and glutathione reductase, GR) increased with no biofilm after 8 days of exposure (article 2).

In contrast with our results that indicate that biofilms may temporally reduce arsenic toxicity, under similar arsenic exposure conditions Magellan et al. (2014) concluded that the presence of biofilms increased arsenic toxicity. Biofilms may retain arsenic and biotransform it into less or more toxic forms. Compared to Magellan et al. (2014), who reproduced a simplified fluvial system with periphytic biofilm and fish, in our experiment we used higher fish density (2 times more) and added another component (sediments) that was responsible for arsenic retention, making arsenic concentration almost two times lower (50-70 µg/L). On the other hand, sediment also reduced water phosphate concentration (2 times lower or 5-6 µg/L) in spite of the fact that fish density was higher. The concentration of phosphate may also influence the biotransformation of arsenic. While arsenic is expected to methylate under low-P conditions, this transformation is not completed if P availability increases (Figure 2). Differences in P availability between our experiment and the experiments of Magellan et al. (2014) could have led to differences in the dominant biotranformation pathways and thus toxicity to

fish; however, this hypothesis requires a validation since arsenic speciation was not measured.

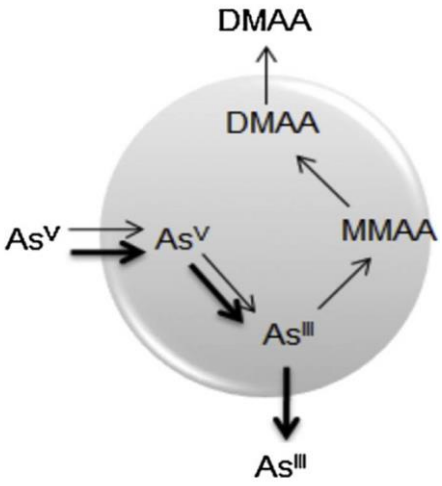

Figure 2. *Arsenic biotransformation depends on phosphorus availability. In P-deficient conditions (represented by narrow line) As^{V} is uptaken by algae and reduced to As^{III}, further methylated so the final production will be methylated arsenic (less toxic than the inorganic form). But in P –replete condition (represented by thick line) As^{V} is uptaken and reduced but could not all be methylated, so algae excrete it as As^{III} considered to be more toxic than As^{V} (Hellweger et al., 2003).*

The influence of biofilm on arsenic toxicity was not seen in the end of the experiment (chronic exposure) **(article 2)**, since arsenic-affected biofilm had lost its role in water purification with poor oxygenation and high conductivity (Graphic abstract, article 2). Focusing on the effects of arsenic on periphytic biofilm (article 1), the effects observed were similar to those reported in Barral-Fraga et al. (2016). This paper describes the effects of arsenic exposure that the experiment previously described in Magellan et al. (2014) had on periphyton. In this experiment arsenic and phosphate concentrations were higher than in our experiment, but the As/P ratio, which is assumed to be a better toxicity predictor than arsenic concentration (Wang et al., 2013), was similar to ours (around nine in both cases) and the effects on periphyton were also similar: the photosynthetic activity was inhibited (making it less phototrophic, Figure 3) and algal groups were in both cases affected. However, in our experiment diatoms were selectively affected by arsenic and adaptation was less evident (the percentage of diatoms, measured as % F_0 browns decreased at the end of the experiment). This

difference may be attributed to the duration of the experiment (60 days compared to 13 days) and also to phosphate concentration. As suggested in Rodríguez-Castro et al. (2015), adaptation of periphyton to arsenic exposure may require phosphate since the mechanism of arsenic excretion (membrane pumps) is ATP dependent (Leyy et al., 2005; Rosen, 1999).

It is important to highlight that the microbial community contributed to nutrient cycling and that arsenic influenced this role. We can therefore suggest that if a toxic compound affects nutrient dynamics, it will influence the whole ecosystem, including fish (Figure 3).

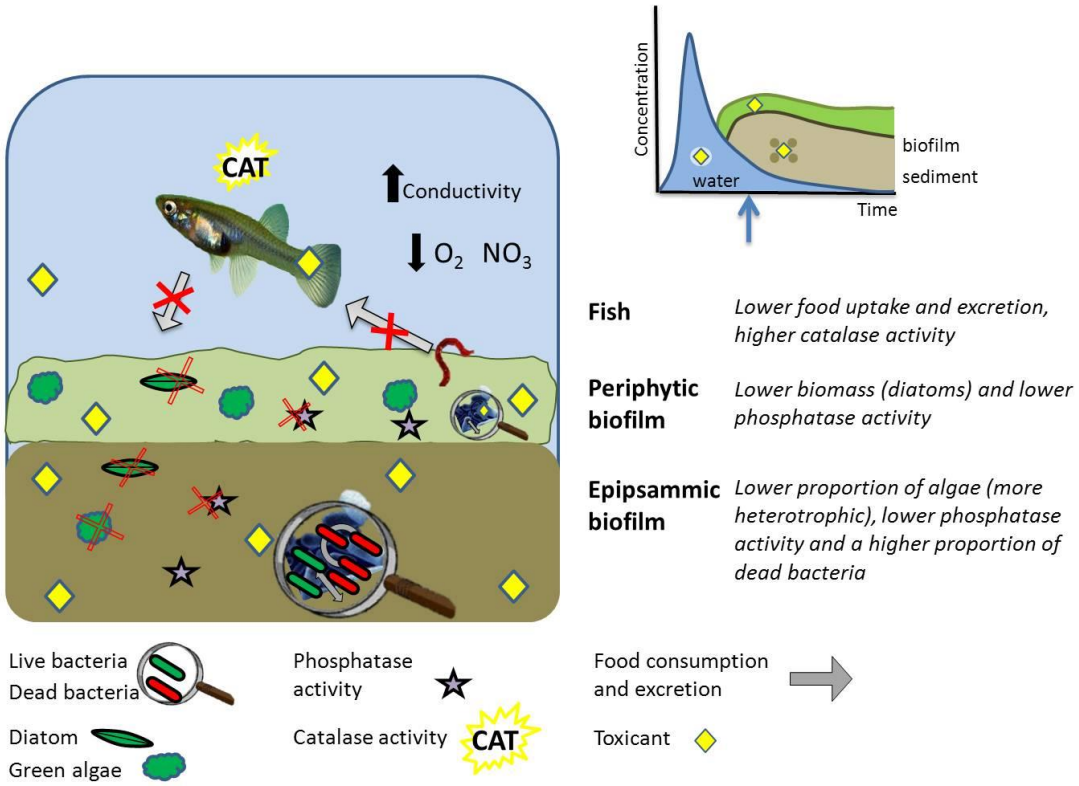

Figure 3. Summary of chronic effects of arsenic on biofilm, fish and fish-biofilm interactions detailed in chapters 1 and 2. Changes in the structure of periphytic biofilm (less diatoms and more green algae), a decrease in bacterial viability in the epipsammic biofilm and alkaline phosphatase activity in both. Fish excretion reduced as a result of lower food consumption and lower oxygen concentration and higher water conductivity due to photosynthesis inhibition. The upper graph illustrates the fate of arsenic to different river components (from water to sediment and biofilm) and the approximate time of exposure (marked with a blue arrow).

But what can we expect in terms of nutrient cycling if a toxic compound affects mainly fish? The role that fish play as nutrient subsides may partially be impaired and hence affect the structure and function of the microbial community. A reduction in nutrient availability may also influence toxicity for compounds like arsenicals since phosphorus deficiency has a strong influence on arsenate toxicity to algae.

We would also like to focus on the contrasting effects of arsenic with biofilm (comparing the treatment with biofilm with the treatment with biofilms and arsenic) and without (comparing the treatment without biofilm with the treatment whit no biofilm and arsenic, Figure 4) since this comparison gives information about the effects expected in streams where both biofilm and fish are present. Under natural conditions, fish may play an important role as nutrient subsides, whereas biofilm will actively uptake nutrients and this tight interaction be a key aspect in the ecosystem functioning. Figure 4 illustrates how toxicity may affect this tight interaction since treatment effects on the AEAs are more severe with biofilm (B & B+As) than without (C& As).

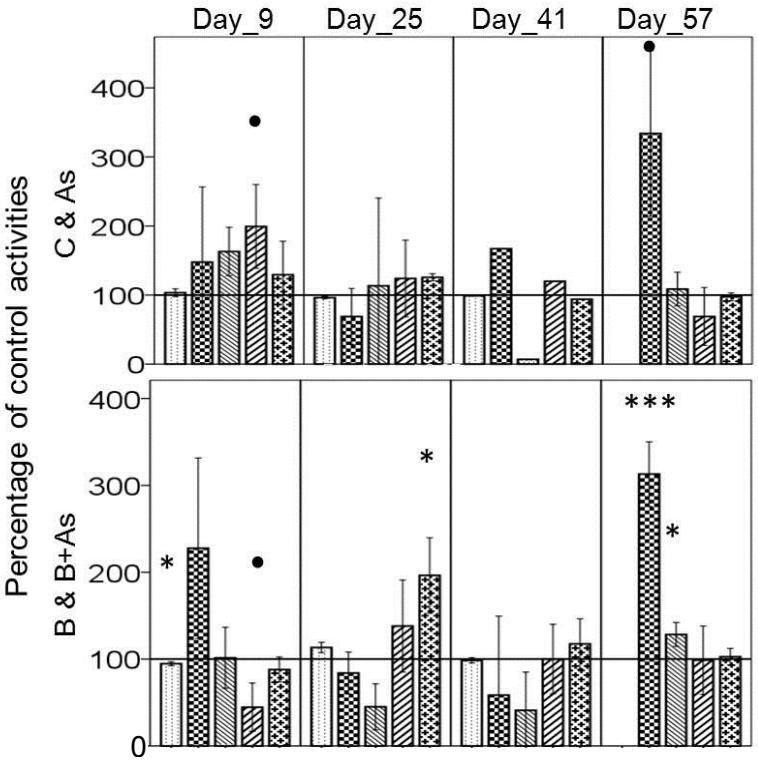

*Figure 4. Antioxidant enzyme activities (AEA): superoxide dismutase (SOD) in dots, catalase (CAT) in squares, ascorbate peroxidase (APX) in up-down left lines, glutathione reductase (GR) in up-down right lines and glutathione-S-transferase (GST) in diamond in liver of fish in different treatments in % of their respective controls: C & As (Arsenic compared to control), B & B+As (Biofilm plus arsenic compared to biofilm). The symbols (• when p<0.1 and * when p<0.05) were atta-ched presenting statistical results.*

Protecting aquatic organisms and their normal functioning in their natural habitat is challenging to conservation of aquatic systems. Therefore, our research highlights the interest and application of incorporating some of the complexity of natural systems in ecotoxicology.

Arsenic in fluvial systems and its effects on aquatic communities

Risk assessment is based on the fact that a toxic compound will cause environmental concern when the range of potential toxicity (based on laboratory studies) and the range of real exposure overlap. In our experiment, the average exposure concentration (37 µg/L with range between 28 and 120 µg/L) was in the range of river water arsenic concentrations (see Table 1, Introduction) and had several effects on biofilm, fish and their functions (Article 1, 2). In European polluted rivers, arsenic concentration ranges between 4 and 45 µg/L, and under the influence of mining activities, it may reach up to 7900 µg As/L with an average value around 140 µg As/L (Bundschuh et al., 2012; Smedley and Kinniburgh, 2002). Our results do not support the criterion continuous concentration of As (estimate of the highest concentration of a substance in surface water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable effect value of 150 µg/L proposed by the USEPA (2014)), highlighting that this threshold should be updated.

Arsenic exposure thresholds for environmental health are 15 times higher than those established for human health (10 µg/L, Council Directive 98/83/EC). This big difference is probably related with the long human lifespan and the difference between most toxicity data (obtained from shot-term tests) and the effects expected after chronic or repetitive exposures. The results of this thesis indicate that fish can be affected at low

concentrations of arsenic, probably due to their comparatively long lifespans (about 2-10 years) and because they continually inhabit the receiving water and integrate their chemical, physical and biological histories (Benejam, 2008). Arsenic effects on fish antioxidant enzyme activities were obvious after chronic exposure (8 weeks). The increase of antioxidant enzyme activities in fish liver, probably indicates that a detoxification mechanism was working. Potential impacts of chronic exposure may be immunological changes and cancerous effect, since arsenic is a well known carcinogen (Smith et al., 1992). Huff et al. (2000) suggested that inorganic arsenic must be assumed as carcinogenic in animals, but more research is need to confim it.

Our results are in agreement with recent investigations focused on the effects of low concentrations of arsenic on biofilms (Barral-Fraga et al., 2016; Rodriguez Castro et al., 2015) in oligotrophic conditions, but disagree with other investigations compiled in the ECOTOX database, where NOEC (no observed effect concentration) values for aquatic organisms range between 10 and 180000 μg/L arsenate and between 300 and 9800 μg/L arsenite (Figure 5).

The effects of environmentally realistic concentrations of arsenic to periphyton could be much related with the bioavailability of phosphorus since our experiments were done under oligotrophic (low-phosphate) conditions, contrasting with the lack of effects on periphyton reported in Rodríguez-Castro et al. (2015), in treatments with phosphate.

While the effects caused to algae (growth inhibition, selection pressure on the species composition and a reduction of P uptake) under low-P conditions (Rodríguez-Castro et al., 2015) will presumably affect (indirectly) fish, a different response is expected under high-P conditions. In this case indirect effects of arsenic on nutrient cycling are not expected (since algal growth is not affected and the P uptake is low and

not affected by arsenic) but direct effects of arsenic exposure on fish may be higher since biofilm activity could be driving the biotransformation of arsenic (transforming As^{V} into As^{III} which is more toxic).

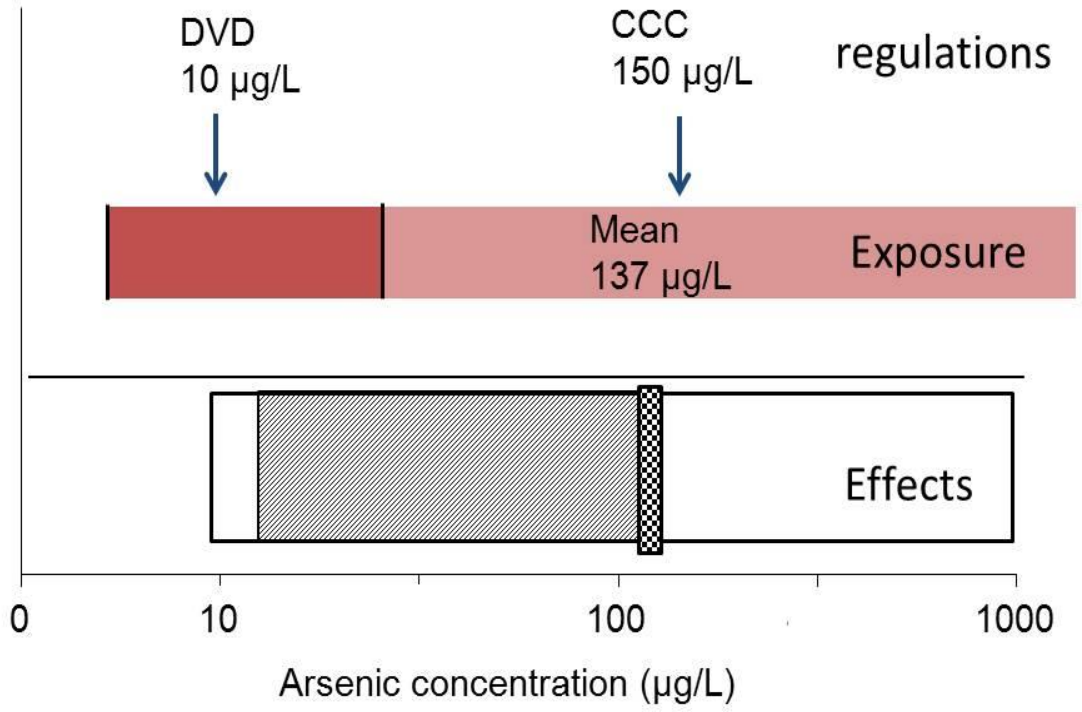

Figure 5. Arsenic concentration in river water (exposure) and effects on aquatic organisms. Blue arrows mark the EU Drinking Water Directive (DVD), and the US-EPA Criterion of Continuous Concentration (CCC). The red bar represents the range of concentrations in European polluted rivers reviewed by Smedley and Kinniburgh (2002) and the pink bar represents the range in rivers near mining zones. The longest horizontal white bars represents the range of NOEC values for aquatic organisms (ECOTOX database), the horizontal bar with slanting lines represents the range of concentrations used in this study, and the narrow vertical bar with dots other periphyton toxicity studies (Barral-Fraga et al., 2016; Rodríguez-Castro et al., 2015).

Upscaling experimental results to real exposure

Experimental conditions in ecotoxicology will always contrast with real aquatic systems characterised by having a mixture of chemicals, many fish species and different environmental conditions causing high uncertainty. Minimal harmful or lethal concentration of a toxic compound is not necessarily a fixed quantity but may vary

greatly with the environmental experimental conditions, duration of exposure, the chemical composition of the water, the concentration of other substances having either synergetic (additive) or independent effects (de Zwart and Posthuma, 2005; Ricart, 2010), and the species and age of the organisms (Kroupova et al., 2005). The evaluations of chemical effects on ecosystems are complicated by the multitude of organisms within a given system, their interaction with each other and the physical/chemical aspects of the varied types of ecosystems (Duke and Mount, 1991). Interactions of biological population can have effects modifying toxicity of chemicals, for example if a prey population is much sensitive to a toxicant then it will indirectly affect to a predator population. The analysis of covariance in this thesis (chapter 3) showed that not only fish species sensitiveness is different among them, but also their response to toxicants may be dependent on the chemicals' type and solubility in water. This observation is in agreement with the literature supporting the general assumption that the biological effects of pollutants differ greatly among species (Posthuma et al., 2001; Vaal et al., 1997, 2000). It was also observed that chemicals differ a lot in their toxicity to fish and that it was impossible to identify a general trend. These observations illustrate how difficult is to upscale the results of the experiments presented in this thesis, although being relatively complex.

Antioxidant enzyme activities were able to inform about fish biochemical changes, particularly an increase of catalase activity caused by low concentration, chronic arsenic exposure (**article 2**). But at higher concentrations an inhibition of catalase activity is commonly reported (Altikat et al. 2013; Zahran and Risha, 2014). Here we showed that the response of fish to a low concentration of toxicant was highly dependent on the functioning of other interacting components of aquatic ecosystems, such as fluvial biofilms. It has been reported that catalase activity in different tissues of the freshwater

fish *Oreochromis niloticus* exposed to different metals was found to be variable depending on tissues, metals and their concentrations (Atli et al., 2006). On the other hand, an increase in the CAT activity is commonly found in metal polluted environments (Filho et al., 2001; Vlahogianni et al., 2007; Viana et al., 2013). Therefore the antioxidant enzyme activity does not seem to be a good surrogate for arsenic exposure in the field. Antioxidant enzyme activities, while being early signals of chemical exposure, are not specific and may not have unequivocal response to arsenic exposure in the field and therefore should be considered in the framework of a multimetric approach. This approach may include other informative variables such as histological changes or arsenic accumulation (as they more easily differentiated from controls on most of the studies summarised in the meta-analysis), among others. It is important to remind that the results obtained with random forests and covariance analyses (article 3) support the general use of biomarkers in the assessment of environmental risk of chemicals on fish. Early signals of fish physiological and biochemical changes used to derive NOEC values provided similar results (similar ranking of fish species) than mortality (LC_{50} values) showing a general link between early signals and real effects on population dynamics (mortality).

It can be concluded that monitoring is needed to provide information on the real effects of pollution, having always in mind that effects may occur at lower concentration than expected. If fish species differ in their sensitivity, according to the effect culmination theory of Liess et al. (2013), predicted effects for sensitive fish populations may occur at concentrations even lower than those used in our experiments. Liess et al. (2013) showed that over several generations to repeated pulses of low concentrations of neonicotinoid insecticide exposure, the more sensitive species continuously declined and did not recover in the presence of a less sensitive competing

species. In the absence of a competitor, the sensitive species recovered more rapidly after a contamination event. It indicates that in the environment, species interactions are important and indirect effects complicate ecological assessments, therefore a community ecotoxicological approach (Clements and Newman, 2002) needs to be considered.

It is important to provide information on biological interactions, not only among fish species but also interactions among different trophic levels and their role in ecosystem function. Extracellular enzyme activities are commonly investigated to assess the role of microbial communities on carbon and nutrient cycling in aquatic ecosystems (Romaní and Sabater, 2001). Our results showed that in biofilm exposed to arsenic, total phosphatase activity decreased due to the decrease of biofilm biomass, but β-glucosidase activity increased significantly, probably due to increased bacterial efficiency in the use of available polysaccharides released from decaying algae (**article 1**), agreeing with some literature (Haack and McFeters, 1982; Middelboe et al., 1995). Those changed extracellular activities may influence water purification capacity (organic matter degradation) of biofilm and eventually cause indirect effects of toxicants to fish. It has been shown that biofilm may rapidly adapt its enzyme activities in response to changes in water nutrient availability (Artigas et al., 2015) but information on how the adaptation changes under toxicant influence is scarce. So it is also indicating the interest of investigating biofilm enzyme activities responses to chemical exposure in field investigations.

Perspectives

Many regions in the world, for instance Mongolia, where mining activities are increasing but little is known about the environmental impacts of this activity, needs monitoring of the environmental impact of arsenic and other metals and metalloids. Recently, arsenic survey in drinking water (wells) was done in the southern Gobi region of Mongolia (Purevdorj, 2012) and noted that arsenic concentration ranged between 0-0.159 mg/L. The research, conducted in the rivers and wells near copper and gold mines to determine the impact of mining activities on the environment, declared that Mo, As, Al, Cu, Mn, Fe, Pb, U, Zn and Cd concentrations exceed both the WHO drinking water guidelines (2011) and the maximum concentration allowable in Mongolia (MNS 4586:1998) (Inam et al., 2011; Battogtokh et al., 2013).

These few scattered study results indicate that an extensive survey is needed to assess river water contamination with metals and metalloids and their environmental effects since, as demonstrated in this thesis, arsenic and other heavy metals and metalloids not only fate to the sediment and concentrate in biofilm but also influence their structure and function at low, environmentally realistic concentrations. How do sublethal effects (e.g fish antioxidant enzyme activities) alter individual fitness and broadcast to the population and fish community is still unclear and more information is needed for its application in biomonitoring. Biofilm results may provide the guidelines for an effective monitoring strategy in Mongolian rivers.

This monitoring would provide the basis for developing a regional and national wide multivariate bioassessment tool based on the structural and functional response of fluvial biofilms to metal pollution, including criterion concentrations not only in water but also in the biofilm. In Eastern Canada, Lavoie et al. (2006) developed a diatom

based index that integrated the effects of multiple stressors on streams. The diatom index was based on correspondence analysis to characterise a chemistry free reference community (Grenier et al. 2006) and the index value indicates the distance of each diatom community from its specific reference community. In Mongolia, several researches have been done for lake diatom assemblages (e.g Shinneman et al., 2009), trying to develop an approach for water quality assessment (Bukhchuluun and Soninkhishig, 2006). Nevertheless, a multivariate analysis conducted by Griffith et al. (2002) showed that analyses of species abundances were sensitive to effects associated with nutrients, substrates, and riparian vegetation, whereas analyses of periphyton metrics were sensitive not only to these nutrient and physical habitat effects but also to toxicological effects associated with metals. Therefore both species abundance analyses and periphyton metrics can together be appropriate to evaluate water pollution. If time and research facilities are possible, generating a combined data matrix based on species abundance data from different taxonomic groups (Mueller et al., 2014) could be an important tool for monitoring ecosystem and community change. This approach is urgently needed in order to establish regional and national baselines to be used to assess early effects of mining and take some measures before fluvial systems degrade irreversibly.

CONCLUSIONS

CONCLUSIONS

1) We analysed the effects of environmentally realistic exposure to arsenic on biofilms and fish and its fate in water, sediment, and biota of experimental channels. Sediments play an important role for arsenic sinks in natural systems, while biofilm accumulates high concentrations of arsenic, allowing field monitoring of contaminant exposure.
2) During arsenic exposure, structural changes occurred in the periphytic biofilm, with a reduction of the relative abundance of diatoms and an increase of green algae. Structural changes in the epipsammic biofilm were not only characterised by the formation of a more heterotrophic biofilm, but also by a reduction of bacterial viability.
3) The reduction of algal growth and activity under arsenic exposure further influenced biofilm contribution to nutrient cycling and water chemical parameters, such as water conductivity and dissolved oxygen.
4) Arsenic-affected biofilm lost its role in water purification and this indirect effect was reflected in the antioxidant enzyme activities of fish.
5) Smaller fish gained less weight and bigger fish gained more weight in presence of arsenic, revealing potential effects on fish fitness and size structure.
6) Our results highlight the interest and application of incorporating some of the complexity of natural systems in ecotoxicology and support the use of a criterion continuous concentration (CCC) for arsenic lower than 150 μg/L and closer to the water quality criteria to protect aquatic life recommended by the Canadian government (5 μg As/L).

7) Modern data analysis techniques such as Random Forests can have high predictive power because of the complexity of toxicological processes and the multitude of factors and interactions that mediate them.
8) The five most important variables to predict LC_{50} with Random Forests were the chemical substance (i.e. CAS number), log *P*, pollutant prioritization, ECOSAR classification, and fish species. Similarly for NOEC the most important variables were CAS number, fish species, log *P*, ECOSAR classification, and water temperature.
9) Our results suggest that log *P* is a good correlate of toxicity but that species × chemical substance × log *P* and species × chemical substance interactions are pervasive and important and thus only using log *P* in non-tested substances can be highly inaccurate.

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SUPPLEMENTARY INFORMATION

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Methods used for the meta-analysis of arsenic effects on fish

1. Literature search

We conducted a literature search in the Web of Knowledge database (http://webofnkowledge.com) to find papers on arsenic effects on fish using the keywords (arsenic fish effect) in the topic and all publication years. Initially 1625 papers, which contained arsenic and fish combinations in their title, key words or abstract, were found. Studies satisfying the following criteria were included in the meta-analysis:

i) the study was an experiment evaluating the effects of arsenic on fish (including all species and all growth stages) using any response variable (e.g. growth, survival, or biochemical changes).

ii) the experiment had control group (without arsenic added) and one or more treatment groups (with different exposure periods and/or with different concentrations of arsenic).

iii) the study clearly reported the duration and concentration of arsenic exposure. Therefore, field studies and studies with indirect exposures, such as transferred by fish diet were excluded from the compilation.

iv) Experimental studies consisting of more complex treatments (e.g., N-acetylcysteine + arsenic) were not included because we were insterested in the unique effects of arsenic.

Following the above mentioned criteria, we extracted 2225 cases from 54 papers using the software GetData Graph Digitizer (http://www.getdata-graph-digitizer.com/). Our database included: i) information on the fish species and biological information

including fish length, weight, growth stage, and sex when available; ii) information on the exposure including arsenic species and exposure duration and concentration, whether whole fish individuals or fish cells were used, and temperature and oxygen during exposure if available; iii) response information including affected organ and measured response variables or biomarker (means, standard deviations or standard error or confidence limits in control and treatment groups, and number of replicates); and iv) the publication reference. The standard errors or confidence limits obtained were converted to the standard deviations, when the latter where not reported.

2. Data analyses

We divided the data into two sections: acute toxicity (if exposure duration was less than or equal to 96 hours) and chronic toxicity (if the exposure duration was more than 96 hours). Acute tests are designed to evaluate the effects of toxicants on survival and other effects following exposures for a short period of their lifespan. Animals used in these tests are normally exposed for 24, 48, 72, or 96 hours in order to estimate acute toxicity. In contrast, chronic toxicity tests evaluate effects over a significant portion of the organism's life span (Hoffman et al. eds 2002). These tests often evaluate sublethal effects on reproduction, growth, and behavior, as well as mortality. When a response variable was measured at different times (within each the acute or chronic exposure data set), we only used the final measurement in the data analyses. To compare the effects of arsenic on the response variables for control and treatment groups we calculated the standardized mean difference or Hedges *d*, as a measure of effect size (Rosenberg et al. 2000) using the "metafor" package (Viechtbauer 2010) in the R environment (R Core Team, 2015). Hedges' *d* is a unit-free index which ranges $-\infty$ to $+\infty$; negative values

of *d* show decrease of that specific response due to the effects of arsenic and viceversa. Following Rosenberg et al. (2009), we calculated *d* as:

$d = \frac{\bar{X}_T - \bar{X}_C}{S_{within}}$.

In the numerator $\bar{X}_T$ and $\bar{X}_C$ are the sample means in the treatment and control groups respectively. In the denominator S_{within} is the within groups standard deviation, pooled across the groups, and is calculated:

$$S_{within} = \sqrt{\frac{(N_T - 1){S_T}^2 + (Nc - 1){S_C}^2}{N_T + N_C}}$$

Where N_T and N_C are the sample sizes in the two groups, and S_T and S_C are the standard deviations in the treatment and control groups. In the metafor package the standardized mean difference or Hedges' *d* is automatically corrected to Hedges' *g* for its slight positive bias (Hedges and Olkin 1985) using a weighting factor (*J*) that depends on the number of replicates for the treatment and control groups. *J* is calculated:

$$g = J \times d$$

$$J = 1 - \frac{3}{4\,(N_T + N_C - 2) - 1}$$

The variance of *g* was calculated as:

$$V_g = \left(\frac{N_T + N_C}{N_T\,N_C} + \frac{d^2}{2(N_T + N_C)}\right) \times J^2$$

3. Aggregating dependent effect sizes

Toxicological experiments usually have a design with multiple treatments, thus differing from conventional data where meta-analysis is generally applied. Our goal was to compute a mean effect of arsenic exposure (aggregating effect sizes for all the concentrations used in same study) on each response variable. The mean effect size of arsenic effect for a response variable of every study was computed as aggregating the effect sizes at different concentrations using the "RcmdrPlugin.MA" package (Del Re, 2013). The correlations among the within-study outcomes, as default, were assumed 0.50 (Wampold et al., 1997).

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Antioxidant enzyme activity (AEA) analyses

CAT activity was measured at 240 nm according to Aebi (1984). The reaction mixture (250 µL) contained 206.3 µL of potassium phosphate buffer (final concentration 80mM, pH 7.0), 18.7 µL H_2O_2 (400 mM), and 25 µL of enzyme extract (4 µg protein). The H_2O_2 consumption was determined by measuring the decrease in absorbance at 25°C for 3 min. CAT activity was calculated as µmol H_2O_2 per micro gram (µg) protein per minute (extinction coefficient of H_2O_2=0.039 cm^2/µmol).

APX activity was measured as monitoring the decrease of absorbance at 290nm at 25°C for 2 min due to ascorbate oxidation, according to Nakano and Asada (1981). 250µL of reaction mixture contained 170 µL of potassium phosphate buffer (final concentration 80mM, pH 7.0), 30µL H_2O_2 (30 mM), 25 µL Na-ascorbate (1.5 mM) and 25 µL of enzyme extract (4µg protein). APX activity was calculated as µmol ascorbate per µg protein per minute (extinction coefficient ε: 2.8M cm^{-1}).

GR activity was measured as monitoring the decrease of absorbance at 340 nm at 25°C for 2 min (Schaedle and Bassham, 1977). 200 µL of reaction mixture was obtained adding tris hydrochloride buffer (pH 7.5, 100 mM final concentration) and EDTA (1mM), oxidized glutathione: GSSG (1 mM final concentration), NADPH (0.15 mM final concentration) and enzyme extract (4 µg protein). GR activity was calculated as µmol NADPH per µg protein per min.

GST activity was measured monitoring the decrease of absorbance at 340 nm at 25°C for 4 min (Grant et al., 1989). 200µL of reaction mixture was obtained adding potassium phosphate buffer (pH 7.4) (100mM final concentration), CDNB (1-chloro-2.4-dinitrobenzene) (50 mM final concentration), reduced glutathione: GSH (5 mM final concentration), and enzyme extract (4 µg protein). GST activity was calculated as

μmol CDNB conjugate per μg protein per min (extinction coefficient ε: 9.6 M cm^{-1} and path length was 0.524 cm) after subtracting the Δ340 min^{-1} for the blank reaction from the Δ340 min^{-1} for each sample reaction.

SOD activity was measured at 450 nm according to the Peskin and Winterbourn (2000) method. The 200μL reaction mixture contained the following substances in the final concentration: potassium phosphate buffer (50 mM, pH 8), diethylene triamide (0.12 mM), hypoxanthine (0.1 mM), water-soluble tetrazolium salt (WST-1 (0.075 mM), and enzyme extract (4 μg protein). 8 μL xanthine oxidase was added to start the assays. WST -1 produces water-soluble formazan dye upon reduction with a superoxide anion (O_2^-). SOD competes with WST-1 for superoxide anions generated by the xanthine/xanthine oxidase system. WST-1 reduction was measured at 450nm and 25ºC for 10 min. One unit of SOD was defined as the amount of sample required for 50% inhibition of WST-1 reduction. The activity was calculated as U (unit) per μg protein after calculating the specific SOD activity (U) using the formula: (%inhibition*100/50)/(protein concentration * volume of enzymatic extract).

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