UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS
Departamento de Química Analítica

TESIS DOCTORAL

Modelo alternativo al TEST OECD 305 para la bioacumulación de especies y nanopartículas metálicas

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Ana López-Serrano Oliver

Directores

Riansares Muñoz Olivas
Carmen Cámara Rica

Madrid, 2014

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HACEN CONSTAR:

Que el presente trabajo titulado “Modelo Alternativo al Test OECD 305 para la bioacumulación de nanopartículas y especies metálicas” ha sido realizado bajo nuestra dirección por Dª Ana López-Serrano Oliver, constituyendo la Tesis Doctoral de la autora.

Y para que conste, firmamos la presente en Madrid a

Fdo: Dra. Riansares Muñoz Olivas

Fdo: Prof. Carmen Cámara Rica

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Resulta difícil escribir en unas hojas la enorme gratitud que siento hacia toda la gente que ha estado a mi lado durante este trabajo, tanto que creo que me daría para escribir una tesis que solo contuviese agradecimientos. Como eso no va a poder ser, porque ya bastante ha sido, intentaré comprimir en estas líneas mi enorme afecto. A toda la gente que ha estado a mi lado en estos casi cinco años, GRACIAS.

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Glosario

GLOSARIO

Glosario

AC GIH: American Conference of Governmental Industrial Hygienists (Conferencia Americana Gubernamental de Higienistas Industriales)

AAS: Atomic Absorption Spectroscopy (Espectroscopía de Absorción Atómica)

AF4: Assimetric Flow Field Flow Fraction (Fraccionamiento en Flujo con campo de Flujo Asimétrico)

AFS: Atomic Fluorescence Spectroscopy (Espectroscopía de Fluorescencia Atómica)

AE: Assimilation Efficiency (Eficiencia de Asimilación)

ASTM: American Society for Testing and Materials (Sociedad Americana de Ensayo de Materiales)

BCF: Bioconcentration Factor (Factor de Bioconcentración)

BCF_k: Bioconcentration Factor defined from k_1/k_2 ratio (Factor de Bioconcentración definido por la relación entre k_1/k_2)

DBT: DiButylTin (Dibutilestaño)

DLS: Dynamic Light Scattering (Espectroscopía de Dispersión cuasi-elástica de la Luz)

EFSA: European Food Safety Authority (Autoridad Europea de Seguridad Alimentaria)

EC_{50}: Half maximal effective concentration (Concentración efectiva media)

EU: European Union (Union Europea)

FAAS: Flame Atomic Absorption Spectroscopy (Espectroscopía de Absorción Atómica con Llama)

FAO (UN): Food and Agriculture Organization of the United Nations (Organización de las Naciones Unidas para la Agricultura y la Alimentación)

FDA: Food and Drug Administration (Administración de Fármacos y Alimentos)

GC-FPD: Gas Chromatography coupled to Flame Photometric Detection (Cromatografía de Gases acoplada a Detección por Fotometría de Llama)

HG-AAS: Hydride Generation Atomic Absorption Spectroscopy (Espectroscopía de Absorción Atómica con Generación de Hidruros)

HPLC: High- Performance Liquid Chromatography (Cromatografia de Líquidos de Alta Eficacia)

ICP-MS: Inductively Coupled Plasma Mass Spectrometry (Espectrometría de Masas con fuente de Plasma Acoplado Inductivamente)

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry (Espectrometría de Emisión Optica con fuente de Plasma Acoplado Inductivamente)

IR: Ingestion rate (Tasa de ingestión)

K_e: Elimination Rate Constant (Constante de Eliminación)

K_{ow}: Octanol-Water Partition Coefficient (Coeficiente de Reparto Octanol-Agua)

K_{uf}: Uptake rate constant from food (Constante de absorción desde la dieta)

LC_{50}: Median Lethal Concentration (Concentración media letal)

MBT: MonoButylTin (Monobutilestaño)

MOD: Moderately Hard Water (Agua de dureza moderada)

OECD: Organization for Economic Co-Operation and Development (Organización para el Desarrollo y Co-operación Económica)

OTCs: Organotin compounds (Compuestos organoestánnicos)

PVP: PolyVinyl Pyrrolidone (Polivinil pirrolidona)

QSAR: Quantitative Structure Aactivity Relationship (Relación Cuantitativa Estructura-Actividad)

REACH: Registration, Evaluation and Authorisation of Chemicals (Registro, Evaluación y Autorización de Sustancias Químicas)

UV: UltraViolet (Ultravioleta)

SPE: Solid Phase Extraction (Extracción en Fase Sólida)

SPME: Solid Phase Micro Extraction (Micro-Extracción en Fase Sólida)

TEM: Transmission Electronic Microscopic (Microscopía de Transmisión Electrónica)

TBT: TriButylTin (Tributilestaño)

TBTO: TriButylTin Oxide (Óxido de tributilestaño)

VHW: Very Hard Water (Agua muy dura)

VSW: Very Soft water (Agua muy blanda)

U.S. EPA: United States Environmental Protection Agency (Agencia de Protección Medioambiental de los Estados Unidos)

WHO: World Health Organization (Organización Mundial de la Salud)

ZGF-AAS: Zeeman Graphite Furnace Atomic Absorption Spectrometry (Espectrometría de Absorción Atomic por Cámara de Grafito con corrector Zeeman)

Abstract

1. ABSTRACT

Abstract

Over the last couple of decades, environmental pollution has became an increasing relevant issue to our society. Aquatic media is one of the most affected environmental compartments. The application of a variety of chemicals used by the industry may lead to a direct or indirect release of classic and emergent contaminants into the environment and they are rapidly entering into the aquatic environment increasing their derive risks. Bioaccumulation, along with persistence and acute toxicity, is used for aquatic environmental hazard identification to determine the potential for adverse effects to biota. Hazard identification is the determination of the adverse effects that a substance may pose based on its intrinsic properties. Because it is based on the substance inherent properties, hazard identification criteria should be independent of exposure conditions. Specific issues that may be encountered locally and regionally are not considered in hazard identification but they are dealt within risk assessment, which integrates hazard identification, dose–response assessment, and exposure assessment. In addition to its use as a criterion for hazard identification, bioaccumulation can also be a component of other regulatory toolboxes and is used in many jurisdictions for prioritization and risk assessment.

In June 2007 entered the new European Directive REACH (Registration, Evaluation and Authorisation of Chemicals) whose main aim is to increase our knowledge about the effect that different chemicals may have on human health or the environment in any of their life cycle steps (manufacture, packing, distribution, commercialization, use and removal). REACH Directive establishes that the bioaccumulation potential of a chemical substance by an organism is among the most important characteristics influencing their risk for the environment, and thus remarks that the bioaccumulation potential of any chemical produced in more than 100 Ton/year should be evaluated.

Presently, the validated methods for determining the bioaccumulation in waters (Bio-concentration test OECD 305, and EPA's ASTM E1022-94) are based on the measurement of the accumulation of a compound dissolved in water over a large period of time in fish. In order to establish a bioconcentration factor for a certain chemical, a very complex experiment should be carried out, whose cost rises over 100.000 € per chemical compound, and which requires over 100 adult fish. Due to the

high cost of this practice and the sacrifice of fish, some alternative methods have been developed (QSAR methods, correlations with the K_{ow}, in vitro methods with cell lines). However, results obtained from these experiments have proven to be difficult to replace the OECD/305 test .

REACH and other regulations related with the environment have expressed the need of having simple and fast methods for ecotoxicological assays of a large number of compounds. The Scientific Community is now directing its interest towards the use of fish embryos and eleutheroembryos of the zebrafish animal model, since the important advantages that present over other vertebrates model systems. On the other hand, conventional tests are normally designed without considering the physico-chemical characteristics of the teste compound: stability, bioaccumulation, or potential synergies with other compounds. The result of this situation is the possibility of overlooking the risk for environmental and human health.

The aim of this research work has been the development of specific tools for a complete and unequivocal assessment of the toxic effects of chemical pollutants, including emerging contaminants (e.g. nanoparticles) in the environment. These tools will account all the parameters not normally considered in conventional toxicity tests. As an example, the characterization of the compound/s under the experimental conditions that will be used in the bioaccumulation study.

Therefore, bioaccumulation studies of classic and emerging contaminants in zebrafish eleutheroembryos have been carried out by the development of analytical methods for the determination of such analytes in these biological microsamples. The specific analytical methodology developed have provided important advantages over existing ones, requiring no or very few sample treatment. After method validation they shall provide fast and economic alternatives to test series using adult fish. For the purpose, a range of contaminants have been selected, in particular some metallic species, (arsenic, tributyltin, silver and titanium) and two of the most comercialized nanoparticles (silver and titanium dioxide). Below it is described the analytical methodology developed and optimized, as well as the bioaccumulation results obtained after their application for each analyte studied in this research project .

Arsenic and tributyltin

The analytical methodology here developed has been applied to calculate the bioconcentration factors (BCFs) of both contaminants measured as arsenic and tin. The method is based on the use of an ultrasonic probe assisted extraction for accelerating the sample treatment followed by detection using graphite furnace atomic absorption spectrometry with Zeeman correction (ZGF-AAS). Results obtained for the BCFs values are in good agreement with previously reported data on freshwater aquatic organisms. In the case of inorganic arsenic, after exposing eleutheroembryos to concentrations of 5 and 50 μg L^{-1}, very low BCFs were observed (between 2.2 and 9.5); while for tributyltin (TBT), the BCFs observed were within the range 840-1280 after exposure to concentrations of 0.2 and 2.0 μg L^{-1}, respectively. This study shows that the use of zebrafish eleutheroembryos together with the proposed analytical approach is a promising alternative to the OECD 305 test.

Furthermore, the capability of zebrafish eleutheroembryos to depurate TBT was also studied. The evaluation of TBT transformation into its degradation products during both accumulation and depuration phases was studied. The analytical method was based on the use of the ultrasonic probe mentioned above and derivatization by ethylation, followed by detection using Gas Chromatography with a Flame Photometric Detector (GC-FPD). Validation of this method was carried out using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and ZGF-AAS. Speciation analysis demonstrated the capability of zebrafish eleutheroembryos to metabolize TBT into monobutyltin (MBT) and likely inorganic tin. However, the presence of dibutyltin (DBT) was not detected.

Ionic silver and AgNPs

In this study, the chemical stability and behavior of silver nanoparticles (AgNPs) in aquatic system was studied assessing the effect of different physicochemical parameters such as stabilizing agents, temperature, time, and type of container walls. AgNPs suspensions were previously fractionated by ultracentrifugation and ultrafiltration experiments. Once the stability conditions were established, zebrafish eleutheroembryos were exposed to ionic silver and to AgNPs and BCFs were

calculated. To determine the silver concentration in both the eleutheroembryos and the exposure media, an analytical method consisting in ultrasound assisted extraction, followed by ICP-MS and ZGF-AAS detection, was optimized. Total silver content was determined by ZGF-AAS and ICP-MS in flow injection operation mode. Once the concentrations at the selected accumulation and depuration times were determined, two different procedures were used to determine bioconcentration factors using the experimental data. The results revealed that ionic silver was more accumulative than AgNPs at the levels tested.

Furthermore, in collaboration with the Govern Center United States Geological Survey (Menlo Park, CA) it was studied the effect of water hardness and humic acids on the bioaccumulation and toxicity of AgNPs coated with polyvinilpirrolidone (PVP) by the invertebrate model system snail *Lymnaea stagnalis* after dietary exposure. We have showed that bioaccumulation and toxicity of Ag from PVP-AgNPs is unaffected neither by water hardness nor by the presence of humic acids. Snails efficiently assimilated Ag from the PVP-AgNPs mixed with diatoms but rate constants of Ag uptake from food (k_{uf}) did not vary among the different water hardness and in the presence of humic acids, demonstrating the lack of effect of water chemistry on the dietary uptake of Ag by snails. These results suggest that regulations aimed to minimize the bioavailability and toxicity of AgNPs to aquatic environments would be important if based on the characteristics of waterborne exposures of AgNPs.

Ti and TiO_2NPs

Bioaccumulation of dissolved titanium and titanium oxide nanoparticles (TiO_2NPs) by the model organism *zebrafish* eleutheroembryos after water exposure was compared in order to investigate the effect of particle size on the toxicity. Firstly, the stability of both chemical forms was fully characterized for further bioaccumulation studies. Several stabilizing agents were evaluated to avoid aggregation of two types of TiO_2NPs (rutile and anatase). TiO_2NPs suspensions were previously fractionated by ultracentrifugation and ultrafiltration. Total titanium was determined by flow injection coupled to ICP-MS detection. A focused ultrasonic probe was employed for leaching the analytes from the eleutheroembryos incubated in the exposure media. Zebrafish

eleutheroembryos incubated in the exposure media spiked with TiO_2NPs were digested with HNO_3/HF/HBO_3/H_2O_2 to extract total titanium. It was demonstrated that the presence of humic acids stabilized an important percentage of TiO_2NPs. However, the presence of *zebrafish* eleutheroembryos in the exposure media increased the aggregation of TiO_2NPs. BCFs values lower than 100 obtained here showed that there was no significant bioaccumulation of both chemical forms by the *zebrafish* eleutheroembryos.

Summarizing, the results presented here have demonstrated that the test developed using *zebrafish* eleutheroembryos is adequate to evaluate the biaccumulation of chemicals resulting in a promising alternative to the Bioconcentration Test OECD 305. The BCFs values obtained for all chemical tested here were in good agreement with values reported in scientific bibliography using OECD 305 guidelines.

2. OBJETIVOS

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Los protocolos normalizados de bioacumulación de contaminantes requieren el uso de más de 100 peces adultos por ensayo, lo cual implica un elevado coste (alrededor de 100000 euros por cada test y cada compuesto) e importantes problemas éticos. Por ello, el presente trabajo se ha centrado en el desarrollo de un Test de Bioconcentración alternativo al Test OECD 305, empleando larvas de pez cebra (*Danio Rerio*). En esta fase de crecimiento, no son considerados sistemas *in vivo* desde un punto de vista legal proporcionando considerables ventajas, tanto en relación a las consideraciones éticas, como por la notable reducción de costes. El test propuesto se ha aplicado a contaminantes clásicos: "As (III), TBT, Ag iónica y Ti disuelto", y emergentes: "nanopartículas de Ag y de TiO_2". Para evaluar la validez del protocolo propuesto ha sido necesario:

1. Desarrollo de nuevas metodologías analíticas para la determinación de contaminantes emergentes en muestras de larvas del pez cebra, así como adaptación a las características de las muestras a tratar de métodos existentes para los contaminantes clásicos.
2. Evaluación de la estabilidad en el medio de exposición de la concentración de cada sustancia estudiada en cada uno de los ensayos desarrollados. El alto contenido en sales de este tipo de muestras puede originar efectos de matriz que se traducen en la disminución o incluso eliminación de la señal de medida.
3. Estudios de caracterización de las nanopartículas, de las que poco se conoce acerca de sus mecanismos de acción y toxicidad para los organismos acuáticos. Se ha evaluado su estabilidad y capacidad de agregación en el medio de exposición.
4. Determinación de la bioacumulación observada en las larvas de pez cebra mediante el cálculo de BCFs. Estudio de la transformación dentro del pez cebra de las sustancias altamente bioacumulables (TBT).
5. Evaluación de la influencia de la química del agua (dureza y presencia de crecientes concentraciones de ácidos húmicos) en la bioaccesibilidad/toxicidad de las nanopartículas de plata para el caracol *Lymnaea Stagnalis.*

3. INTRODUCCIÓN

La presencia de contaminantes en el ecosistema acuático, en especial en ríos, lagos y aguas subterráneas ha aumentado constantemente desde el origen de la Revolución Industrial, hace aproximadamente unos 200 años, con el incremento de la población y el surgimiento de la actividad industrial. El medio acuático ha sido durante todo este tiempo el receptor directo e indirecto de desechos derivados de la actividad antropogénica.

La contaminación del agua se produce a través de la introducción directa o indirecta en los cauces o acuíferos de sustancias sólidas, líquidas, gaseosas, así como de energía calórica, entre otras. Muchas de estas sustancias no son consideradas tóxicas, si bien pueden alterar las características organolépticas del agua, perturbar el ecosistema severamente y/o ser directamente nocivas para el ser humano. En la actualidad, se estima en más de un millón las sustancias que se introducen en las aguas naturales a través de vertidos antropogénicos (Förstner et al. 1993) lo que se traduce en grandes alteraciones de la calidad de las aguas con los subsiguientes riesgos para la salud de la población. Desde la industrialización los esfuerzos para lograr la eliminación de los contaminantes generados por el hombre no han sido capaces de ajustarse ni al ritmo de incremento en la cantidad de desechos industriales, ni al crecimiento demográfico. Todo ello ha provocado a menudo la transformación de ríos, lagos y costas en depósitos de residuos en los que el equilibrio natural se ha perturbado gravemente (Förstner et al. 1981).

La presencia de ciertos contaminantes puede suponer daños irreversibles en los organismos vivos del medio acuático y pueden constituir un peligro para la salud tanto de las personas como de los animales. Se considera *sustancia peligrosa* aquella que es capaz de producir efectos adversos en cualquier organismo. El impacto que una sustancia peligrosa puede tener depende de las propiedades fundamentales e inherentes de tal sustancia, así como de las condiciones de exposición en las cuales suponga un riesgo para el entorno en el que haya sido liberada. El *riesgo* de que una sustancia peligrosa produzca un efecto perjudicial se entiende como la probabilidad de que ocurra un efecto adverso tanto a nivel individual o poblacional por la exposición a una concentración dada o dosis de agente peligroso, siendo la *toxicidad* una medida del peligro inherente de la sustancia. Por tanto, para evaluar el riesgo que puede

suponer una sustancia peligrosa habrá que evaluar el efecto combinado de dosis-tiempo de exposición bajo unas condiciones definidas, las posibles vías de exposición a la misma, la forma química en la que acceda al organismo considerado y por último, los efectos adversos consecuencia de tal exposición (Vegter et al. 1981).

3.1. Legislación Ambiental

El ser humano ha tomado conciencia en las últimas décadas de la necesidad de proteger el medio ambiente como consecuencia de los efectos en los ecosistemas derivados de su actividad antropogénica. En la segunda mitad del siglo XX, la preocupación por el medioambiente se ha extendido a todos los ámbitos políticos, sociales y científicos. Para la protección del medioambiente se han adoptado numerosas iniciativas legales de todo tipo. Las más extendidas son las disposiciones que permiten tomar medidas restrictivas y sancionadoras contra los que con sus acciones degradan la Biosfera. Aunque gran número de países han promulgado leyes, la necesidad de dedicar grandes recursos económicos es, la mayoría de las veces, la excusa para no aplicar y/o no legislar convenientemente otras iniciativas que pueden resultar incluso relativamente menos gravosas.

Las primeras acciones de protección del medio ambiente a nivel europeo comenzaron en 1972 durante la "*Cumbre de París*", en la que los Estados miembros de la Unión Europea se pusieron de acuerdo sobre la importancia de su protección. Se crearon programas de acción que han ayudado a integrar aspectos ecológicos y ambientales en todas las áreas de las políticas de la Comunidad Europea. Desde entonces, se han implementado seis programas de acción en este sentido (Crocket et al. 1991). Sin embargo, el ámbito del medioambiente no fue jurídicamente vinculante hasta 1987 a través del marco del "*Acta Única Europea*": tratado internacional firmado en Luxemburgo por los 12 países que en ese momento formaban la Comunidad Europea (Bonet et al. 1988). Si bien el "*Acta Única Europea*" es reconocida como el punto clave de la política ambiental europea, en la actualidad está en vigor el "*VI Programa de Acción en Materia de Medioambiente de la Unión Europea"*

(Medioambiente 2010: el futuro está en nuestras manos) http://ec.europa.eu/environment/newprg/index.htm , que contempla una visión general de las medidas necesarias para aplicar el "P*rincipio del Desarrollo Sostenible*", referido al uso racional de los recursos naturales y a la protección del ecosistema mundial en las figuras de los ciudadanos (respeto al medioambiente, cambio de hábitos), ciencia (conocimientos y soluciones) y poderes públicos (legislación y cooperación con otros países).

El control de la contaminación de las aguas fue uno de los primeros aspectos que se incluyeron en la política de Medioambiente de la UE. En la actualidad se cuenta con una Directiva Marco (Directiva 60/2000/CE del Parlamento Europeo y del Consejo 22/12/2000), extendida al campo de la protección acuática de todas las aguas que establece como objetivo para el año 2015, conseguir un "*buen estado*" de las aguas europeas y que el "*uso sostenible del agua*" esté asegurado en toda Europa. Esta Directiva establece un marco comunitario de actuación para la protección de las aguas superficiales continentales, de transición, costaneras y subterráneas, para prevenir o reducir su contaminación, promover su uso sostenible, proteger el medioambiente, mejorar el estado de los ecosistemas acuáticos y atenuar los efectos de las inundaciones y las sequías. Para alcanzar estas metas, la norma requiere la cooperación entre los distintos estados miembros, así como la participación de las entidades locales y ONGs en las actividades de gestión del agua. Además, esta directiva reafirmó el principio según el cual "*el que contamina, paga*", para evitar así que la política de protección del medio ambiente se costee con cargo a fondos públicos y recaiga, en definitiva, sobre todos los contribuyentes. Dicha Directiva se caracteriza por presentar una visión global y un marco de acción local. En ella, se especifican las medidas a tomar para conseguir la protección integrada del agua y la calidad química y ecológica de ésta, mediante la reducción progresiva de la contaminación existente y el cese de vertidos, emisiones y fugas de sustancias peligrosas. Las sustancias que esta directiva establece como de interés prioritario en el agua se encuentran recogidas en una lista dinámica (Decisión No. 2455/2001/EC) que se revisa cada cuatro años. Aparte del control de los compuestos incluidos en esta lista, los aspectos biológicos e hidromorfológicos toman relevancia en la diagnosis integrada de la calidad.

La incorporación a la Unión Europea de España en 1985 favoreció el desarrollo de una política ambiental en nuestro país, asumiendo las directivas comunitarias sobre la protección de la calidad de aguas superficiales. Desde el momento de su adhesión tuvo lugar su aplicación inmediata, fecha en la que entró en vigor la Ley de Aguas (Ley 29/1985, de 2 de agosto; BOE, 1986). Las medidas legislativas que, con distinto rango normativo, se han ido adoptando progresivamente con la finalidad de proteger los recursos hídricos existentes y de armonizar nuestra legislación con la europea han sido numerosas. Así pues, la normativa vigente en materia de aguas se encuentra, hoy en día, dispersa en una amplia variedad de herramientas legislativas que presentan distintos: a) niveles de competencia: europeo (directivas), nacional (real decretos, órdenes, etc.) o autonómico (leyes, decretos legislativos); b) ámbitos de aplicación (aguas de consumo humano, aguas subterráneas, aguas destinadas a la producción de agua potable, etc.); c) aspectos a regular (parámetros de calidad, frecuencias de muestreo y análisis, etc.).

No obstante, en los próximos años, está previsto que muchas de las normas actuales queden derogadas por la Directiva 2000/60/CE.

3.2. Normativa REACH

En enero de 2007 entró en vigor en la Unión Europea una nueva normativa de Registro de Productos Químicos, REACH. Esta normativa está basada en el principio preventivo de que los fabricantes, importadores y usuarios tienen que probar que sus productos fabricados no presentan efectos adversos sobre la salud y el medioambiente, durante todo su ciclo de vida (fabricación, envasado, distribución, transporte, comercialización, utilización y eliminación) antes de permitir su venta y uso para otros fines.

El Reglamento REACH regula el registro, la evaluación, la autorización y la restricción de las sustancias y los preparados químicos, con el objetivo de proteger la salud humana y del medio ambiente, así como la libre circulación de sustancias en el mercado interior, fomentando al mismo tiempo su competitividad y su innovación. A su

vez, este Reglamento fomenta el desarrollo de métodos alternativos para evaluar los peligros que plantean las sustancias. La aplicación del Reglamento se basa en varios procedimientos aplicables en función de la peligrosidad de la sustancia, del volumen de fabricación, de la utilización (mayor de 100 Tm/año), suponiendo que existe una relación directa entre la cantidad de sustancia producida y la magnitud de la exposición. Principalmente hay que tener en cuenta tres grupos de sustancias para llevar a cabo el cumplimiento del Reglamento REACH: sustancias altamente preocupantes; sustancias sujetas a autorización y sustancias con restricciones.

La tabla 1 recoge las sustancias que ya están reconocidas como especialmente peligrosas sujetas a autorización. Ha sido elaborada por agencias gubernamentales con diferentes objetivos (*Extremely Hazardous Substance List* 1994, *Priority Substances List*. 1999, Reglamentos UE No 143/2011, Nº 125/2012, y No 348/2013) Estas sustancias se clasifican en: Tóxicas, Persistentes y Bioacumulables; Carcinógenas; Mutágenas y tóxicas para la Reproducción; Disruptores Endocrinos; Sensibilizantes.

Tabla 1. Sustancias peligrosas sujetas a autorización para cumplimentar la Normativa REACH, Abril 2013.

Sustancia	NºCE	NºCAS	Fecha Expiración	Fecha límite de revisión de solicitud	Propiedades intrínsecas contempladas en el artículo 57
Fosfato de Tris (2-chloroetilo) (TCEP)	204-118-5	115-96-8	21/08/2015	21/02/2014	Tóxico para la reproducción
Tricloroetileno	201-167-4	79-01-6	21/04/2016	21/10/2014	Carcinógeno
Dicromato de sodio	234-190-3	7789-12-0; 10588-01-9	21/09/2017	21/03/2016	Carcinógeno Mutágeno y Tóxico para la reproducción
Cromato de sodio	231-889-5	03/11/7775	21/09/2017	21/03/2016	Carcinógeno; Mutágeno y Tóxico para la reproducción
Dicromato de potasio	231-906-6	7778-50-9	21/09/2017	21/03/2016	Carcinógeno; Mutágeno y Tóxico para la reproducción
Cromato de potasio	232-140-5	7789-00-6	21/09/2017	21/03/2016	Carcinógeno; Mutágeno
Amarillo de sulfocromato de plomo (C.I. Pigmento Amarillo 34)	215-693-7	1344-37-2	21/05/2015	21/11/2013	Carcinógeno; Tóxico para la reproducción
Rojo de cromato, Molibdato Sulfato de plomo (C.I. Pigmento Rojo 104)	235-759-9	12656-85-8	21/05/2015	21/11/2013	Carcinógeno; Tóxico para la reproducción
Cromato de plomo	231-846-0	7758-97-6	21/05/2015	21/11/2013	Carcinógeno ; Tóxico para la reproducción
Hexabromociclododecano (HBCDD) α- hexabromociclododecano β-Hexabromodiciclodecano γ- Hexabromodiciclodecano	221-695-9, 247-148-4	3194-55-6, 25637-99-4, 134237-50-6, 134237-51-7, 134237-52-8	21/08/2015	21/02/2014	Persistente, Bioacumulable y Tóxico
Ftalato de di-isobutilo (DIBP)	201-553-2	84-69-5	21/02/2015	21/08/2013	Tóxico para la reproducción

Ftalato de dibutilo (DBP)	201-557-4	84-74-2	21/02/2015	21/08/2013	Tóxico para la Reproducción
Trióxido de diarsénico	215-481-4	1327-53-3	21/05/2015	21/11/2013	Carcinógeno
Pentóxido de diarsénico	215-116-9	1303-28-2	21/05/2015	21/11/2013	Carcinógeno
Trióxido de cromo	215-607-8	1333-82-0	21/09/2017	21/03/2016	Carcinógeno; Mutágeno
Ftalato de bis(2-etilhexilo) (DEHP)	204-211-0	117-81-7	21/02/2015	21/08/2013	Tóxico para la reproducción
Ftalato de bencilo y butilo (BBP)	201-622-7	85-68-7	21/02/2015	21/08/2013	Tóxico para la reproducción
Dicromato de amonio	232-143-1	2151163	21/09/2017	21/03/2016	Carcinógeno; Mutágeno; Tóxico para la reproducción
Ácidos derivados de Trióxido de Cromo y sus oligómeros El Grupo incluye: Ácido Crómico, Ácido dicrómico; Oligomeros del Ácido crómico y del Ácido dicrómico	231-801-5, 236-881-5	7738-94-5, 13530-68-2	21/09/2017	21/03/2016	Carcinógeno
5-terc-butil-2,4,6-m-xileno (Azmicle de xileno)	201-329-4	81-15-2	21/08/2014	21/02/2013	Muy persistente y muy Bioacumulable
4,4´-Difenilmetano (MDA)	202-974-4	101-77-9	21/08/2014	21/02/2013	Carcinógeno
2,4 – Dinitrotolueno (2,4-DNT)	204-450-0	121-14-2	21/08/2015	21/02/2014	Carcinógeno

CAS : Número de Registro asignado por la Sociedad Americana de Química

CE: Número de Registro asignado por las autoridades europea

En la clasificación e incorporación a la lista de sustancias sujetas a autorización se ha de evaluar el riesgo que cada una de ellas presenta para la salud y el medioambiente, valorando algunas de sus propiedades intrínsecas como son su ecotoxicidad, movilidad, persistencia y degradabilidad, potencial de bioacumulación, toxicidad acuática, etc. Particularmente, esta nueva normativa europea REACH considera que una de las características más importantes e influyentes en el riesgo medioambiental es el *potencial de bioacumulación* de una sustancia química por un organismo por ser uno de los parámetros claves indicativos que, dependiendo de los datos sobre la exposición y efectos adversos, permite una distinguida valoración del riesgo global (Franke et al. 1994).

3.3. Bioacumulación/ Bioconcentración

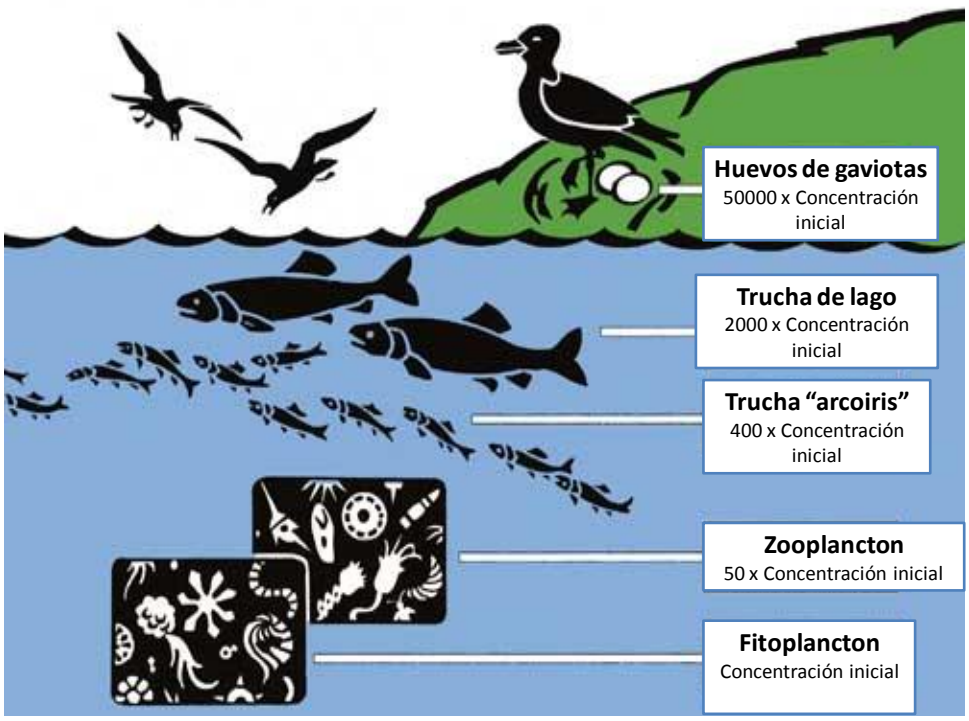

Figura 1. Esquema de bioacumulación en los distintos niveles de la cadena trófica.

(Figura adaptada de:

http://wikiecologyproject.wikispaces.com/file/view/41.jpg/289004489/41.jpg)

Bioacumulación, junto con persistencia y toxicidad, es un término comúnmente empleado para la identificación del peligro potencial que supone una sustancia sobre un ecosistema ya que representa los posibles efectos adversos para la biota. Se define como el proceso de acumulación de sustancias químicas en organismos vivos de forma que adquieren concentraciones más elevadas que las del medio ambiente donde se desarrollan o de los alimentos que consumen (Beek et al. 2000). Se trata de un parámetro a menudo difícil de evaluar que engloba la acumulación de la sustancia peligrosa considerando todas las rutas posibles de exposición del entorno que rodea al organismo acuático: aire, agua, suelo/sedimentos y alimentos. La concentración de las sustancias propensas a la bioacumulación aumenta a medida que se avanza en el nivel trófico de la cadena alimenticia. Dicha acumulación puede producirse en función de cada sustancia a partir de fuentes abióticas (suelo, aire, agua), o bióticas (otros organismos vivos) siendo las principales vías la respiratoria y la digestiva.

El criterio para evaluar y clasificar la bioacumulación de una sustancia se establece a partir de su biomagnificación y bioconcentración por un organismo produciendo una carga corporal en el mismo que puede resultar tóxica o no.

Bioconcentración se define como el resultado neto de captación, transformación y eliminación de una sustancia en un organismo debido a la exposición prolongada a la misma en el medio abiótico en el que habita; este parámetro es más fácil de determinar que el de bioacumulación, que incluye todas las rutas de exposición (aire, agua, sedimento/suelos y dieta). El grado en el cual una sustancia tóxica se concentra en un organismo acuático viene dado por el *factor de bioconcentración*, BCF, calculado como el cociente entre la concentración máxima de la sustancia testada, alcanzado el estado estacionario en el tejido del organismo o planta considerado y la encontrada en el medio de exposición a la misma.

Biomagnificación, es otro término a través del cual puede expresarse la bioacumulación y se define como la acumulación y traslado de sustancias a través de la dieta, incrementando su concentración en los organismos de manera que se avanza a niveles superiores de la cadena trófica (Gray et al. 2002).

Biodisponibilidad es otro parámetro de interés, definido como la afinidad de un contaminante para ser absorbido o adsorbido por un organismo vivo, atravesando las

barreras biológicas (membranas celulares o epitelios), o bien mediante procesos biológicos activos o procesos pasivos de tipo físico o químico (Anderson et al. 2008). No obstante, algunos científicos diferencian entre disponibilidad externa o *bioaccesibilidad* y *biodisponibilidad interna (*Caussy et al. 2003*,* Peijnenburg et al. 2003). Se considera una sustancia bioaccesible aquella que puede entrar en contacto con el organismo y ser absorbida, y las sustancias biodisponibles son aquellas que tras ser absorbidas por el organismo pueden interactuar con las moléculas u órganos diana. Algunos factores fisicoquímicos como la temperatura, la salinidad, la presencia o ausencia de agentes complejantes, la presencia o ausencia de metales y factores propios de los organismos, como el grado de impermeabilidad superficial, la etapa nutricional, etc., determinan la biodisponibilidad de las sustancias peligrosas hacia los organismos. La biodisponibilidad de cualquier sustancia química depende de la especie.

Cualquier sustancia propensa a acumularse tendrá unas determinadas propiedades intrínsecas químicas de hidrofobicidad y lipofilidad que determinarán su capacidad de absorción por un organismo determinado, ya que dependerá de la partición termoquímica entre el medio acuático y la fase lipídica del organismo acuático en cuestión. Por lo general existe una relación directa entre el BCF y el coeficiente de partición octanol-agual de la sustancia. Por tanto, para la medida del potencial de bioacumulación de una sustancia química se ha asumido el coeficiente de distribución octanol/agua como criterio de armonización para obtener el *factor de bioconcentración* (Mc Geer et al. 2003).

El factor de bioconcentración dependerá además de (Caussy et al. 2003):

i) el grado de bioaccesibilidad/biodisponibilidad**,**

ii) las respuestas fisiológicas de los organismos utilizados en las experiencias,

iii) un valor constante de la concentración de exposición del compuesto en estudio,

iv) la duración de la exposición,

v) el metabolismo corporal del organismo designado y la excreción del mismo.

Para estudiar y evaluar los posibles efectos adversos producidos por contaminantes tóxicos de origen antropogénico se utilizan los *bioensayos*. Se trata de herramientas que permiten realizar medidas experimentales de la relación concentración-respuesta que genera un efecto tóxico en los organismos de ensayo, bajo condiciones controladas "in situ" o en laboratorio. Los ensayos de toxicidad con organismos acuáticos son métodos reconocidos por la comunidad científica internacional y empleados en muchos países, para el monitoreo y control de la contaminación hídrica. Los bioensayos de laboratorio posibilitan tener un primer acercamiento al posible impacto que producen estos efluentes y se implementan bajo estándares generados por la comunidad científica, con la finalidad de obtener datos comparativos entre diferentes organismos (Schipper et al. 2010).

Existe consenso general en la consolidación de los datos obtenidos de estos bioensayos, los cuales han de obtenerse preferentemente aplicando métodos de ensayo normalizados. Algunos bioensayos estandarizados, comúnmente denominados Guías de Ensayo, utilizan la bioconcentración para evaluar el riesgo que una sustancia puede poseer en el medioambiente. Las Guías de ensayo normalizadas, tanto nacionales como internacionales, desarrollan tests de bioconcentración con organismos acuáticos con el objetivo de evaluar la bioacumulación de un determinado agente contaminante. El criterio de clasificación se establece a partir del BCF y su valor debe mantenerse constante en una serie determinada de condiciones.

Se han documentado y adoptado diferentes directrices de ensayos para determinar experimentalmente la bioconcentración en peces. Las que se aplican con carácter más general son la Directriz 305 de la OECD (OECD, 1996) y la guía normalizada ASTM (ASTM E 1022-94) dictada por la US EPA (United States Environmental Protection Agency) (Burkhard et al. 2012). Los principios de las Directrices OECD 305 y ASTM son similares, pero sus condiciones experimentales difieren, especialmente en:

a) el método de suministro del agua de ensayo (estático, semiestático o dinámico);

b) el requisito de realizar un estudio de depuración;

c) el método matemático para calcular el BCF;

d) la frecuencia del muestreo: número de mediciones en agua y de muestras de peces;

e) la necesidad de medir el contenido en lípidos del pez;

f) la duración mínima de la fase de absorción

Aunque se permiten regímenes semiestáticos (ASTM E 1022-94), siempre que se cumplan los criterios de validez sobre mortalidad y mantenimiento de las condiciones de ensayo, se prefieren regímenes de ensayo dinámicos (OECD 305, 1996). Cuando se trata de sustancias lipofílicas (log K_{ow} > 3), se prefieren los métodos dinámicos. Por esta razón el bioensayo de toxicidad seguido aquí surge como alternativo al Test de Bioconcentración de la OECD 305 y se ha llevado a cabo teniendo en cuenta una serie de pautas seguidas que se detallan a continuación. De acuerdo con esta Directriz surge la normativa REACH para evaluar el potencial de bioacumulación de sustancias peligrosas cuya producción supere las 100 t/año atendiendo a las respuestas a estímulos externas de cada uno de ellos en unas concentraciones determinadas dependiendo del valor de su LC_{50}.

3.4. Test OECD 305

La Guía de Ensayo 305 de la OECD (OECD, 1996) describe un procedimiento normalizado para la evaluación del potencial de bioacumulación/bioconcentración de cualquier sustancia química en peces tras su exposición a través del agua o de la dieta, bajo unas condiciones definidas de régimen de flujo. Generalmente, se trata de un ensayo con peces que consta de dos fases: una de exposición a la sustancia de estudio (absorción) y otra de post-exposición (depuración). Durante la depuración, los peces se transfieren a un medio exento de la sustancia de ensayo. La concentración de ésta en el pez deberá determinarse en distintos tiempos de muestreo durante cada una de las fases de ensayo.

Esta guía de ensayo propone el estudio de la bioconcentración de la sustancia evaluada al menos con dos niveles de concentración. Normalmente estas concentraciones son elegidas en base al valor del LC_{50} a las 96 horas proporcionado para la sustancia, resultando la concentración más alta del ensayo un 1% del valor de su LC_{50} y la concentración más baja el 0,1%. Además de estas dos concentraciones testadas, se debe desarrollar un ensayo paralelo en idénticas condiciones pero en ausencia de la sustancia de estudio, para establecer comparaciones entre los posibles efectos adversos derivados de la exposición a la sustancia y los efectos encontrados en el grupo control.

El Test de Bioconcentración OECD 305 estima la duración de la fase de absorción o contaminación en base al valor del K_{ow} de la sustancia a testar. Generalmente esta fase suele durar 28 días, a menos que el estado de equilibrio haya sido previamente alcanzado. Si no se alcanza dicho estado estacionario en ese tiempo establecido, la fase de absorción debe ser ampliada hasta que se alcance. La fase de depuración comenzará en el momento que ese estado se ha alcanzado y su duración se estima como la mitad del tiempo de la fase de absorción.

Este test establece como necesidad evaluar la bioconcentración de cada sustancia química individualmente, ya que pueden existir efectos sinérgicos que incrementen o disminuyan la toxicidad de las sustancias de estudio produciendo resultados erróneos que no permitan una apropiada valoración de su impacto.

La bioconcentración de la sustancia de estudio vendrá expresada por unos determinados parámetros consolidados que se calculan a partir del modelo matemático que mejor ajuste la concentración de la sustancia en el pez respecto a la concentración de la sustancia en el medio de exposición. Además, en la Guía de ensayo OECD 305 la bioconcentración debe ser expresada en relación al contenido lipídico de la sustancia contaminante en estudio y al peso del organismo en cuestión.

3.5. Modelos matemáticos para el estudio de la bioconcentración

Se necesita elegir, por tanto, un modelo matemático que exprese en términos comparativos la relación dosis-respuesta para el análisis de los resultados obtenidos en los ensayos de bioconcentración y sus variables. Estos modelos se basan en la premisa de que la bioconcentración de los contaminantes no es más que la consecuencia de equilibrios termodinámicos entre las especies presentes en el medio de exposición y en el tejido del organismo considerado. El equilibrio se establece por difusión pasiva, y cualquier proceso de regulación interna puede provocar desviaciones del mismo. Estos modelos implican que controlando los niveles de contaminantes en el medio de exposición, se puede predecir en base a los equilibrios de partición las concentraciones en los organismos. A menudo, estos modelos presentan deficiencias para explicar la biodisponibilidad de los compuestos en los ecosistemas, pues no tienen en cuenta procesos que pueden reducir las concentraciones biodisponibles tales como los efectos de competencias entre las especies, las reacciones de complejación, los procesos de transferencia a través de la membrana biológica y las múltiples estrategias de las que los organismos disponen para asimilar o eliminar la sustancia contaminante (Worms et al. 2006).

3.5.1. Bioconcentración a través del medio acuático

El modelo matemático utilizado en este trabajo para el estudio de la bioconcentración a través del agua es el más utilizado para definir la cinética de este proceso y está basado en un sistema de un compartimento que representa al organismo. Consiste en el balance entre la asimilación y la eliminación de los contaminantes, sin considerar su posible biodegradación. Estos procesos siguen una cinética de primer orden y están caracterizados por tasas constantes que definen la bioconcentración según:

$$\frac{dCorganism}{dt} = k_1 C_A - k_2 C_{organism}$$

donde $C_{organism}$ y C_A son las concentraciones de contaminante en el organismo considerado y en el medio de exposición y k_1 y k_2 las constantes de absorción y depuración de primer orden, respectivamente. Considerando C_A constante en el tiempo, t, e integrando la ecuación anterior:

$$C_{organism} = \left(\frac{k_1}{k_2}\right) C_A (1 - e^{-K_2 t})$$

La bioconcentración de un compuesto aumentará con el tiempo de exposición, t, hasta que se alcance el equilibrio donde:

$$C_{organism} = \left(\frac{k_1}{k_2}\right) C_A \quad o \quad \frac{dC_{organism}}{dt} = 0$$

La relación k_1/k_2 o $C_{organism}/C_A$ es conocida como el Factor de Bioconcentración o BCF. De esta manera cualquier estimación del BCF requerirá que el equilibrio se haya alcanzado para la sustancia de interés y/o que se conozca el tiempo en el cual ocurre.

3.5.2. Bioconcentración a través de la dieta

Otro modelo matemático de bioconcentración más complejo empleado en este trabajo ha sido un modelo biodinámico simplificado que contempla la acumulación de la sustancia testada, únicamente a partir de la dieta (Luoma et al. 2005). La concentración acumulada por un organismo, $C_{organism,}$ viene expresada como un balance de masas entre su capacidad para acumularla y para eliminarla:

$$C_{organism} = k_{uf} \times C_{food} - k_e \times C_{organism}$$

donde k_{uf} es la constante de absorción desde la comida; C_{food}, es la concentración de la sustancia de estudio en la comida, y k_e, la constante de eliminación.

Este modelo sigue una cinética de primer orden y consta de los parámetros que definen la bioconcentración: la constante de absorción desde la comida, k_{uf}, y dos parámetros fisiológicos, la tasa de ingestión a través de la dieta, IR, (cantidad ingerida por unidad de tiempo) y la eficiencia de asimilación, AE, (fracción total de sustancia retenida en el animal una vez completadas su digestión y asimilación). Estos $Kuf = AE \times IR$, y pueden ser obtenidos experimentalmente a partir de los datos obtenidos en las condiciones controladas de ensayo.

$$AE = \frac{(C_{organism})}{(C_{organism} + C_{feces})} \times 100$$

$$IR = \frac{(C_{food} + C_{feces})}{C_{food} \times wt_{organism} \times T}$$

donde C_{feces}, es la concentración de sustancia retenida en las heces, $wt_{organism}$ es el peso seco del organismo considerado y T, es el tiempo de exposición a la sustancia.

3.6. Sistemas biológicos modelo

Estos test normalizados de ensayo para evaluar el nivel de toxicidad y peligrosidad de las sustancias químicas contaminantes precisan un número muy elevado de pruebas reguladas que son normalmente llevadas a cabo con organismos vivos como por ejemplo en organismos acuáticos (peces, moluscos, etc)., en vertebrados (ratas y conejos) y, en algunos casos, en primates no humanos. Además de ser estudios muy costosos y que requieren mucho tiempo, implican el sacrificio de

un número muy alto de animales de laboratorio. Desde hace unos pocos años se están desarrollando nuevos métodos alternativos en experimentación animal que cumplan el principio de las tres “R” (Reemplazar, Reducir y Refinar). Estos métodos reclaman la sustitución de los ensayos con animales *in vivo* por la realización de estudios *in vitro,* que son llevados a cabo principalmente en líneas celulares, en especies microbianas unicelulares como bacterias o levaduras, u organismos acuáticos en fases juveniles de crecimiento, reduciendo los tiempos de exposición y el empleo de seres vivos. Estos organismos se han convertido en objetos de estudio centrales de la investigación biológica y en la actualidad son piezas clave de la investigación biomédica. Se les podría llamar organismos simples o de experimentación.

Un *organismo modelo* en biología es una especie muy estudiada para entender fenómenos biológicos particulares, que puedan darnos una idea de cómo funcionan esos procesos en otros organismos. Se refiere a aquellas especies que han sido intensamente estudiadas y que se usan para investigar múltiples aspectos de la biología. A pesar de sus diferencias de tamaño y forma de vida, todos estos organismos modelo producen proteínas que realizan las mismas funciones básicas que los seres humanos: le indican al organismo cuándo y cómo crecer, reproducirse, combatir el estrés y eventualmente morir. Por tanto, un organismo constituye un sistema modelo si se usa como un análogo o es ilustrativo para algo que por sí mismo no está bajo estudio directo, como la totalidad de un grupo de animales o un ancestro extinguido irreparablemente (Weber et al. 2005).

Los organismos modelos son también utilizados para analizar las causas de enfermedades humanas y posibles tratamientos ya que la experimentación en humanos es contraria a la bioética. Esta estrategia ha sido posible debido a la relación evolutiva de todos los organismos vivientes que comparten diversos mecanismos metabólicos y biológicos, así como material genético.

Los organismos modelo han sido ampliamente utilizados y han permitido la obtención de una ingente cantidad de información. En la elección de un determinado organismo modelo en el laboratorio debe tenerse en cuenta su abundancia y su facilidad de cultivar/criar y manipular en el laboratorio. Además, deben presentar características distintivas (embriones de gran tamaño, linaje celular fijo, transparencia,

etc.) interesantes para el estudio de determinados procesos. Cualquier animal seleccionado para un estudio es un modelo si es tomado como representante de algunas características de un grupo mayor (género, familia, orden).

Dentro de las especies modelo más estudiadas se encuentran ciertas bacterias entéricas, la levadura de cerveza, el moho, algunos nemátodos y gasterópodos, la mosca de la fruta, ciertas ranas y ajolotes, el pollo, el ratón, el pez cebra….(Lozada et al. 2012). Dentro del grupo de los vertebrados candidatos a ser considerados organismos modelo para muchas de las disciplinas de la biología, se encuentran los peces teleósteos (peces óseos) que ocupan una de las primeras posiciones. Agrupan aproximadamente unas 18.000 especies y colonizan hábitats muy diversos, por lo que han tenido que desarrollar estrategias evolutivas muy variadas (Walter et al. 2001). La Tabla 2 ilustra algunos de los organismos modelo de vertebrados acuáticos más empleados en investigación, así como el uso para los que se han realizado los ensayos.

Tabla 2. Organismos modelo de vertebrados acuáticos.

Nombre Común	Género y especie	Usos comunes
Pez dorado Carpa común Pez cebra	*Carassius auratus* *Cyprinus carpio* *Danio rerio*	Neurociencia Acuicultura Biología del desarrollo Enfermedades géneticas Cáncer Toxicología de Aguas Peces transgénicos Inmunología comparada Fisiología Comparada
Anguila Pez de zanja Bagre de canal Trucha	*Electrophorus electricus* *Fundulus heteroclitis* *Ictalurus punctatus* *Oncorthynchus mykiss*	Neurociencia Endocrinología Acuicultura Toxicología de Aguas Peces Transgénicos
Salmón	*Salmon salar y Oncorhynchus spp.*	Inmunología comparada Acuicultura Fisiología Comparada Peces transgénicos
Tilapia	*Tilapia spp y Oreochomiss sp*	Acuicultura
Medaka	*Oryzias latipes*	Biología del desarrollo Estudios oncológicos Peces transgénicos
Raya eléctrica	*Torpedo californica*	Neurociencia
Pez platy	*Xiphophorous maculatus*	Estudios oncológicos

Los peces como animales de experimentación exhiben las siguientes ventajas:

1. Ofrecen una gran cantidad de modelos alternativos. Su divergencia evolutiva y extrema diversidad proporciona una fuente de genomas distintos importante donde buscar y encontrar todas las posibles combinaciones estructura-función que han ocurrido en la naturaleza durante los últimos 400 años (Walter et al. 2001).

2. Son adecuados tanto para la experimentación de campo como la de laboratorio (Powers et al. 1989). Se adaptan fácilmente a la vida en cautividad y pueden mantenerse en los acuarios sin demasiados requerimientos técnicos.

3. Su adquisición y mantenimiento son mucho más económicos que la de cualquier ave, anfibio, o mamífero utilizado en experimentación.

De las especies de peces que se han utilizado y aún se utilizan en investigación con distintos fines, nos vamos a centrar en una especie perteneciente al grupo de los peces teleósteos: el pez cebra, que en estos momentos está considerada, junto con el ratón, el modelo por excelencia en el estudio de la biología de vertebrados.

A continuación se comentan algunas de las características y ventajas más importantes del pez cebra respecto a su uso en experimentación. Además, también se describen las características y ventajas de otro organismo modelo de invertebrados de uso frecuente, la especie caracol *Lymnaea Stagnalis,* como indicador de la contaminación por metales en aguas de ríos y que también ha sido empleado en este trabajo.

3.6.1. Pez cebra *(Danio rerio)*

Figura 2. Pez cebra (*Danio rerio*).

(Recuperado de http://i44.tinypic.com/11vqosg.jpg)

El pez cebra (*Danio rerio* o *Brachydanio rerio*) pertenece a la familia *ciprinidae* emparentado con las carpas y los barbos, originario del Sudeste Asiático y está distribuido de forma natural en las cuencas del Ganges y el Brahmaputra y las aguas dulces tropicales de las regiones monzónicas de la India, Bangladesh, Nepal, Pakistán, Bután, Tailandia y el norte de Myanmar. Se trata de una especie de uso frecuente en acuarios y en investigación debido a su homología genética con el hombre ya que

comparten más del 80 % del genoma (Barbazuk et al. 2000). Esto permite que resultados obtenidos de fármacos y numerosos compuestos testados en estos animales sean potencialmente extrapolables al ser humano.

Son peces alargados, fusiformes, con una única aleta dorsal, boca dirigida hacia arriba y un par de finas barbillas que son difíciles de ver salvo que el animal esté parado. El color de fondo es dorado o plateado, y presentan de 5 a 9 bandas de color azul oscuro distribuidas longitudinalmente que comienzan detrás del opérculo y llegan hasta el final del animal (incluyendo la cola), dándole un aspecto cebrado del que toma el nombre. El opérculo es azulado y la zona ventral de un tono blanquecino rosado. Alcanza 5 cm como talla máxima.

El pez cebra vive en pequeños bancos que van desde cinco a veinte individuos; se reproducen y desovan de manera asincrónica; sobre el sustrato depositan sus huevos, que no son cuidados por los padres después del desove. Como otros peces de regiones monzónicas, la época de lluvias marca el inicio de la estación de reproducción, aunque se han observado hembras con hueva madura durante la época de sequía, lo que permite suponer que la reproducción está correlacionada con la disponibilidad de alimento, y se ve intensificada con las lluvias. La fecundación es externa y los huevos, dependiendo de las condiciones ambientales, liberan larvas que nadan libremente después de cuatro a siete días de ser fecundadas. Los machos son territoriales sobre los posibles sitios de desove y adoptan tácticas para perseguir a las hembras. De su ciclo de vida sólo se tienen datos de laboratorio, donde llegan a vivir hasta cinco años.

Respecto a su dieta, estos peces son generalistas, es decir, consumen una amplia variedad de crustáceos del fondo marino, además de gusanos y larvas de insectos, si bien muestran una marcada preferencia por las larvas de los dípteros, por lo que se ha propuesto su uso para el control de mosquitos.

La tabla 3 resume las características fisicoquímicas de su hábitat, caracterizado por pequeños y tranquilos cursos de agua con abundante vegetación; incluso se le puede encontrar en los arrozales y otras tierras que se inundan temporalmente.

Tabla 3. Características fisicoquímicas del hábitat preferido por el pez cebra (Lozada et al. 2012).

pH	8.0
Dureza	100 mg L^{-1} $CaCO_3$
Salinidad	0.4 – 0.6 ng L^{-1}
Temperatura del agua	16 a 33 ºC
Actividad	Inmóvil en corrientes de 0.1 m s^{-1}
Sustrato	Barro, lodo, grava

El pez cebra es un modelo práctico y común para estudios dedicados al desarrollo de vertebrados y función génica. Entre sus ventajas destacan su elevada capacidad de producción de un gran número de huevos (cada hembra puede poner hasta 200 huevos de una vez), junto con las particularidades tanto de una fertilización externa y desarrollo embriológico también externo (pasando de huevo a larva en 3 días); son grandes, robustos y transparentes, características que facilitan la manipulación experimental en microinyección de ADN (para obtener integración y la generación de animales transgénicos), transplante celular y observación con diferentes técnicas de microscopía. Es posible visualizar todas las estructuras internas de embriones y larvas con una simple lupa de disección y el desarrollo embrionario puede observarse sin ninguna intervención. La docilidad en el manejo de sus embriones y peces adultos permite realizar sofisticados experimentos genéticos. También es posible inducir mutaciones o afectar la expresión de genes específicos y analizar las consecuencias de dichas perturbaciones puesto que se pueden visualizar los productos de la expresión génica *in situ*, sin necesidad de seccionar el tejido. El genoma de *Danio rerio* está secuenciado, lo que permite aislar y manipular cualquier gen de este organismo.

Además, y a pesar de la distancia evolutiva entre esta especie y el ser humano, el desarrollo de los órganos es similar, tal y como ocurre a nivel del sistema nervioso, páncreas, timo o vasos sanguíneos. El pez cebra tiene la habilidad de regenerar aletas, piel, corazón, cerebro u otros órganos. Su estudio está permitiendo a la comunidad científica entender los mecanismos de curación y regeneración en vertebrados (Dooley et al. 2000).

Su pequeño tamaño hace fácil su almacenaje, ya que caben hasta un centenar de animales en contenedores de un litro del medio de cultivo. Su sencillo mantenimiento hace que sea una de las preferencias de los científicos como animal de laboratorio en el siglo XXI.

En resumen, este modelo animal ofrece una poderosa combinación de características embriológicas, genéticas y celulares, que lo hacen el candidato ideal como organismo de estudio para entender y comparar diferencias y similitudes entre distintas especies de vertebrados.

En este trabajo se ha desarrollado un nuevo y simplificado Test de Bioconcentración alternativo al Test OECD 305 (OECD 1996) aplicado a contaminantes clásicos (arsénico inorgánico, tributilestaño, plata y titanio iónicos) y emergentes (nanopartículas de plata y nanopartículas de dióxido de titanio) en larvas de peces cebra.

3.6.2. Caracol *Lymnaea stagnalis*

Se trata de un organismo acuático que actualmente está siendo utilizado como organismo modelo en la investigación de contaminantes medioambientales para elucidar los mecanismos de defensa en invertebrados y ha sido propuesto como organismo test modelo por la OECD (Ducrot et al. 2010).

La especie caracol *Lymnaea stagnalis* habita en aguas dulces estancadas, lagos y ríos ricos en vegetación, pantanos, acequias, charcas temporales, riachuelos, etc. Se puede encontrar en Europa, el Norte de Estados Unidos y parte de Asia por lo

que puede ser considerado como un elemento indicador en la evaluación de la calidad de las aguas (Gomot et al. 1998). Presenta una concha en espiral que acaba en punta pronunciada de color marrón oscuro, raras veces gris oliva con manto de colores grisáceos oscuros. Sus antenas son triangulares y aplanadas (aspecto de orejas puntiagudas), al contrario que otros caracoles que las tienen tentaculiformes. Suelen tener una longitud de entre 2-5 cm, incluyendo su concha espiral y normalmente se encuentra en las aguas superficiales.

Figura 3. Caracol *Lymnaea stagnalis*

(recuperado de http://ep01.epimg.net/sociedad/imagenes/2009/11/27/actualidad/1259276432_850215_0000000000_sumario_normal.jpg)

Es un importante componente de los sistemas de aguas dulces ya que representa la fuente de alimentación de muchos pescados y cangrejos de río (Lodge et al. 1986). Además, esta especie es casi exclusivamente herbívora, le gusta disponer de abundante vegetación donde encuentra alimento y refugio siendo capaz de asimilar eficientemente determinados tipos de algas (Brönmark et al. 1989), por lo que su presencia tiene efectos positivos sobre el crecimiento y supervivencia de macrófagos, dado que puede retirar una gran capa de algas. A veces se desarrolla fuera del agua sobre la vegetación de ribera, aunque la mayor parte del tiempo se encuentra sumergido.

La respiración es a través de la piel, es por ello que su desarrollo está influenciado por el contenido de oxígeno del medio acuático en el que habita. La locomoción de esta especie de caracol se realiza principalmente por la coordinación de adelante hacia atrás mediante movimientos cíclicos de inflexión de su concha espiral y

cuerpo que implican contracciones musculares (Winlow et al. 1992). Son capaces de desplazarse grandes distancias, por ejemplo, desde la superficie del agua hasta lo más profundo del estuario en el que reside para conseguir el alimento alternando estos ciclos de locomoción con su respiración.

Se adapta fácilmente a las condiciones del entorno, pues son capaces de vivir en cualquier tipo de acuario, con o sin calefacción, con o sin filtración o aeración, siempre que las aguas sean duras y algo alcalinas. Si se mantiene en aguas ácidas y blandas su concha sufrirá severos desperfectos y el animal acabará muriendo. Su temperatura óptima se sitúa en torno a los 20-25ºC aunque pueden llegar a soportar temperaturas entre los 3 y los 35 ºC. Se reproduce y consigue su fuente de alimento principal en medios con plantas frondosas. Por su carácter pacífico son compatibles con cualquier otra especie que no se alimente de moluscos.

Es ovíparo, hermafrodita de reproducción cruzada y para que se reproduzca se necesitan al menos dos individuos. En su apareamiento un individuo actúa como macho y otro como hembra. A menudo, tiene lugar una inversión de los papeles sexuales por el mismo par de caracoles. Tras la cópula depositan unas bolsas de huevos gelatinosos y transparentes que eclosionan al cabo de pocas semanas. Los lugares preferidos para la puesta de huevos son plantas naturales, troncos o los mismos cristales del acuario. Durante la puesta de los huevos, producen masas gelatinosas de huevos constituidas por 100 huevos o más en su interior. Se reproducen fácilmente bajo condiciones de laboratorio (Bohlken et al. 1982). Por todo esto resulta una especie particularmente adecuada para la investigación de posibles efectos adversos causados por exposición a contaminantes donde un gran número de organismos son requeridos.

Esta especie ha sido empleada en este trabajo durante una estancia en colaboración con el centro de investigación localizado en Menlo Park, California, USGS (United States Geological Survey), para evaluar la influencia de algunas condiciones medioambientales, como la dureza del agua y la presencia de sustancias comunes naturales de ríos, ácidos húmicos, sobre la bioconcentración de nanopartículas de plata.

3.7. Sustancias peligrosas de alto impacto medioambiental: Contaminantes Clásicos y Emergentes

Este trabajo se incluye en un proyecto de Investigación Fundamental que tiene por objetivo primordial el desarrollo de herramientas específicas para evaluar los efectos tóxicos de diversos contaminantes químicos. Se han estudiado algunos contaminantes emergentes en el medioambiente como son nanopartículas de plata y dióxido de titanio y algunos contaminantes clásicos (metales y especies organometálicas).

Como *contaminantes clásicos* nos referiremos a aquellos que desde los años 40 se han empleado en la industria química como estabilizantes, pesticidas, biocidas, etc., y se han ido acumulando en el medio natural durante varias décadas. En concreto, se han estudiado arsénico, tributilestaño, plata y titanio. A continuación, se detallan algunas de las características más relevantes de cada uno de ellos.

Por otro lado, consideramos *contaminantes emergentes* como aquellos previamente desconocidos o todavía no reconocidos como tales, que aún no están incluidos en la legislación e implican riesgo por su potencial toxicidad, persistencia y bioconcentración, y que por la actividad antropogénica se están liberando al medioambiente de forma relevante en los últimos años. Según esta definición en este trabajo se incluirán dos tipos de nanopartículas metálicas ampliamente utilizadas: nanopartículas de plata y de dióxido de titanio.

3.7.1. Metales pesados y especies metaloides

Los *metales pesados* se consideran agentes contaminantes de alto riesgo para el ecosistema acuático, por tratarse de sustancias no biodegradables difíciles de eliminar por procesos naturales. Los más importantes son: Arsénico (As) (especie metaloide), Cadmio (Cd), Cobalto (Co), Cromo (Cr), Cobre (Cu), Mercurio (Hg), Níquel (Ni), Plomo (Pb), Estaño (Sn), Zinc (Zn), Aluminio (Al) y Plata (Ag). Tienen tendencia a

formar asociaciones con diversos aniones presentes en las aguas (carbonatos, sulfatos, etc.) y en mayor grado con sustancias orgánicas mediante fenómenos de adsorción, quelación, intercambio iónico, etc, por lo que se acumulan en el medioambiente, principalmente en los sedimentos de ríos, lagos y mares (Harte et al. 1991, Fergusson et al. 1990).

Una vez que los metales pesados entran en los ecosistemas acuáticos se transforman a través de procesos biogeoquímicos y se distribuyen entre el material particulado (>0,45 μm), coloidal (1 nm-0,45 μm) y especies disueltas (≤1 nm). En estas últimas especies se incluyen los iones metálicos hidratados, considerados como la fracción metálica más biodisponible, así como los complejos metálicos orgánicos e inorgánicos formados por su reacción con ligandos de origen natural o antropogénico, potencialmente biodisponibles. Además, la materia particulada y coloidal, tanto orgánica como inorgánica, desempeña un papel clave en la coagulación, sedimentación y en los procesos de adsorción, los cuales influyen en los tiempos de residencia y transporte de los metales traza a los sedimentos. Una vez que han alcanzado los sedimentos los metales pesados pueden quedar inmovilizados en los mismos o redisolverse posteriormente (Prokop et al. 2003).

Dado que un elemento metálico puede encontrarse formando diferentes especies (en distintos estados de oxidación o coordinado con otras especies), su toxicidad y características depende no sólo de su concentración sino de las especies en que estarán presentes en el medio. Por todo ello, las especies presentes metal/metaloide se transformarán en función de las condiciones biofisicoquímicas del medio, y por tanto, su forma química puede variar en función del tiempo y del espacio. Variaciones en la proporción de las especies presentes afectan a la biodisponibilidad de los metales traza, a su grado de adsorción en los coloides y partículas, y en general, a su movilidad en el medio acuático y sus tasas de transferencia a través de la interfase agua-sedimento (Tercier-Waeber et al. 2003).

Los metales pesados son elementos potencialmente tóxicos, cuya presencia en el medioambiente ha aumentado exponencialmente en las últimas décadas, fundamentalmente por causas antropogénicas. Grandes cantidades de estos metales y otros contaminantes son vertidos a la atmósfera, suelo, agua y, finalmente, penetran

en los organismos vivos mediante alguna de las múltiples vías del ciclo de los nutrientes. La contaminación por metales pesados supone un desafío medioambiental importante para los seres vivos, ya que existen diversos metales que son micronutrientes esenciales los cuales se convierten en tóxicos a concentraciones elevadas; otros, sin embargo resultan tóxicos a dosis mínimas. Por tanto, son considerados sustancias peligrosas presentes en los diferentes ecosistemas ya que pueden provocar la degradación de ríos, así como la y muerte de la vegetación, animales e incluso, afectar directamente al hombre en muchas regiones del mundo (Robert et al. 2010).

La bioconcentración de metales y metaloides como un indicador de exposición reviste especial interés expresando el carácter persistente de los mismos. El proceso de la bioconcentracion de una especie resulta un tema complejo al estar afectado por las múltiples vías de exposición y los efectos geoquímicos que afectan a la bioaccesibilidad. Los metales pesados han sido y están siendo ampliamente estudiados por sus efectos tóxicos, su capacidad de absorción y acumulación en organismos, y por la capacidad de ser metabolizado y depurado por parte del organismo en estudio (Valavanidis et al. 2010).

A continuación, se describen algunas características relevantes de las especies metálicas que han sido seleccionados para este estudio.

3.7.1.1. Arsénico inorgánico

El arsénico es un elemento de propiedades intermedias entre los metales y los no metales, aunque por su electronegatividad y energía de ionización predominan las características de no metal y forma más fácilmente aniones que cationes. Por su posición en la tabla periódica, este elemento presenta un comportamiento químico similar al del fósforo, hecho que conlleva múltiples implicaciones tanto a nivel edáfico como sobre la toxicidad del arsénico para las plantas.

El arsénico puede presentarse como: As (V), As (III), As (0) y As (-III). Algunas especies de As como: arseniato, arsenito, y sus derivados orgánicos que son las especies más encontradas en los pescados como la arsenobetaína, arsenocolina y otros arsenoazúcares son de gran interés medioambiental. Las estructuras químicas de estos compuestos se muestran en la figura 4.

El arsénico y sus compuestos han sido utilizados con fines homicidas a lo largo de la historia, principalmente en forma de anhídrido arsenioso (polvo blanco, insípido e inodoro, llamado “polvo de sucesión” y “rey de los venenos”). Entre los siglos XVI y XIX el trióxido de diarsénico (As_2O_3) fue la sustancia preferida por los envenenadores. Entre los que hicieron uso de él se encuentran los Borgia, la marquesa de Brinvilliers, la Voisin, madame Lafarge y Jeanne Gilbert. Se cree asimismo que Napoleón murió envenenado con arsénico.

Actualmente, el arsénico se usa comercial e industrialmente como un agente en la fabricación de transistores, láseres y semiconductores, así como también en la fabricación del vidrio, textiles, papeles, adhesivos de metal, conservantes en alimentos y madera, municiones (con el plomo permite la fabricación de perdigones) procesos de bronceado, y en la industria de curtiduría y peletería. También se aplica en la elaboración de insecticidas, herbicidas, raticidas, fungicidas, aunque cada vez se utiliza menos con estos fines debido a su probada toxicidad.

Algunas sales de arsénico se utilizan especialmente como colorante de ciertas pinturas y papeles, en cerámica y vidriería. Además, algunos compuestos orgánicos trivalentes del arsénico (sulfarsenol, amebarsone, cacodilatos, etc) son usados para la elaboración de productos farmacéuticos, por ejemplo, para el tratamiento de enfermedades de la piel como la psoriasis.

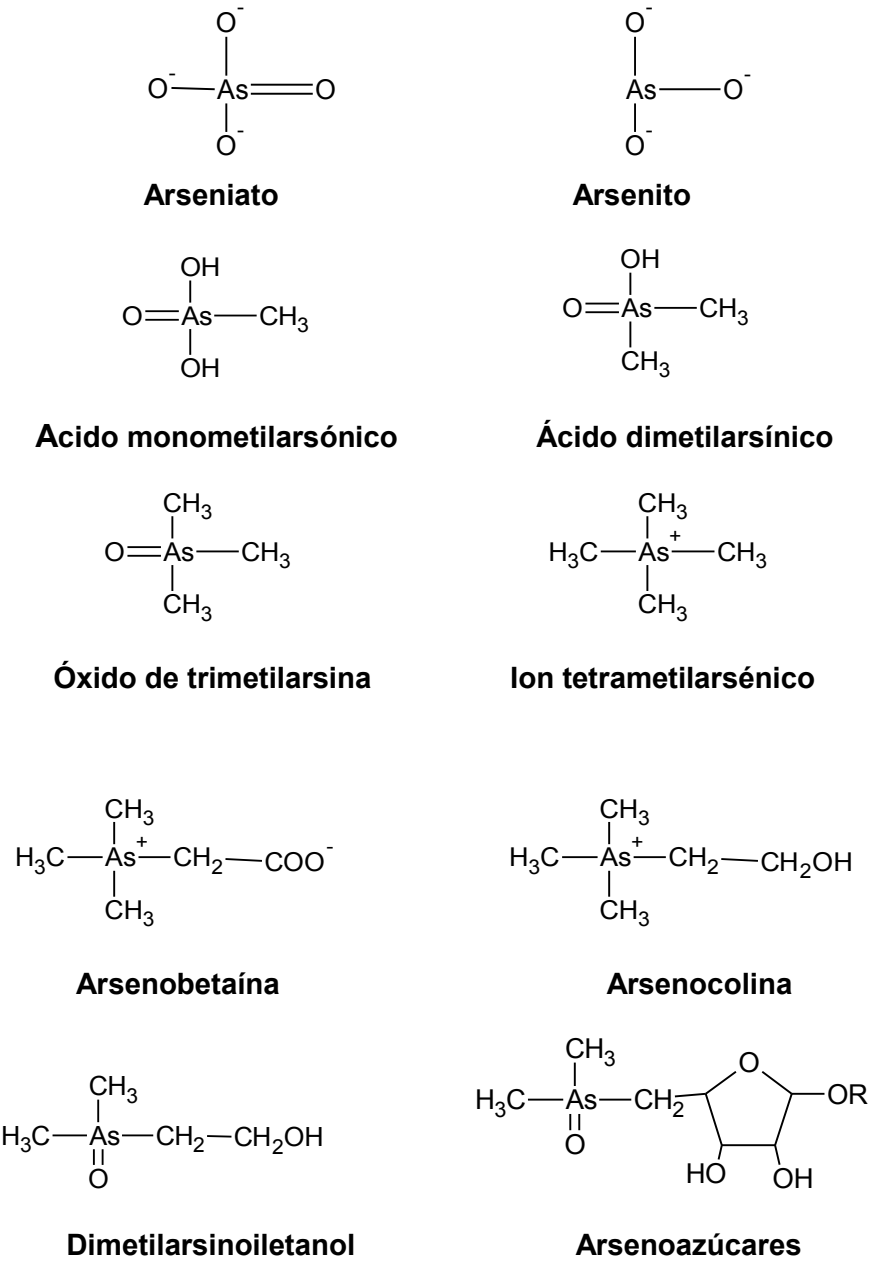

Figura 4. Estructuras químicas de las especies de arsénico predominantes en el medioambiente

Arsénico en el medio ambiente: El arsénico es un elemento omnipresente que ocupa el vigésimo puesto en abundancia en la corteza terrestre, el decimocuarto en el agua de mar y el duodécimo en el cuerpo humano. Es ubicuo en el medio ambiente ya que aunque en pequeñas cantidades, se encuentra en las rocas, en el polvo, en las cenizas, en los suelos, en el aire y en el agua (Miyashita et al. 2009). Además, es un

elemento químico inocuo o tóxico, en función de su forma química y su nivel de concentración.

En el medioambiente, raramente se encuentra el arsénico en su forma libre, ya que por lo general se encuentra combinado con sulfuros, oxígeno y hierro. Su estado de oxidación juega un importante papel en sus características, determinando además su comportamiento de absorción y movilidad (Matschullat et al. 2000).

El arsénico se encuentra en aguas naturales en diferentes formas químicas siendo las formas hidratadas las más tóxicas, mientras que las especies complejadas fuertemente o asociadas con partículas coloidales suelen ser menos tóxicas. La concentración de arsénico en las aguas naturales depende de la composición geológica y del grado de contaminación del ambiente (Jain et al. 2000). Las concentraciones de As en el agua son usualmente menores a 80 μg L^{-1} mientras que en sitios cercanos a minas o a lugares contaminados con minerales arsenicales, fluctúan entre 200 y 1000 μg L^{-1}. En aguas oceánicas presenta valores muy bajos entre 1-10 μg L^{-1}. En ríos, su concentración es muy variable y se han determinado concentraciones que oscilan desde 0,01 mg L^{-1} hasta 1 mg L^{-1} (Mandal et al. 2002).

El aporte de As al organismo se ve afectado por la presencia de minerías en zonas próximas por la capacidad de pasar a las aguas, peces y otros alimentos. Las concentraciones de As en el aire en sitios alejados de actividades antropogénicas son de 1 a 3 ng m^{-3}, mientras que, en áreas industriales emisoras de As, los niveles de este elemento pueden ser de hasta 300 ng m^{-3}. Las concentraciones de As en el suelo varían de 1 a 40 mg Kg^{-1} y, en áreas agrícolas, pueden ser más altas por la presencia de residuos de plaguicidas arsenicales (Carbonell et al. 1995). La figura 5 ilustra el ciclo geoquímico del arsénico en el medioambiente.

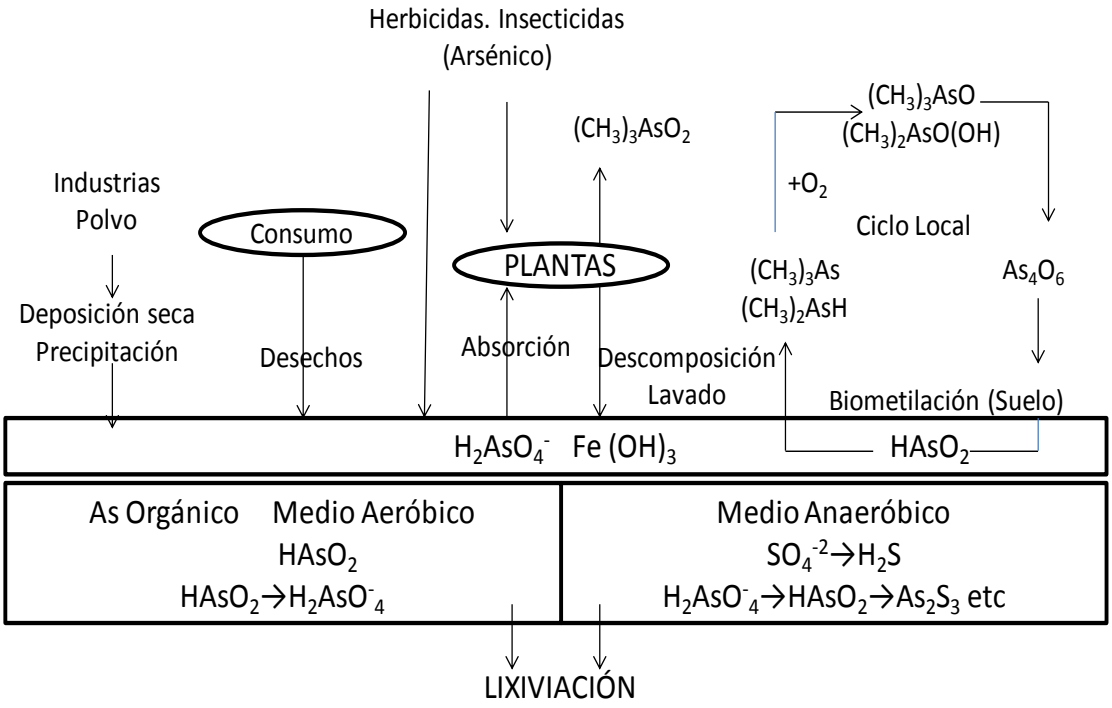

Figura 5. Ciclo geoquímico del As. (Carbonell et al. 1995)

Los crustáceos y peces marinos comestibles constituyen el principal aporte de As a la dieta, pues pueden llegar a acumular concentraciones entre 0.1 y 90 μg g^{-1} (Derache et al. 1990). Sin embargo, el As presente en este tipo de alimentos es fundamentalmente arsenobetaína, que se considera un compuesto no tóxico. La ingestión de As en el agua de bebida (principalmente arsénico inorgánico) es una fuente de exposición importante para algunas poblaciones, por lo que puede ser un problema grave de salud pública ya que la ingesta en pequeñas cantidades de arsénico inorgánico provoca problemas crónicos por su acumulación en el organismo, pudiendo además provocar cáncer (Derache et al. 1990).

Toxicidad del arsénico: La toxicidad de algunas de las especies más comunes de arsénico varía en el orden: arsenito > arseniato > monometilarsénico (MMA) > dimetilarsina (DMA) > arsenocolina > arsenobetaína (Haw-Tarn et al. 2008). A excepción del arsénico elemental, la absorción de los compuestos arsenicales se realiza a través de la piel, los pulmones y el tracto gastrointestinal. Debido a la biomagnificación de este elemento a lo largo de la cadena trófica puede resultar un

problema para la salud humana y causar efectos adversos muy variados que pueden ir desde lesiones de la piel hasta cuadros clínicos graves de los sistemas gastrointestinal, circulatorio periférico y nervioso (Ratnaike et al. 2003). El gran centro de atención actual en la toxicología del arsénico inorgánico, se centra en su potencial cancerígeno (Derache et al. 1990). La ingestión, a largo plazo, de agua de bebida rica en arsénico inorgánico (del orden de mg L^{-1}) puede inducir cáncer de piel, como se han detectado en países de América del Sur (Argentina, Chile), así como en el Sudeste asiático (Taiwán, India, Bangladesh).

La toxicidad de los compuestos trivalentes de arsénico se debe a su afinidad por los grupos sulfihidrilo de las proteínas. El arsenito As (III), se distingue por su tendencia a reaccionar rápidamente con los grupos tiol, especialmente con los ditioles, tales como el ácido lipoico. Al bloquear a los enzimas oxidativos que precisan del ácido lipoico, el arsenito provoca la acumulación del piruvato y de otros α-cetoácidos (Metzler et al. 1981). El arsenito en plantas es tan tóxico que simplemente destruye todos los tejidos con los que entra en contacto, probablemente por reacción con los grupos sulfhidrilos de las proteínas e incluso muerte celular y rápida necrosis si el contacto ha sido foliar (Wauchope et al. 1983).

Respecto a las especies de As (V) resulta difícil evaluar su contribución tóxica puesto que se reduce rápidamente a As (III) en el organismo. Puede sustituir a grupos fosfato en reacciones que son catalizadas enzimáticamente, como la producción de ATP y la síntesis de ADN. Esta especie exhibe tres formas de biotransformación: transformaciones Redox entre el arsenito y el arseniato; reducción y metilación del arseniato; y biosíntesis de compuestos organoarsenicales. En plantas, el arseniato desacopla la fosforilación en la mitocondria e inhibe la absorción foliar de otros elementos químicos, y tiene un profundo efecto en los sistemas enzimáticos causando degradación de membranas (Derache et al. 1990).

En organismos acuáticos, el trióxido de arsénico resulta tóxico en algunas especies de peces, los valores de LC_{50} a las 96 horas oscilan entre 50-100 mg L^{-1} para el pez dorado; 30-40 mg L^{-1} para la carpa, 20-25 mg L^{-1} para la trucha (Liao et al. 2007). Los arsenitos penetran en los tejidos del pez más rápidamente que los arseniatos y son más tóxicos. El As también causa daños neuromotores (McGeachy

et al. 1992), daño en la reproducción de diferentes organismos acuáticos después de la administración a los mismos de sales inorgánicas de As (Naqvi et al. 1990) e incluso daños en la médula ósea con la consecuente disminución de la producción de células rojas (Oladimeji et al. 1984).

3.7.1.2. Tributilestaño

El estaño es un metal pesado natural en la corteza terrestre que puede dar lugar a diferentes compuestos: - cuando está combinado con oxígeno, cloro o azufre se le conoce como un compuesto inorgánico de estaño, pero - cuando se combina con sustancias que contienen carbono se le conoce como compuestos orgánicos de estaño o *compuestos organoestánnicos (OTCs)* que generalmente son producidos por la acción de microorganismos. Los compuestos organoestánnicos son aquellos en los que existe al menos un enlace estaño-carbono, donde el estaño suele presentar un estado de oxidación de +4. Estas sustancias se utilizan en diferentes aplicaciones industriales, y poseen una estructura que responde a las siguientes fórmulas: R_4Sn, R_3SnX, R_2SnX_2 y $RSnX_3$; donde, R es un grupo orgánico, como por ejemplo el grupo metilo ($-CH_3$), etilo ($-C_2H_5$), butilo ($-C_4H_9$) u octilo ($-C_8H_{17}$), mientras que X es un sustituyente inorgánico, por lo general, cloruro (Cl^-), fluoruro (F^-), hidróxido (OH^-), carboxilato (COO^-) o tiol

(-SH). En general, son incoloros, o con un color amarillento leve, y tienen un olor desagradable. Son poco solubles en agua, aunque pueden serlo en agua caliente, variando su solubilidad entre 1 y 100 mg L^{-1} en función del pH, la temperatura o de los aniones presentes. Por ejemplo, en agua de mar y en condiciones normales, el tributilestaño (TBT) existe como tres especies: hidróxido, cloruro y carboxilato, que permanecen en equilibrio. A pH < 7 predominan las formas $Bu_3SnOH_2^+$ y Bu_3SnCl; a pH alrededor de 8, Bu_3SnCl, Bu_3SnOH, y $Bu_3SnCO_3^-$ y a pH > 10, Bu_3SnOH and Bu_3SnCO^{3-}. Su coeficiente de reparto octanol - agua (K_{OW}) se encuentra entre 3,19 y 3,84 para agua destilada y es de 3,54 en agua de mar (Laughlin et al. 1986).

La estructura del cloruro de tributilestaño viene representada en la figura 6.

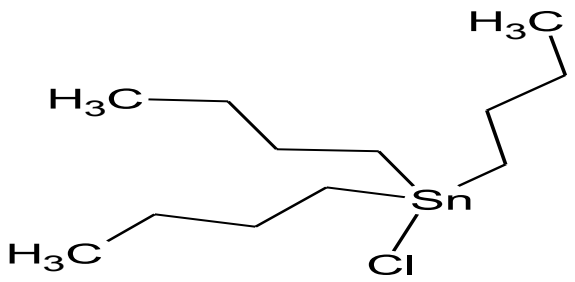

Figura 6. Fórmula molecular del cloruro de tributilestaño

Los OTCs han sido utilizados como catalizadores, estabilizadores en polímeros, insecticidas, fungicidas, bactericidas, preservantes de la madera, … (Wang et al. 1999). El dibutilestaño (DBT) se utiliza como estabilizante en la producción de policloruro de vinilo (PVC). El TBT se ha utilizado generalmente como biocida y como aditivo en pinturas anti-incrustantes para prevenir el crecimiento de moluscos y otros organismos. Actualmente, se estima que la producción anual de estos compuestos organoestánnicos es de aproximadamente 40.000 toneladas (Graf et al. 2000), de las que un 20% corresponde a derivados trisustituidos aplicados como biocidas, mientras que la mayoría de la producción corresponde a derivados mono y disustituidos que se aplican como estabilizantes de plásticos (76%) y catalizadores (5%).

Entre los principales compuestos registrados que tienen como componente al TBT se encuentran el óxido, cloruro, adipato, dodecilsuccinato, sulfuro, acetato, acrilato, fluoruro, metacrilato y resinato de tributilestaño (USEPA 1999).

El enlace Sn-C no se rompe debido a la acción del agua, del oxígeno atmosférico ni se degrada térmicamente hasta 200ºC, por lo que es estable en condiciones ambientales. Sin embargo, la radiación ultravioleta (fotolisis), la presencia de ácidos fuertes o agentes electrófilos producen su ruptura. Las propiedades físico-químicas de los compuestos organoestánnicos dependen del número y de la longitud del sustituyente alquílico, siendo la solubilidad en agua una de las más importantes, ya que regula su distribución en el medioambiente.

Tributilestaño en el medio ambiente: los compuestos But_3SnX pueden biodegradarse en el agua por la presencia de microorganismos y convertirse en derivados di- y monobutilados de estaño de menor toxicidad. Su vida media varía desde unos cuantos días hasta varias semanas en agua, aunque cuando se trata del TBT en sedimentos, la descomposición es más lenta (Santos et al.2010). En ausencia de oxígeno el TBT permanece estable durante varios años. Por tanto, en las aguas cuyos fondos están muy sedimentados, como es el caso de los puertos y estuarios, existe el riesgo de que la contaminación por TBT dure varios años (Pereira et al.1999). El TBT se degrada a DBT, éste a su vez a MBT y finalmente a estaño inorgánico Sn (IV) (Morcillo et al. 1997). El TBT adsorbido en sedimentos pasa a tener coeficientes de partición del orden de 102 a 104. Por todo esto, en octubre del 2001, la Organización Marítima Internacional (IMO) prohibió el uso de pinturas anti-incrustantes conteniendo TBT y estableció como fecha límite el 2008 para su total remoción del casco de los barcos. El Convenio internacional sobre el control de los sistemas anti-incrustantes perjudiciales de la Organización Marítima Internacional (OMI), de la que España forma parte, incluye la prohibición, a partir del 1 de enero de 2003, de la aplicación o reaplicación de compuestos de estaño que actúan como biocidas en sistemas anti-incrustantes de los buques. Todo ello ha conducido a la publicación de la Directiva 2008/105/CE de 16 de diciembre.

Toxicidad de los OTC's: la toxicidad aguda de los compuestos orgánicos de estaño depende de la longitud de sus cadenas alquílicas. En general, la toxicidad de los compuestos orgánicos de estaño está más influenciada por los sustituyentes alquílicos que por el sustituyente iónico (Tesfalidet et al.2012).

La exposición prolongada de los organismos vertebrados a los compuestos orgánicos de estaño ha producido daños en el conducto biliar en diversas especies de mamíferos y se ha demostrado que el TBT puede ser un potente agente inmunotóxico. En los mamíferos, elevados niveles del óxido de tributilestaño, pueden afectar las glándulas endocrinas, alterando los niveles hormonales de la pituitaria, las gónadas y la tiroides. El tributilestaño se puede considerar como moderadamente tóxico para las aves (Solectis et al. 1992).

Dosis relativamente elevadas de TBT han demostrado dañar el sistema reproductivo y el sistema nervioso central, la estructura ósea y el conducto biliar del hígado de los mamíferos. Además, pueden ser tremendamente irritantes pudiendo producir a elevadas concentraciones quemaduras por contacto directo en sólo unos pocos minutos, especialmente en los folículos del cabello y en la piel. Otras membranas mucosas como las de los ojos y las vías nasales también se pueden irritar con la exposición a esta sustancia (Schweinfurther et al. 1987). El TBT es tóxico para organismos invertebrados a muy bajas concentraciones con valores de LC_{50} que oscilan entre 3 - 50 μg L^{-1} para ciertos organismos de agua dulce (WHO 1990).

El TBT es lipofílico y tiene gran capacidad de acumulación en ostras, mejillones, crustáceos, moluscos, peces y algas para los cuales exhibe una toxicidad elevada (Guérin et al. 2007). Para estos organismos, el contacto con bajos niveles de TBT puede causar cambios estructurales, retardo del crecimiento, e incluso la muerte. Las larvas de las langostas inhiben su crecimiento con sólo 1 μg L^{-1} de TBT. Los moluscos, utilizados como indicadores de la contaminación del TBT por su alta sensibilidad a estas sustancias químicas, reaccionan de modo negativo a niveles muy bajos de TBT (0,06-2,3 μg L^{-1}). Las ostras bioacumulan fácilmente compuestos de TBT, alcanzando una respuesta de equilibrio poco después de la exposición y liberan con lentitud esta sustancia química. Los factores de bioconcentración son muy elevados con valores de 1000 a 6000 para ostras. El salmón juvenil acumula TBT rápidamente tras su exposición a este compuesto (Short et al. 1986).

Por lo general, el TBT se elimina muy lentamente desde los organismos que lo han absorbido. La exposición al TBT en concentraciones de 0,05 μg L^{-1} ha generado el fenómeno imposex, el desarrollo de características masculinas en las hembras, en varias especies de caracoles (Bryan et al. 1993, Oberdörster et al. 2002). También se ha observado imposex en los caracoles de fango, y en moluscos con menos de 3 ng L^{-1} de TBT. La toxicidad del TBT en el campo puede subestimarse sustancialmente en los estudios de laboratorio porque se adhiere a las paredes laterales de los contenedores y al plancton. En general, las larvas de todas las especies son más sensibles a la exposición del TBT que los adultos (Maguire et al. 2000). Por último, se ha demostrado

que el TBTO, inhibe la supervivencia de las células de las algas marinas unicelulares variando el LC_{50} tras 72 horas de exposición entre 0,33 µg L^{-1} a 1,03 µg L^{-1}.

3.7.1.3. Plata

La plata (Ag), es un metal lustroso de color blanco-grisáceo. Desde el punto de vista químico se considera un metal pesado y noble; desde el punto de vista comercial, es un metal precioso. Se conocen 25 isótopos de este elemento. Sus masas atómicas fluctúan entre 102 y 117.

Algunas veces se encuentra en la naturaleza como elemento libre (plata nativa) o mezclada con otros metales, aunque la mayor parte de las veces se encuentra en minerales formando distintos compuestos. Los principales minerales de plata son la argentita, la cerargirita o cuerno de plata y varios minerales en los cuales el sulfuro de plata está combinado con los sulfuros de otros metales. El 75% de la plata producida es un subproducto de la extracción de otros minerales, sobre todo, de cobre y de plomo.

Aunque la plata es uno de los elementos nobles más activo químicamente, su actividad no es superior a la de otros metales. No se oxida fácilmente (como el hierro), pero reacciona con el azufre o el sulfuro de hidrógeno para formar la conocida plata deslustrada. La plata no reacciona con ácidos diluidos no oxidantes (ácidos clorhídrico o sulfúrico) ni con bases fuertes (hidróxido de sodio). Sin embargo, los ácidos oxidantes (ácido nítrico o ácido sulfúrico concentrado) la disuelven formando el ion positivo de la plata, Ag^{+}. Aunque la plata no se oxida cuando se calienta, puede ser oxidada química o electrolíticamente para formar óxido o peróxido de plata, un agente oxidante poderoso. Por esta actividad, se utiliza mucho como catalizador oxidante en la producción de ciertos materiales orgánicos.

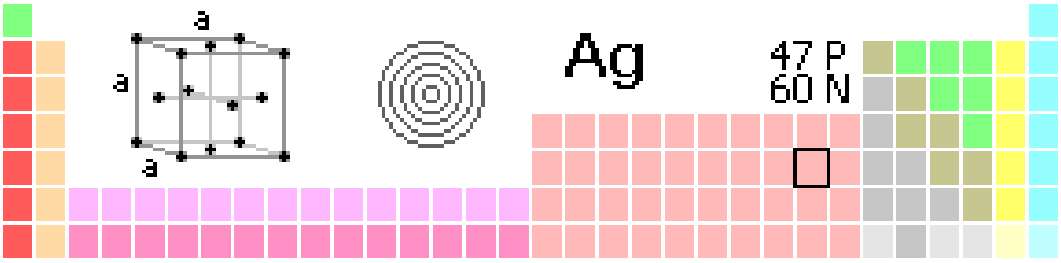

Figura 7. Plata

(Recuperado de http://enciclopedia.us.es/index.php/Plata_(elemento_qu%C3%ADmico)

Los usos de la plata han sido asociados desde mucho tiempo atrás con la comida y la bebida. En civilizaciones que datan desde los fenicios la usaban en cubertería de alta calidad para servir el agua y el vino. Muchos de sus usos se pensaron para prevenir el deterioro de los productos alimenticios.

Su uso en medicina tiene una larga historia: desde los tiempos de Hipócrates se conocía su efecto germicida y se han comercializado y aún hoy día se comercializan, diversos remedios para muchas dolencias. Aunque no existen estudios clínicos y análisis médicos que puedan demostrar la utilidad de la plata como antibiótico, se ha empleado con fines análogos. Desde 1884 los alemanes introducían el nitrato de plata como agente útil en la cura contra enfermedades causadas en el ojo en bebés nacidos de madres que tenían la gonorrea (Eisler et al. 1996). Los compuestos de plata fueron extensivamente usados durante la primera Guerra Mundial para prevenir enfermedades infecciosas; se encontraba en cáusticos, germicidas, antisépticos y astringentes, presumiblemente como desinfectante. Con los avances de antibióticos selectivos como la penicilina y la cefalosporina declinaron la mayoría de los usos médicos de la plata. Una mezcla de plata y fármacos sulfurados (por ejemplo, sulfadicina de plata en cremas) permanece en la actualidad como tratamiento antibacteriano estandarizado para combatir quemaduras de alto grado.

Plata en el medio ambiente: la plata pura no existe en la naturaleza propiamente sino los complejos de plomo, zinc, cobre y plata, que se encuentran en yacimientos donde no provocan ningún daño medioambiental. Sin embargo, en el proceso de

obtención de la plata pura a partir de complejos de plata presentes en minerales, su extracción genera una elevada contaminación atmosférica, ya que se liberan polvos en suspensión que se desplazan varios kilómetros antes de depositarse (Eisler et al. 1996). Además, esta extracción requiere grandes cantidades de agua y el empleo de sustancias muy nocivas (ácidos) que floculan y sedimentan los demás metales, permitiendo la obtención del metal más puro. Una vez que la plata se ha extraído, la contaminación se genera por el carácter ácido de subproductos y el resto de metales implicados en la reacción de extracción que normalmente se depositan en lodos potencialmente peligrosos y pueden terminar siendo vertidos a los efluentes de ríos destruyendo la biota allí existente de forma irreversible.

Toxicidad de la plata: mucho se ha debatido en el mundo científico sobre la conveniencia de la extensa aplicación de la plata en la vida de los seres humanos, por razones de su toxicidad. Aunque la plata en sí misma no es tóxica, muchas de sus sales son venenosas y pueden ser cancerígenas. Algunos de los compuestos que tienen plata pueden ser absorbidos por el sistema circulatorio, depositándose en varios tejidos causando lo que se conoce como *argiria*, una dolencia que ocasiona una coloración grisácea en la piel y algo de mucosa. Hollinger y otros autores demostraron algunos posibles efectos adversos (cicatrización de heridas retrasada, absorción dentro del sistema circulatorio y toxicidad localizada en las células) derivados del uso de la plata como agente antimicrobiano en cremas para prevenir y tratar las infecciones de las quemaduras de tercer y segundo grado (Hollinger et al. 1996).

Algunos estudios realizados con animales de laboratorio han revelado que la sobre-exposición crónica a un componente o varios componentes de la plata puede producir daños renales, oculares, pulmonares, hepáticos, anemia y cerebrales (Mayr et al. 2009). Además, la exposición a niveles altos de plata en el aire ha producido problemas respiratorios, irritación de la garganta y el pulmón y dolores de estómago.

La plata iónica es uno de los metales más tóxicos conocidos para los organismos acuáticos investigados en el laboratorio. La U.S. EPA la define como contaminante prioritario por su persistencia y toxicidad en las aguas naturales desde 1977 y por tanto, como uno de los 136 agentes contaminantes cuya liberación al medio

ambiente acuático debe ser regulada. Cuando un organismo acuático ingiere plata iónica después de su exposición en el medio, se perturba la regulación de la mayoría de los iones en las branquias por inhibición de la absorción de sodio ya que provoca una disrupción de los procesos de transporte de membrana. Esta inhibición de la capacidad para regular sodio y cloruros en las branquias perturba la concentración de la mayor parte de iones en la sangre y afecta a la regulación del volumen fluido interno, entre otros procesos fundamentales para la vida (Wood et al. 1999). Los mecanismos por los cuales la plata iónica resulta tóxica en invertebrados son más desconocidos, pero se puede producir disrupción de su metabolismo como consecuencia de la unión de la plata a enzimas ricos en grupos sulfhidrilos traduciéndose en un retraso del crecimiento.

La toxicidad de la plata para plantas y animales acuáticos está correlacionada con la concentración de plata iónica libre. En presencia de algunos ligandos naturales capaces de complejarla, como sulfuros y tiosulfatos, su toxicidad decrece remarcablemente. Hogstrand y Wood demostraron que la toxicidad de la plata estaba fuertemente influenciada por las especies presentes en el medio acuático. El estudio demostraba que las especies más tóxicas eran aquéllas con mayor tendencia a disociarse y liberar ion plata, como cloruro de plata, que presenta valores de LC_{50} de 100 µg L^{-1} para embriones y larvas de D*aphnia magna* tras 96 horas de exposición (Hogstrand and Wood 1998). Sin embargo, la presencia de iones sulfuro y tiosulfatos disminuía al menos en tres órdenes de magnitud la toxicidad de la plata debido a la formación de complejos, para las cuales los valores de LC_{50} a las 96 horas de exposición eran mayores: 100 mg L^{-1}. A pesar de ello, otros datos de LC_{50} publicados para la plata iónica son tan solo del orden de 0,8 µg L^{-1} para ciertos peces de río (Birge et al. 1995).

3.7.1.4. Titanio

El titanio es un metal de transición de color gris plata considerado como el cuarto metal estructural más abundante en la superficie terrestre y el noveno en la gama de

metales industriales. No se encuentra en estado puro sino en forma de óxidos, en la escoria de ciertos minerales de hierro y en las cenizas de animales y plantas. Está presente en titanatos (perowskita), silicatos y en muchos minerales de hierro como ilmenita óxido básico negro, $FeTiO_3$, ésta última constituye la principal fuente comercial del titanio, la esfena y el rutilo. También se encuentra en las plantas y en el cuerpo humano (Somi et al. 2009).

En cuanto a las propiedades físicas del titanio, posee una gran dureza y una muy baja corrosión frente a compuestos químicos y al medioambiente. Presenta importantes características técnicas, como ser un buen conductor de electricidad y calor, y se caracteriza por ser bastante más ligero que la mayoría de los metales. Su utilización se ha generalizado con el desarrollo de la tecnología aeroespacial, donde es capaz de soportar las condiciones extremas de frío y calor que se dan en el espacio y en la industria química, por ser resistente al ataque de muchos ácidos. Asimismo, este metal tiene propiedades biocompatibles, es inerte al contacto con entes biológicos, por lo que el cuerpo humano no lo rechaza, al contrario, los tejidos del organismo toleran su presencia. Como consecuencia se emplea en el implante de muchas (Niinomi et al.2008).

Entre sus compuestos uno de los más estudiados es el dióxido de titanio, TiO_2, que suele encontrarse en una forma de color negro castaño conocida como rutilo. El dióxido de titanio está en la naturaleza en varias formas alotrópicas: rutilo (tetragonal), anatasa (octahédrico) y brookita (ortorómbico) (figuras 8 a, b y c). Tanto el rutilo como la anatasa puros son de color blanco y son las dos formas de dióxido de titanio utilizadas comercialmente como pigmento blanco en pinturas exteriores por ser químicamente inerte, por su gran poder de recubrimiento, su opacidad al daño por la luz UV y su capacidad de autolimpieza. Ha sido ampliamente usado en la industria química y en el medio ambiente por su elevada actividad fotocatalítica. La versatilidad del TiO_2 se debe en parte a sus propiedades, ya que incluso siendo insoluble en agua, resulta químicamente inerte para sistemas biológicos además de ser de bajo coste. El dióxido de titanio también se ha empleado como agente blanqueador y opaco en esmaltes de porcelana, dando un acabado final de gran brillo, dureza y resistencia al ácido (Watanabe et al.1999).

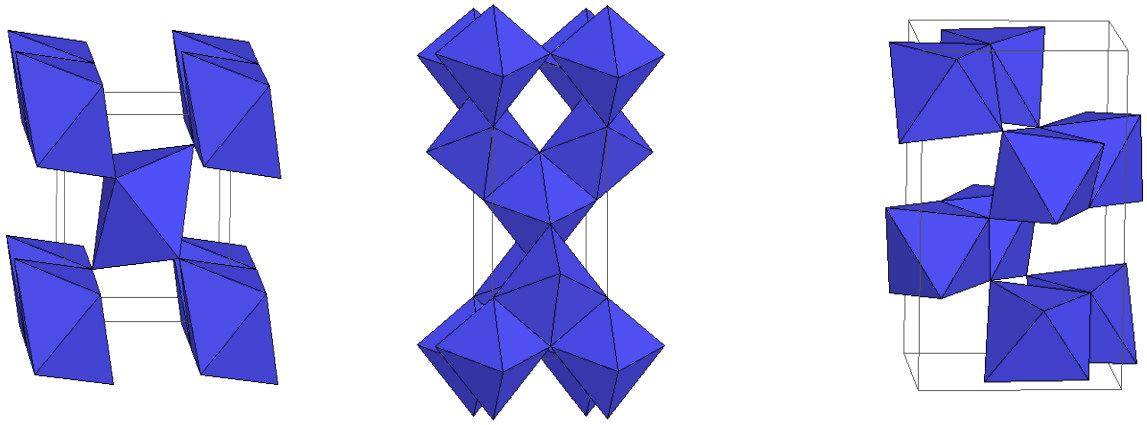

Figura 8: Estructuras cristalinas del TiO_2: a) rutilo; b) anatasa; c) brookita

Las excepcionales propiedades químicas, ópticas, dieléctricas y semiconductoras del TiO_2, han extendido sus aplicaciones a muy diversos campos científicos y tecnológicos: fabricación de aparatos electrónicos tales como sensores de gas, células fotovoltaicas y dispositivos de memoria y en la construcción de superficies autolimpiadoras (recubrimiento de salas de hospitales, componente de parabrisas, escaparates y espejos) (Scheneider et al. 1997, Jacoby et al. 2003).

Numerosas líneas de investigación que se han centrado en el estudio de enfermedades pulmonares han experimentado con titanio usándolo como marcador inerte en estudios de limpieza de pulmones por su bajo nivel bajo de toxicidad (Ferin et al. 1985). Su uso como marcador inerte en ensayos de nutrición en peces es bastante frecuente (Richter et al. 2003).

Titanio en el medio ambiente: El titanio, puede ser extraído de la corteza terrestre mediante un proceso bastante simple ya que no se encuentra a grandes profundidades. La presencia de los minerales de titanio es frecuente en rocas ígneas y metamórficas ya sea como minerales primarios o como minerales accesorios y en rocas sedimentarias y sedimentos no consolidados como minerales detríticos. En general los minerales de titanio son resistentes al ataque químico, características que

favorecen su concentración en zonas específicas dentro de horizontes sedimentarios o en arenas de ríos y playas. Tradicionalmente, el titanio se obtenía a partir del rutilo, sin embargo, la disminución de las reservas de rutilo, ha dirigido las investigaciones a obtener el titanio a partir de la ilmenita, $FeTiO_3$, cuya abundancia en la naturaleza es muy superior (Hansen et al.1995). Es uno de los metales más respetuosos con el medio ambiente. Los desechos de titanio resultantes de los procesos de fabricación son totalmente reciclables. El dióxido de titanio es anfótero y muy estable químicamente, pues no es atacado por la mayoría de los agentes orgánicos e inorgánicos, disolviéndose en ácido sulfúrico concentrado y en ácido fluorhídrico.

Toxicidad del titanio: a pesar de su gran inocuidad, se han detectado algunos efectos adversos causados por sobre-exposición *al polvo de titanio*. La inhalación del polvo puede causar tirantez y dolor en el pecho, tos, y dificultad para respirar en humanos; su contacto con la piel y los ojos puede provocar irritación.

El rutilo y la anatasa, dos de las formas alotrópicas del TiO2 son en principio materiales inertes (Zitting et al. 1972). Sin embargo, las partículas de dióxido de titanio han sido clasificadas como un polvo "molesto" (AC GIH, *American Conference of Governmental Industrial Hygienists* 1984) para el cual el valor umbral límite establecido es de 10 mg m^{-3} en total o 5 mg m^{-3} como máximo polvo respirable.

Se ha observado en experimentos con animales ciertos efectos tóxicos causados por el polvo de titanio metálico y el dióxido de titanio. Varios estudios han probado que una exposición prolongada a polvo que contenga titanio puede provocar enfermedad pulmonar crónica de carácter leve (fibrosis) (Rode et al. 1981). Por ejemplo, la inhalación de TiO_2 anatasa, puede causar fibrogénesis, consecuencia de su actividad hemolítica resultando citotóxica debido a una más lenta eliminación desde los pulmones. Algunos estudios con animales de laboratorio (ratas) expuestos a dióxido de titanio por inhalación han demostrado pequeñas áreas localizadas de polvo oscuro depositado en los pulmones que pueden estar asociados con cambios fibrógenos en los mismos (Maata et al. 1963).

El dióxido de titanio presenta una baja toxicidad en peces, con valores de LC_{50} a las 96 h ≈ 1000 mg L^{-1} para *Pimephales promelas*. Lo mismo que en invertebrados acuáticos *Daphnia magna*. En plantas acuáticas presenta una relativa mayor toxicidad siendo la concentración mínima capaz de originar un efecto tóxico, EC_{50}, de 61 mg L^{-1} en *Pseudokirchneriella subcapitata* (alga verde).

3.7.2. Nanopartículas metálicas

Son materiales de tamaño comprendido entre 1 y 100 nm que tienen extraordinarias propiedades en cuanto a dureza, estabilidad térmica, baja permeabilidad y alta conductividad (Mohanpuria et al. 2008). Actualmente se están utilizando en un gran número de diferentes aplicaciones industriales, como por ejemplo, la industria cosmética (Lens et al. 2009), biomedicina (Barnett et al. 2007), el sector de las construcciones, la electrónica (Kachynski et al. 2008), etc. El sector alimentario ha mostrado también su interés por el empleo de nanopartículas. La dimensión *nano* incrementa las propiedades antibacterianas de algunas partículas metálicas, o favorece la absorción a nivel intestinal de algunos compuestos. El desarrollo de suplementos alimentarios de compuestos en forma nano y de envases para alimentos que incorporan nanopartículas metálicas para incrementar la vida útil de un alimento son dos de las aplicaciones más comunes de las nanopartículas en este campo (Weir et al. 2012). Las consecuencias medioambientales de su uso no están aún bien establecidas pero hay indicios de una toxicidad potencial. No se han obtenido datos que pongan claramente de relieve su ecotoxicidad con la concentración ni cómo pueden verse afectadas por otros factores medioambientales que puedan inducir cambios en su estado de agregación, disolución, etc. (Navarro et al. 2008, Liu et al. 2010). Las propiedades extraordinarias de las NPs hacen que su comportamiento sea bastante imprevisible. Esto implica que la detección y la identificación de eventuales efectos tóxicos no sea evidente. De hecho, no existen de momento métodos oficiales para determinar la presencia de nanopartículas en una matriz y tampoco existen ensayos de toxicidad estándar aplicables. En la sección experimental se describen algunos ensayos realizados en diferentes organismos para los cuales se ha demostrado la

capacidad de ciertas NPs de producir efectos tóxicos adversos en la biota. La U.S.EPA inició hace unos años un programa de investigación y la EFSA *(European Food Safety Authority*) está empezando a reglamentar el uso de nanopartículas y nanotecnología en alimentación, aunque no exista de momento ninguna legislación específica que permita su utilización expresamente.

Los problemas que generan, se deben a que las nanopartículas se acumulan en diversos órganos porque el organismo no las elimina. Al ser tan pequeñas, además, pueden alcanzar cualquier parte del cuerpo, incluso atravesar las células, en cuyo interior interfieren con organismos subcelulares (Asharani et al. 2009). Hasta ahora, se había creído que las nanopartículas no eran tóxicas porque no producían reacciones químicas. Sin embargo, este descubrimiento alerta de su peligrosidad.

A continuación se describen algunos de los aspectos más relevantes de dos de los tipos de nanopartículas más comercializadas que han sido estudiadas en este trabajo: nanopartículas de plata y nanopartículas de dióxido de titanio.

3.7.2.1. Nanopartículas de plata

Las nanopartículas de plata (AgNPs) se encuentran entre las categorías emergentes de más rápido crecimiento y están siendo utilizadas en multitud de aplicaciones consecuencia de su elevada relación superficie/volumen que les confiere importantes propiedades específicas respecto a la plata metálica (Figura 9). Debido al cambio de escala, las nanopartículas de plata presentan nuevas funciones y propiedades entre las que destacan una alta conductividad térmica y eléctrica, superficie mejorada de dispersión Raman, estabilidad química, actividad catalítica y comportamiento óptico no linear (Fabrega et al. 2011). Es importante destacar que estas propiedades dependen de la morfología, tamaño y distribución de las NPs así como también del medio en el que se encuentran. Además, la dependencia de sus propiedades ópticas con el medio que las rodea abre el camino a la preparación de dispositivos que las incluyan y sean sensibles al medio.

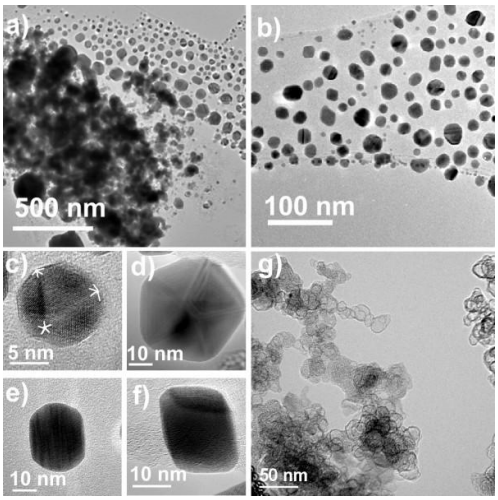

Figura 9. Micrografía de nanopartículas de Ag. (Elechiguerra et al. 2005).

Presentan un potencial atractivo en una amplia gama de productos que las contienen como son tintes y dispositivos pertenecientes al campo de la microelectrónica e imagen médica. Sin embargo, su amplio espectro de aplicaciones surge como consecuencia de su importante actividad bactericida, que junto con el relativo bajo coste que suponen las hace extremadamente populares en una gran diversidad de materiales de consumo como plásticos, jabones, pasta de dientes, y material textil (http://www.nanotechproject.org/inventories/silver/). En la base de datos "The Woodrow Wilson" aparece una lista de 1015 productos de consumo que contienen nanopartículas en su composición, de los cuales 259 contienen AgNPs (http://www.nanotechproject.org). Actualmente, las investigaciones relacionadas con las AgNPs tienen entre sus objetivos su uso en formulaciones antimicrobianas, lo que constituye un nuevo y promisorio campo de estudio**.** Están siendo empleadas en el empaquetamiento de alimentos y productos relacionados, adicionándose a polímeros para preservar por más tiempo los mismos ya que inhiben el crecimiento de los microorganismos. Se han aplicado a superficies interiores de refrigeradores para prevenir el crecimiento microbiano y mantener un ambiente limpio e higiénico dentro del mismo. También se han incorporado dentro de recubrimientos activos antimicrobianos, como por ejemplo en baterías de cocina, vajillas y en tablas de cortar.

(http://www.nanotechproject.org/inventories/consumer/browse/products/fresherlonger_miracle_food_storage/).

El sector textil es otro de los sectores en el cual las nanopartículas de plata han cobrado enorme importancia. En efecto, su producción y empleo se ha multiplicado por 10 entre los años 2009 y 2012 empleándose en prendas, tejido de hogar, sector médico, deporte, campo militar y textiles industriales. Muchas marcas de calcetines y de medias y prendas de deporte integran esta tecnología. Presentan también un potencial atractivo como componente de tejidos de uso médico ya que muchos fabricantes de vendajes las han puesto en el mercado y se venden libremente en farmacias (Wilkinson et al.2011).

La medicina es otra de las áreas de uso de las AgNPs. Al igual que ocurre con la plata metálica, son capaces de matar a bacterias y virus al impedir el transporte de los electrones en los microbios; el inconveniente es que los altos niveles de iones plata pueden matar también a las células. Se ha demostrado que las AgNPS tienen efecto en bacterias Gram negativas como *Escherichia coli*, *Vibrio cholera*, y *Pseudomonas aeruginosa* (Morones et al. 2005, Sondi et al. 2004), Gram positivas como *Bacillus subtilis, Staphylococcus aureus* (Shrivastava et al. 2007). En 2005, en el "Journal of Nanotechnology" se publicó un artículo, que investigó la actividad antiviral de nanopartículas metálicas. Todos estos autores demostraron que, sin ser citotóxicas, las AgNPS son capaces de inhibir la infección por VIH-I a cultivos celulares in vitro actuando en la etapa de fusión del virus a la célula (Elechiguerra et al. 2005.).

Toxicidad de las nanopartículas de plata: debido al crecimiento exponencial de la producción de nanopartículas de plata, existe una gran preocupación por el impacto que las mismas pueden tener una vez sean descargadas al medio como productos de desecho. Por ello, el estudio de la predicción del peligro y riesgo que las mismas presentarán para la salud y el medio ambiente está siendo objeto de muchas líneas de investigación.

Hasta el momento, dado que la Nanotecnología es un sector emergente, no existe una amplia bibliografía de estudios relacionados a la toxicidad de las AgNPs en

comparación con sus aplicaciones potenciales. Algunos estudios realizados *in vitro* con células humanas demuestran que pueden producir estrés oxidativo de las células, paro del ciclo celular, inflamación, genotoxicidad, apoptosis y necrosis (Lim et al. 2012, Asharani et al. 2009). Por otro lado se ha propuesto que pueden interaccionar con el sistema inmune y producir un efecto inmunomodulador, bien mediante estimulación, bien mediante inhibición (Zolnik et al. 2010).

Algunos estudios *in vivo* con ratas a las cuales se les administraron nanopartículas de diferentes metales vía intravenosa, intraperitoneal e intracerebral mostraron que nanopartículas de diferentes tamaños eran capaces de cruzar la barrera hematoencefálica, modificando su permeabilidad y produciendo daños sobre la misma (Sharma et al. 2010). En dicho trabajo los animales expuestos a AgNPs presentaban edema cerebral en mayor magnitud comparado a los animales expuestos a nanopartículas de otros metales.

Numerosos trabajos desarrollados en organismos acuáticos modelo han demostrado los efectos adversos de las AgNPs: alteraciones o retardo en alguna de sus etapas de crecimiento, así como su deposición en células del núcleo, en el cerebro, sistema nervioso y en la sangre (Asharani et al. 2008, Yeo et al. 2008, Chae et al. 2008). Algunos estudios revelan la habilidad de las AgNPs para acumularse en órganos como en branquias e hígado afectando a la capacidad reactiva de los peces frente al hábitat que presentan niveles bajos de oxígeno e induciendo el estrés oxidativo (Bilberg et al. 2010, Griffitt et al. 2008).

Actualmente, uno de los retos de las líneas de investigación es evaluar su comportamiento una vez alcanzan el medioambiente ya que las AgNPs pueden formar agregados/aglomerados, oxidarse a plata iónica, o incluso formar complejos modificando su accesibilidad para los organismos acuáticos y del mismo modo su toxicidad. Ya existen algunos datos que constatan que se encuentran en aguas superficiales a concentraciones del orden de ng L^{-1} provenientes de productos de consumo y se espera que esta concentración incremente exponencialmente en respuesta a su creciente uso (Benn et al. 2008). La especiación de las AgNPs, una vez hayan sido liberadas al medio será crítica para evaluar su toxicidad potencial.

En la sección experimental se presenta una revisión bibliográfica en la que se han descrito con detalle algunos ejemplos relevantes sobre el impacto que las nanopartículas de plata poseen sobre los humanos y organismos acuáticos. Además, también se muestran algunos otros ejemplos ilustrativos de los problemas que se están encontrando actualmente en su determinación y en la interpretación y comprensión de sus mecanismos de toxicidad.

3.7.2.2. Nanopartículas de TiO_2: anatasa y rutilo

Las nanopartículas de TiO_2 constituyen otro grupo que presentan multitud de aplicaciones por su importante capacidad de absorber la radiación UV y por su actividad fotocatalítica (Gao et al. 2001). En 2006-2010 se estimó una producción mundial de 5000 toneladas y se espera que entre 2011-2015 esta cantidad se incremente hasta 10000 toneladas alcanzando las 2,5 millones de toneladas para el 2025 (Menard et al. 2011).

En las dos últimas décadas se han utilizado como componentes esenciales en una gran variedad de productos comerciales como son productos de protección solar, por su gran capacidad para reflejar, dispersar y absorber la radiación ultravioleta (UV). De este modo, actúan protegiendo más eficientemente contra los efectos dañinos inducidos por la exposición prolongada a la luz solar. En su forma nanométrica el dióxido de titanio tiene la doble ventaja de ser un protector solar más eficaz contra la radiación UV y además es menos blanco y más transparente que la forma micrométrica. Cuando se formulan con otras sustancias empleadas como filtros de la radiación solar, especialmente con los orgánicos, las nanopartículas de TiO_2 pueden actuar absorbiendo ambos espectros de radiación, el UV-A y el UV-B, convirtiéndose así en un factor esencial para la prevención del cáncer de piel.

Estas nanopartículas utilizan la energía de la radiación absorbida para catalizar reacciones de otras moléculas a temperaturas bajas. La comunidad científica ha clasificado este tipo de nanopartículas como catalizadores de muy alto rendimiento a partir de luz solar.

Además, las nanopartículas de TiO_2 presentan efectos positivos sobre germinación de las semillas y el crecimiento de la raíz de algunas plantas (Zheng et al. 2005, Clément et al. 2013). Se cree que estos efectos positivos son debidos a las propiedades antimicrobianas de la estructura cristalina anatasa que incrementa la resistencia de la planta al estrés medioambiental. También se utilizan en procesos de descontaminación medioambiental de aire, suelos y aguas.

Este tipo de nanopartículas se consideran biológicamente inertes (Jugan et al. 2011) por lo que poseen un importante atractivo en el campo de la medicina, ya que presentan elevada estabilidad y baja citotoxicidad frente a células humanas. Las nanopartículas de dióxido de titanio son capaces de matar virus, bacterias y hongos e incluso células cancerígenas (Kubota et al. 1994, Tsuang et al. 2008). Cuando las nanoparticulas de TiO_2 se irradian con radiación UV se generan pares de electrones excitados y huecos en su banda de valencia que promueven la formación de especies reactivas de oxígeno como peróxido de hidrógeno, radicales hidroxilo y superóxidos capaces de matar a las células cancerígenas. En los últimos años se han aplicado en la fototerapia de células cancerígenas y de bacterias actuando como fotosensibilizador. Sin embargo, su aplicación como agente anticancerígeno presenta todavía algunas limitaciones, ya que la radiación UV no es capaz de penetrar profundamente dentro de los tejidos humanos, resultando sólo efectivas para tumores superficiales. Además, la vida de los radicales de oxígeno generados es corta por lo que no será capaz de provocar la muerte de las células cancerígenas prolongadamente. Por otro lado, las nanopartículas de TiO_2 incrementan la eficiencia de absorción de algunos fármacos empleados en la lucha contra el cáncer por las células para los que la célula ha sido capaz de desarrollar resistencia inhibiendo su acción terapéutica (Song et al. 2006).

Toxicidad de las nanopartículas de TiO_2: como consecuencia de su elevada aplicación, su descarga en el medio ambiente es inevitable y por ello, al igual que con las nanopartículas de plata, son ya numerosos los grupos de investigación que se han centrado en la evaluación del impacto que tendrán sobre la salud y el medioambiente. Los dos tipos de nanopartículas de TiO_2 mayormente comercializadas se diferencian en su estructura cristalina: rutilo (tetragonal) y anatasa (octaédrico). Cada una de estas

formas alotrópicas presenta diferentes propiedades superficiales y reactividad que se traducen en diferencias en su toxicidad asociada.

Algunos estudios han demostrado que pueden ocasionar daños en el ADN; científicos del Johnson Comprehensive Cancer Center de la UCLA (en Estados Unidos) han demostrado que las TiO_2NPs presentes en productos muy comunes, como los cosméticos o los bronceadores, causan daño genético sistémico en ratones al inducir roturas en las cepas del ADN (Trouiller et al. 2009). Además, también causaron daño cromosómico e inflamación, así como aumentaron el riesgo de cáncer en los animales.

Estudios *in vitro* se han llevado a cabo en ratones para evaluar y valorar los posibles efectos que las TiO_2NPs pueden causar en los mismos demostrando que su inhalación causa inflamación pulmonar. Se demostró que las nanopartículas de TiO_2 eran causantes de una reacción inflamatoria, afectando a la fisiología hemostática, al observarse una red de fibrina que recubría las fibras más grandes en los ratones expuestos a ambos tipos de nanopartículas (Oosthuizen et al. 2010).

Las TiO_2NPs pueden alcanzar lagos, ríos y otras fuentes de agua en las que los microorganismos desempeñan un papel fundamental a la hora de mantener un medio ambiente saludable. Gruden y otros autores han observado una disminución significatica en la supervivencia de la bacteria *Escherichia coli (E. coli)* cuando se expone en cultivos de laboratorio a pequeñas concentraciones de nanopartículas en menos de una hora (Mileyeva-Biebesheimer et al. 2010).

Se prevé que las concentraciones medioambientales de TiO_2NPs resulten del orden de $\mu g\ L^{-1}$ y a esas concentraciones no se observe bioacumulación (Mueller et al. 2008). Sin embargo, ya se han demostrado algunos efectos adversos asociados a estas concentraciones medioambientales en la especie mejillón *Mytilusgallo provincialis* tras 96 horas de exposición in vivo a suspensiones de 1, 10 y 100 $\mu g\ L^{-1}$ (Barmo et al. 2013). Los resultados revelan que las TiO_2NPs afectan a la actividad funcional y molecular de las glándulas digestivas de esta especie de mejillón y a sus células inmunes. La mayoría de los trabajos publicados hasta la fecha suelen ser en base a la exposición a TiO_2NPs en concentraciones del orden de $mg\ L^{-1}$ (Wiench et al. 2009). También se han evaluado los efectos subletales sobre la reproducción del pez cebra tras la exposición a estas nanopartículas en concentraciones de 0,1 y 1 $mg\ L^{-1}$. No se

observaron efectos toxicológicos significativos, pero la reproducción sí se vio negativamente afectada (Ramsden et al. 2012).

Recientemente, se ha demostrado que las TiO_2NPs expuestas a la radiación ultravioleta pueden ser tóxicas para los organismos marinos (Miller et al. 2012). Dichos autores señalan que el dióxido de titanio a escala nanométrica presenta la propiedad de generar especies reactivas del oxígeno cuando se exponen a la radiación ultravioleta resultando muy eficiente en los recubrimientos antibacterianos y en la desinfección de las aguas residuales. Sin embargo, por su reactividad a la luz solar y otras formas de radiación ultravioleta, pueden inducir toxicidad para el fitoplancton marino, los productores primarios más importantes de la Tierra.

Hasta la fecha, los resultados publicados sobre la toxicidad causada por nanopartículas son difíciles de comparar, incluso si se trata de la misma sustancia. En el caso de las TiO_2NPs su rápida cinética de agregación se traduce en un cambio importante en sus propiedades fisicoquímicas y como consecuencia afectará a su reactividad, interacciones celulares y en definitiva a su toxicidad. Se ha observado que pueden estabilizarse por la presencia, en los medios acuáticos de los ácidos húmicos que incrementan las repulsiones electrostáticas evitando su aglomeración (Daohui et al. 2012). Otros componentes naturales presentes en el medio acuático son los ácidos fúlvicos, capaces de afectar a la accesibilidad de las mismas provocando fenómenos de agregación (Domingos et al. 2009). La presencia de organismos acuáticos puede influir también en la cinética de aglomeración de este tipo de nanoparticulas (Filella et al. 2008).

Todo lo expuesto pone de manifiesto la importancia de monitorizar la distribución del tamaño de nanopartículas durante el curso de los estudios de toxicidad ya que su forma química se encuentra estrechamente relacionada con su potencial toxicológico. Daohui Lin y otros autores probaron la influencia de la presencia de ácidos húmicos sobre la toxicidad de nanopartículas de TiO_2 para la especie alga *Chlorella sp* demostrando que estos componentes naturales de los ríos disminuían la toxicidad causada por las mismas (Daohui et al. 2012). Esta protección provenía del hecho que los ácidos húmicos prevenían la adhesión de las TiO_2NPs a las células de esta especie debido a un incremento de las repulsiones electrostáticas entre ellas.

Algunos ejemplos representativos sobre la cinética de agregación de TiO_2NPs una vez que son liberadas al medio como otros relacionados con el impacto toxicológico asociado a las mismas se ha explicado con más detalle en la revisión bibliográfica de nanopartículas presentada al principio de la sección experimental.

4. EXPERIMENTAL

4.1. Test de Bioconcentración Alternativo al Test OECD 305

Los trabajos presentados en esta tesis doctoral se llevaron a cabo de acuerdo a la normativa REACH. Se propone el empleo de larvas de pez cebra (eleuteroembriones) como sistema modelo de vertebrados. Para ello, se ha desarrollado un test simplificado, ilustrado en la figura 10, como ensayo de Toxicidad Alternativo al Test OECD 305. Algunas de las características más relevantes de dicho test se describen a continuación:

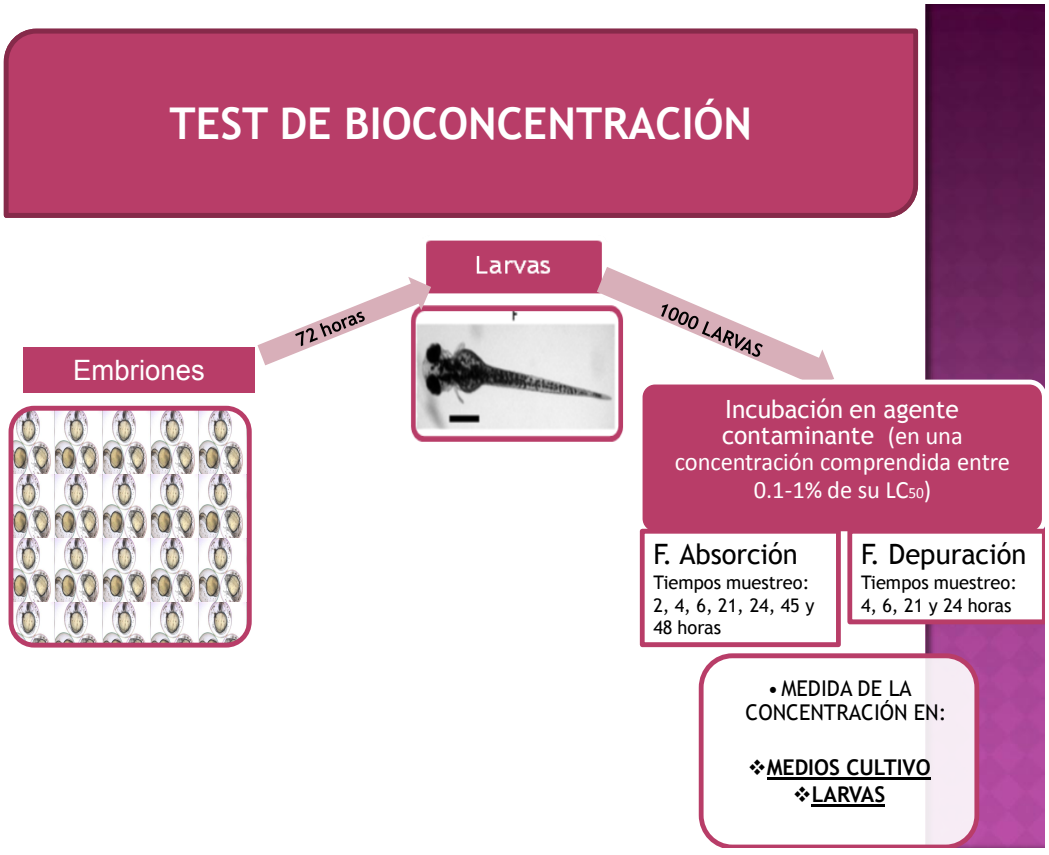

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Figura 10. Test Bioconcentración desarrollado y propuesto como alternativa al Test OECD 305.

Como se ha mencionado en el apartado 3.4, el Test de Bioconcentración de la OECD (OECD/305) es una de las Guías de ensayo que goza de mayor uso y repercusión para evaluar el potencial de bioconcentración por peces adultos de cualquier sustancia química cuya producción exceda las 100 t/año. Debido al alto coste y a la gran cantidad de peces que requiere dicho test, junto con la gran complejidad que implican estos experimentos resulta evidente la necesidad de *desarrollo y validación de nuevas metodologías alternativas*. El bioensayo de toxicidad seguido aquí surge como alternativo al Test de Bioconcentración de la OECD 305 y se ha llevado a cabo teniendo en cuenta una serie de pautas que se detallan a continuación:

El ensayo consiste en dos fases:

Una primera fase de absorción, que termina por lo general en estado de equilibrio, y suele durar 48 horas (salvo que se demuestre que dicho equilibrio se alcanza antes) o hasta un máximo de 72 horas si no se alcanzase dicho equilibrio.

Una segunda fase de depuración, que tiene una duración de aproximadamente la mitad de la primera, unas 24 horas en la que las larvas se exponen al medio exento del compuesto a ensayar. Se considera que tienen capacidad de depuración si la concentración de las larvas se reduce al menos en un 30 % respecto a la máxima obtenida.

Los tanques o recipientes empleados para el ensayo serán un mínimo de seis: tres para la Fase de Absorción (un tanque para el control y dos tanques para la sustancia a testar a dos concentraciones diferentes) y tres para la Fase de Depuración. Las dimensiones de estos tanques deben adaptarse a la tasa de carga, para que no varíe más de un 20 % la concentración de la sustancia a testar a lo largo de todo el ensayo.

La concentración máxima del contaminante al que se expondrán las larvas debe estar alrededor del 1% del LC_{50} agudo asintótico. Para los compuestos cuya bioacumulación es lenta se contempla la posibilidad de aumentar esta concentración para poder obtener datos a tiempos cortos. De igual modo, en compuestos cuyo LC_{50}

esté cerca del límite de detección, se podrá contemplar la alternativa de elevar este índice, por ejemplo hasta el 10%. La segunda concentración a ensayar debe diferir en un factor de 10 de la anterior.

El número de larvas que se deben exponer debe ser al menos un mínimo de 10 individuos por cada 20 mL. Asimismo, la OECD estipula que la densidad de las mismas esté comprendida entre 0.1 y 1.0 gramos de larvas/litro de agua. En nuestro caso, se estimó que debería ser al menos 1 mL de medio de incubación por larva (lo que daría una tasa de carga de 0.7-0.8 g/L).

La variable de alimentación se desestima al tratarse de larvas que no se alimentan del medio exterior.

Por último, es esencial medir los parámetros de calidad del agua (cantidad de oxígeno disuelto, pH, temperatura y dureza) antes de comenzar con los experimentos de contaminación de las larvas. Posteriormente, habrá que medirlos todos los días que dure el experimento de bioconcentración.

4.2. Estudios de bioconcentración de contaminantes clásicos: Arsénico y Tributilestaño

El trabajo descrito en el apartado 4.2.1. se centró en el estudio de la bioconcentración de las especies arsenito, As(III), y tributilestaño, TBT, por el sistema modelo propuesto. De acuerdo a la clásica definición del cálculo del Factor de Bioconcentración, que implica la determinación de la concentración total del analito de estudio en el tejido del pez y en el medio acuoso en el que ha sido expuesto, resultó necesario el desarrollo de una metodología analítica para ambos tipos de muestras: medios de cultivo y larvas del pez cebra. La metodología desarrollada implicó un tratamiento de muestra adecuado a la determinación de los analitos mediante Espectrometría de Absorción Atómica por Cámara de Grafito (ZGF-AAS), proporcionando la sensibilidad, exactitud y precisión necesarias. Los resultados obtenidos para los BCF han demostrado ser consistentes con otros trabajos ya

publicados realizados en peces adultos. En el caso del arsénico, tras la exposición a las larvas a las concentraciones nominales de 5 y 50 μg L^{-1}, los BCFs obtenidos (BCF≈10) evidenciaron que no existía una bioconcentración significativa. Para el TBT, sin embargo, los valores tan altos de BCF obtenidos, (840-1280), para las concentraciones nominales de exposición de 0,2 y 2 μg L^{-1}, permiten clasificar a esta sustancia como peligrosa y altamente bioacumulable para las larvas del pez cebra.

El trabajo descrito en 4.2.2 se planteó a partir de los resultados obtenidos con el TBT en el anterior. Por ello, el objetivo de este trabajo fue el estudio de la posible degradación del TBT como posible mecanismo de detoxificación por las larvas del pez cebra. La determinación de las correspondientes especies derivadas de la degradación de TBT, DBT, MBT, y Sn inorgánico se llevó a cabo por Cromatografía de Gases acoplada a un Detector Fotométrico de Llama, GC-FPD. Se optimizaron los parámetros instrumentales de esta técnica analítica, comúnmente empleada en la determinación de especies organoestánnicas. Los resultados obtenidos por GC-FPD fueron validados mediante el análisis de un material de referencia con valores orientativos para las especies de estaño, así como mediante el empleo de técnicas alternativas como ZGF-AAS e ICP-MS para la determinación del total extraído. Cabe destacar que en este trabajo la extracción de las especies organoestánnicas resultó un reto analítico dada la naturaleza lipofílica de las muestras de larvas de pez cebra. Por otra parte la pequeña cantidad de muestra disponible requería el empleo de métodos analíticos de muy elevada sensibilidad para poder llevar a cabo la especiación de los OTCs.

4.2.1. Zebrafish eleutheroembryos as a model for evaluation of inorganic arsenic and tributyltin bioconcentration (Water Research, 2011)

Los resultados de este trabajo fueron presentados como
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Zebrafish larvae as a model for the evaluation of inorganic arsenic and tributyltin bioconcentration

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ABSTRACT

The European REACH legislation establishes the need to study the toxicity, persistence and bioaccumulation of those chemicals with an exceeding production of 100 tons and/or chemicals considered PBTs substances (Persistence, Bioaccumulation and Toxicity). Currently, the OECD technical guideline 305 is the most used protocol to determine bioconcentration factors of contaminants in aquatic environments. However, this procedure implies high cost and amount of adult fishes. Zebrafish (*Danio Rerio*) has been selected since this animal model has several advantageous features over other vertebrates, mainly fast embryonic development and easy growth. The analytical methodology here developed has been applied to calculate the bioconcentration factors (BCFs) of two contaminants: inorganic arsenic and tributyltin (measured as arsenic and tin). The method is based on the use of an ultrasonic probe assisted extraction for accelerating the sample treatment followed by detection using graphite furnace atomic absorption spectrometry with Zeeman correction (ZGFAAS). Results obtained for the BCFs values are in good agreement with previously reported data on freshwater aquatic organisms. In the case of arsenic, after exposing larvae to concentrations of 5 and 50 $\mu g\,L^{-1}$, very low BCFs were observed (between 2.2 and 9.5); while for tributyltin, the BCFs observed were within the range 840–1280 after exposure to concentrations of 0.2 and 2.0 $\mu g\,L^{-1}$, respectively. This study shows the use of zebrafish larvae together with the proposed analytical approach as a promising alternative to the OECD 305 test to evaluate the BCFs of classical and emergent contaminants.

1. Introduction

Over the last couple of decades, environmental pollution has become an increasing relevant issue to our society. Evaluation of pollution comprises two different approaches: first, the determination of the damage in the environment caused by already polluted areas and the potential remediation of the harmful effects; and second, prevention of the contamination by previous evaluation of the potential impact of chemicals in the environment. This chemical impact has been traditionally evaluated by studying the toxicity of the species. However, studying the toxicity alone is not sufficient to provide a complete environmental impact analysis. Other parameters such as ecotoxicity, mobility, persistence, bioaccumulation, and degradation have to be considered. Actually, the recently approved European regulation REACH (Registration, Evaluation and Authorisation of Chemicals) (European Commission, 2006) requires the evaluation of such parameters for those

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chemicals with a production exceeding 100 tons and/or those substances that are considered PBTs (Persistence, Bioaccumulation and Toxicity).

Although there are different established methods to evaluate the bioaccumulation factor (ASTM E1022-94 from the American Society for Testing and Materials and OPPTS 850.1730 from US EPA), the OECD bioconcentration Test 305 (OECD, 1996) is the most commonly used. It is summarized on the REACH's Test Methods Regulation (European Commission, 2008) as the standard method to calculate bioconcentration. Briefly, this test evaluates the accumulation in adult fish of a dissolved chemical by measuring its final concentration in both, the fish and the surrounding media after an equilibration time. This complex method is expensive and requires a large amount of adult fish (Weisbrod et al., 2007). Thus, the development of an alternative method for establishing the bioconcentration factor (BCF) of a given chemical but reducing the cost of the analysis and the amount of adult animals required would be of great interest.

Taking into account that the European legislation calls for the use of non-animal alternative approaches to replace animal testing wherever possible, several frameworks have been developed in the last years. As a first approach to estimate theoretical BCFs of chemicals, computational methods as Quantitative Structure Activity Relationship (QSAR) (Meylan et al., 1999) or Baseline Models (POPs) (Dimitrov et al., 2005) can be used. Other approaches use in vitro methods such as cell-based assays using liver slices, hepatocytes, cell lines, S9 fractions, microsomes, recombinat enzymes and nuclear receptors (Weisbrod et al., 2009). Recently, a protocol using fish eggs under short-term exposure has become a substitute for the acute fish assay in the toxicity analysis of wastewater in Germany (DIN, 2001).

In Europe (2010/63/EU Directive), fish embryos and larvae are legally considered to be in vitro systems until they become free-feeding larvae. As a result, they are increasingly being used as alternatives to acute fish toxicity tests (Scholz et al., 2008) and other applications (Petersen and Kristensen, 1998). Zebrafish, a small tropical fish native to the rivers of India and South Asia, is an animal of great scientific interest due to the advantageous features over other vertebrate model systems (Teraoka et al., 2003). The small size of larvae and adult zebrafish results in lower test cost. Transparent embryos allow the detection of morphologic and embryonic changes and also help to easily distinguish between dead and living embryos. A high production and fast embryonic development facilitates fast bioaccumulation kinetics (with a maximum bioaccumulation achieved in less than 72 h). The zebrafish also has a high genomic homology with humans (over 80%), which enables a significant correlation of the data obtained between the two species and, in addition, it is one of the model animals recommended by OECD Bioconcentration Test 305 (OECD, 1996).

An alternative strategy for the determination of the BCFs of chemicals might therefore imply the use of fish (especially zebrafish) embryos or larvae as in vitro model (Teraoka et al., 2003; Schreiber et al., 2009). The key criteria identified for judging the reliability of alternative study were established by a workshop of experts from governments, industry, and academia (Parkerton et al., 2008): (1) clear specification of test substances and fish species investigated, (2) analysis of test substances in both fish tissue and exposure medium, (3) no significant adverse effects on exposed test fish, and (4) a reported BCF test reflecting steady state conditions with unambiguous units. To measure the internal concentration of chemicals in fish embryos or larvae for BCF determination, highly sensitive analytical methods are required due to the extremely small sample size.

Heavy metals, such as mercury, cadmium, lead, arsenic and tin are well-known pollutants that can cause evolutionary changes due to their harmful effects on living organisms. Among these, arsenic and tin are relatively toxic to the environment, mainly to aquatic organisms (Chagot et al., 1990; Prieto García et al., 2006; Liao et al., 2008), observed high accumulation factors for both elements in several freshwater species exposed to arsenic or tin at the mg L^{-1} (Bushong et al., 1998). Determination of those analytes in biological samples requires a previous extraction step. The most common methods applied for this purpose are: ultrasound- and microwave-assisted solvent extraction (Bermejo et al., 2004), acid solubilization and SPE/SPME (Pan and Pawliszyn, 1997; Gómez-Ariza et al., 2000). Derivatization steps such as ethylation prior to separation/quantification are also needed for TBT analysis in many cases (Morabito et al., 2000). Finally, determination of these analytes is performed with highly sensitive detectors (ICP/MS, GFAAS, FAAS, GC/MS). One of the problems related to the use of such small samples as zebrafish larvae (wet weight of 0.4 mg) implies that the extraction techniques employed should be capable to use small quantities of extractant. In addition, clean-up and sample preparation procedures should be as simple as possible to avoid analyte loose. Quantification techniques should reach very low limits of detection since small sample volumes at very low analyte concentrations are used.

The present study has been focused on developing analytical methods to evaluate the bioaccumulation factor of inorganic arsenic and tributyltin by zebrafish larvae considering that both species are stable in natural water (Gómez-Ariza et al., 1999; Hall et al., 1999). The instrumental technique selected for determination of the total concentration of both analytes in such samples have been Zeeman corrected graphite furnace atomic absorption spectrometry (ZGFAAS) because of its high sensitivity, low sample consumption, compatibility with organic solvents, and ability to directly analyzed solid samples (Bryszewska et al., 2009).

2. Experimental section

2.1. *Instrumentation*

A Perkin–Elmer 4100 ZL atomic absorption spectrometer with a longitudinal Zeeman background correction, equipped with a transversely heated graphite tube atomizer (THGA) with L'vov platforms was used. The analyte concentration was calculated from the integrated absorbance of the atomic absorption signal. A volume of 20 μL was injected manually. The furnace operation was controlled using the Perkin–Elmer AA Winlab software, Version 4.1 SSP1. A Perkin–Elmer arsenic

electrodeless discharge lamp (EDL) with wavelength 197.3 nm and instrument slit width 0.7 nm was used. A Perkin–Elmer EDL System was used to stabilize the lamp current between 349 and 351 mA. For tin, A Perkin–Elmer hollow cathode lamp (HCL) with wavelength 286.3 nm and instrument slit width 0.7 nm was used.

A Vibra cell VCx130 ultrasonic processor (Connecticut, USA) equipped with a titanium 2-mm diameter microtip and fitted with a high-frequency generator of 130 W at 20 kHz was used for the leaching of the analytes from larvae in deionized water. Centrifugation was carried out in a centrifuge model type: FVL-2400N, Combi-Spin, Boeco (Germany).

2.2. Reagents and standards

Analytical grade chemicals were used for all studies. Tributyltin chloride, CAS: 1461-22-9, (>97%) and Triton X-100, used as surfactant, were obtained from Sigma–Aldrich (Madrid, Spain) and $As_2O_3 \cdot H_2O$ (99.5%), CAS: 1327-53-3, from J.T. Baker (Deventer, Holland). Glacial acetic acid was purchased from Panreac Química S.A. (Madrid, Spain); methanol was supplied by Scharlab S.L. (Barcelona, Spain), toluene was provided by Carlo Erba Reactifs-SDS (Cedex, France) and tropolone (98% purity) from Avocado (Lancashire, UK). Nitric acid was purchased from Merck (Damstadt, Germany) and purified by distillation.

All solutions and samples were prepared using high-purity water with a resistivity of 18.0 MΩ cm obtained from a Millipore (Bedford, MA, USA) ZMFQ 23004 Milli-Q water system. The organotin chloride and arsenic oxide stock solutions containing 1000 mg L^{-1} of tin and arsenic were prepared in pure methanol and deionized water, respectively, and stored at 4 °C in the dark. Working solutions were prepared daily in deionized water with 2% nitric acid. The $Pd(NO_3)_2$ matrix modifier solution was made from a dilution of 10.00 ± 0.03 g L^{-1} Pd solution (Merck, Darmstadt, Germany) with water to the desired final concentration.

2.3. Procedure for larvae exposure

Zebrafish larvae were supplied from ZF Biolabs (Madrid, Spain). The exposure solution was prepared according to the composition of fresh river water. Briefly, 16 mL of concentrated solution (containing 2.9 g of $CaCl_2$, 17.2 g of NaCl, 0.76 g of KCl and 4.9 g of $MgSO_4$ per litre) were diluted to 1 L with distilled water. According to OECD guidelines, conditions of this exposure solution were: 26 ± 2 °C, dissolved oxygen ≥60%, pH 6–8.5 (before and after renewal).

To obtain the zebrafish larvae, it was necessary to develop embryos to 72 h post fecundation (hpf), the moment when the embryos hatched. Zebrafish larvae remain classified as such until another 48 h later (120 hpf) when they are regarded as proper fish, but can be consider non-feeding other 24 h (Westerfield, 2007). An appropriate larvae amount was placed into three tanks for each analyte: one for control (without the addition of the analyte) and two with different concentrations of the target analyte. The test consisted of two phases: absorption, (48 h in a contaminated exposure solution) and depuration (24 h in a clean exposure solution). About 15–25 larvae were removed from the tanks at different time (0, 2, 4, 6, 21, 24, 45, 48, 50, 54 and 72 h), to determine the concentration of the analyte absorbed and accumulated. According to OECD 305 test, the loading rate of larvae at the beginning of the experiments ranged between 0.7 and 0.8 g L^{-1} (wet weight) and the mortality of larvae was lower than 20% at the end of the test.

The two nominal concentrations used to incubate the larvae for each analyte selected, are also dictated by Test OECD 305, which sets they must differ by a factor of ten, being the highest concentration at 1% of LC_{50} value (when detection limits allow the analytical determinations). Literature data indicates 96-h LC_{50} values of around 15–50 mg L^{-1} for arsenic (Qadir-Shah et al., 2009) and 3–40 μg L^{-1} for tributyltin (Dimitriou et al., 2003; Meador, 1997) using adult fishes and 30–50 μg L^{-1} for tributyltin for Zebrafish larvae (Dong et al., 2006). Also, 48-h LC_{50} values for the zebrafish larvae were calculated using several morphological and functional endpoints and using the inverse cumulative distribution (probit) function, values of 3 mg L^{-1} for arsenic and <60 μg L^{-1} for tributyltin were obtained. Thus, nominal concentrations chosen to carry out these bioconcentration experiments were 50 and 5 μg L^{-1} for arsenic and 2 and 0.2 μg L^{-1} for tributyltin. Control (blanks) experiments were carried out in parallel to all contamination studies. Special care was taken to check that mortality rate of larvae was less than 20% (ranging between 5 and 10%), very similar to the results obtained in the control experiments. The exposure solution was changed every 24 h to fulfil the OECD 305 requirement, that nominal concentration of the chemical substance cannot fluctuate more than 20% throughout the whole experiment.

2.4. Analytical procedure

The concentration of the target analytes was determined in two sample types as previously described: exposure solution and larvae. For each sample type and each analyte, different analytical methods were developed. Arsenic determination in the exposure solutions was carried out by adding nitric acid up to a concentration of 5% to eliminate the matrix effects. For TBT, a 25-fold preconcentration step was performed by liquid–liquid extraction adding 200 μL of toluene followed by addition of 25 μL 0.5% tropolone in aqueous acetic glacial. The organic phase was then separated and finally analyzed.

For the analysis of larvae, a leaching process to extract the analytes of interest was required. This step was carry out using an ultrasonic probe (USP) after addition of 15 μL of nitric acid (2.5–5%) followed by 12 μL of triton X-100 (1%) for sample homogenization or 15 μL of acetic acid (5% final concentration) for arsenic and TBT, respectively. After leaching with USP during 90 s and 55% of amplitude, methanol was added to precipitate the lipidic content of the samples whereas the analyte remained in the supernatant.

2.5. Quality assurance

Quality assurance steps included controls, replicate analyses, surrogate recoveries (as no reference material with similar matrix was found) and calibrations. The limits of detection obtained for the whole method (MDLs) were 2.5 ng g^{-1} in larvae and 0.15 ng mL^{-1} in the exposure solution for TBT, and

1.0 ng g^{-1} in larvae and 1.5 ng mL^{-1} in the exposure solution for arsenic. Concentrations of all target compounds in the control samples were below the MDLs. Linearity drift check and spike recovery analyses were carried out using commercial caviar eggs and proper zebrafish larvae. Analyses were carried out in triplicate showing good reproducibility (6–8% for exposure solutions and 13–20% for larvae). Recoveries averaged 95%, and all exceeded 90%. Calibrations showed good linearity ($R = 0.99$).

2.6. Toxicokinetics: bioconcentration factors (BCFs)

The bioconcentration factor is the most employed parameter to evaluate the accumulation capability of a contaminant by living organisms (Tsuda et al., 1998). Bioaccumulation factors accordingly to the OECD guideline 305 are calculated as the ratio between the concentration of the analyte in the larvae and the exposure solution at steady state, BCF_{ss}. Sometimes the steady state is not reached and BCF can be calculated from the ratio of k_1 to k_2 (BCF_k), where k_1 and k_2 are conditional rate constants which mainly depend on the fish species used for the experiments and also on experimental factors such as temperature and pH. They can be obtained from bioaccumulation models. These models describe uptake and depuration process as a first-order kinetic (Eq. (1)) (Gobas and Zhang, 1992)

$$\frac{dC_f}{dt} = k_1 \times C_w - k_2 \times C_f \quad (1)$$

where C_f is the concentration in fish (in ng g^{-1}), t is the exposure time (h), k_1 is the first-order uptake constant (litre per kilogram dry weight per hour), C_w is the concentration of substance in water (ng/mL), and k_2 is the first-order elimination rate constant (per hour). So, assuming that the initial concentration in fish is zero at $t = 0$ and the concentration of chemical in water is constant, Eq. (2) is obtained:

$$C_f = \frac{k_1}{k_2} \times C_w\left(1 - e^{-k_2 t}\right) \quad (2)$$

k_1 and k_2 values can be obtained if the experimental concentrations values obtained in the bioconcentration test fit to this equation. On the other hand, for the depuration phase, where C_w is assumed to be zero, the equation of first-orders kinetics may be reduced to Eq. (3):

$$\frac{dC_f}{dt} = -k_2 \times C_f \quad (3)$$

so Eq. (4) can be expressed as

$$C_f = C_{f,0} \times e^{-k_2 t} \quad (4)$$

This model has been widely employed to calculate BCFs (Gabric et al., 1990; Mortimer and Cornnell, 1993) but sometimes first-order kinetics are not suitable to fit experimental data and more complex models have to be employed (Spacie and Hamelink, 1982; Banerjee et al., 1984).

3. Results and discussion

3.1. Optimization of ZGFAAS determination

The thermal furnace program applied, and the type and amount of chemical modifier were the three main parameters to be optimized in ZGFAAS for each analyte. Several common chemical modifiers, such as $Pd(NO_3)_2$, $Mg(NO_3)_2$ and $NH_4H_2PO_4$, were tested individually or in combination (Daminelli et al., 1998). Injection of 5 μL of 2 g L^{-1} $Pd(NO_3)_2$ was the best option for both analytes. Optimized thermal graphite conditions for both analytes are summarized in Table 1. Graphite tube thermal stabilization with only iridium was applied for the quantification of arsenic in the exposure solution (Ruella de Oliveira and Anchieta Gomes Neto, 2007). Additional chemical modifier was not needed in this case. Determination of arsenic required two different programmes: one for the exposure solution and one for the larvae. The latter required two additional steps to avoid the irreproducibility caused by bubble formation because of the surfactant Triton-X100. TBT, however, could be detected in both types of samples with the same programme. An argon flow rate of 250 mL min^{-1} was used in the furnace cavity in all steps except during atomization.

3.2. Optimization of sample treatment

3.2.1. Exposure solution

Significant matrix effects were observed for both analytes. This effect, in the exposure solution was minimized in the case of As determination using different concentrations of nitric acid, previously described as a matrix modifier

Table 1 – Thermal furnace programs optimized for arsenic and tin determination by GFAAS. As (ES): conditions for arsenic in the exposure solution; As(L): conditions for arsenic in larvae.

Step	T (°C)			Ramp(s)			Hold(s)		
	As(ES)	As(L)	TBT	As(ES)	As(L)	TBT	As(ES)	As(L)	TBT
Dry 1	110	90	110	1	5	10	15	10	15
Dry (L)	–	110	–	–	3	–	–	20	–
Dry 2	130	300	130	5	20	3	15	10	15
Pyrolysis	1200	1100	600	10	30	10	10	20	20
Pyrolysis (L)	–	1200	–	–	1	–	–	2	–
Atomization	2300	2100	2400	0	0	0	5	4	3
Cleaning	2400	2300	2450	1	1	1	3	4	4

(Daminelli et al., 1998). The optimal signal preserving the lifetime of the graphite tube was obtained with 5%. HNO_3. The matrix effects found for tin were significantly more pronounced than for arsenic even in the presence of nitric acid at different concentrations. Glacial acetic acid is currently employed to increase the extraction efficiency of tin in the presence of tropolone as a chelating agent in an organic solvent; thus, its effect on interference removal was tested (Leroy et al., 1998; Bermejo-Barrera et al., 1998; Steve, 1997). Optimal conditions were: 1.25 and 5 mL of exposure solution (for the concentrations of 2 and 0.2 $\mu g L^{-1}$, respectively) treated with 25 μL of 0.5% tropolone in aqueous glacial acetic (2.5%) for tin leaching. Preconcentration was done using 200 μL of toluene as extractant. 20 μL of the organic extract was manually injected after mixing with 5 μL of Pd-based modifier. It is important to point out the high preconcentration factor achieved (up to 25) with the procedure.

3.2.2. *Larvae*

A focused ultrasonic probe (USP) was employed to carry out leaching of the analytes from these samples. Time of ultrasonication was 90 s and the power of the probe used was 55%. In a first approach, each sample containing 15–20 larvae was sonicated with 150–200 μL of 2.5–5% nitric acid in deionized water to accelerate arsenic extraction. Matrix effects were very high due to the high viscosity of the resulting extract. Therefore, triton X-100 (0.04 %) was added to decrease such viscosity (Hernández-Caraballo et al., 2002; Miller-Ihli, 1990; Byrd and Butchert, 1993; Kim et al., 2003). The surfactant combined with a complex or counter ion pair in the slurry could be separated as an organic layer. The high reproducibility showed homogeneity of the resulting suspension. Thus, recovery in the injected sample was complete. Combination of several acids and bases (NH_4OH, HCl, HNO_3) with Triton-X100 were tested. A combination of 5% HNO_3 with 0.04% Triton X-100 was selected as the best option.

The same USP system was employed to leach TBT from larvae. Following the same criteria as for exposure solution, 5% glacial acetic acid was added to promote the process of leaching. This process was carried out in 150–200 μL of deionized water, depending on the amount of larvae. The high lipidic content of these samples resulted in significant matrix interference. Thus, 70 μL of methanol was added and the resulting mixture was vortexed vigorously for one minute and then centrifuged at 4000 rpm for 15 min. The lipid fraction remained precipitated in the bottom of the tube and could be separated from the solution containing the analyte to avoid the interferences mentioned before (Steve, 1997). This procedure was applied twice to obtain quantitative recovery.

3.3. *Bioconcentration experiment*

3.3.1. *Concentration in the exposure solution*

Taking into consideration the possible inorganic arsenic and TBT instability in the exposure solution and how important is to know their real concentration all throughout the bioconcentration experiment, the concentration of both analytes was determined at the different sampling times. The evolution of arsenic concentration for the two concentration chosen (5 and 50 $\mu g L^{-1}$) has been represented in Fig. 1. Both concentrations had similar behaviour and remained practically constant during the absorption phase, and were negligible during depuration. Also concentration in the control exposure solution without adding any arsenic was negligible during the tested time. Results obtained for TBT are showed in Fig. 2. The nominal concentration of 0.2 $\mu g L^{-1}$ remained practically constant, like in the case of inorganic arsenic; however, a significant decrease is observed for the nominal value of 2 $\mu g L^{-1}$ (from 2 to 1.25 $\mu g L^{-1}$). Although this appreciable shut, it is important to signal that it occurred during the first 3 h and then, remained constant for the rest of the bioconcentration assay. As mentioned before, the application of the OECD 305 test requires that the concentration must remain constant during the whole experiment. The concentration measured in the depuration phase and in the control exposure solution without addition of the analytes, remained negligible, as expected.

3.3.2. *Concentration in the larvae*

Results obtained for arsenic accumulation in larvae at both concentrations showed that larvae do not significantly bioaccumulate this analyte, since most of them provided a signal below the method detection limit (maximum concentration of 94 $ng g^{-1}$ for the exposure solution at 50 $\mu g L^{-1}$ and 40 $ng g^{-1}$ for the one at 5 $\mu g L^{-1}$ were obtained at 48 h). On the other hand, bioconcentration of tributyltin, was very significant at

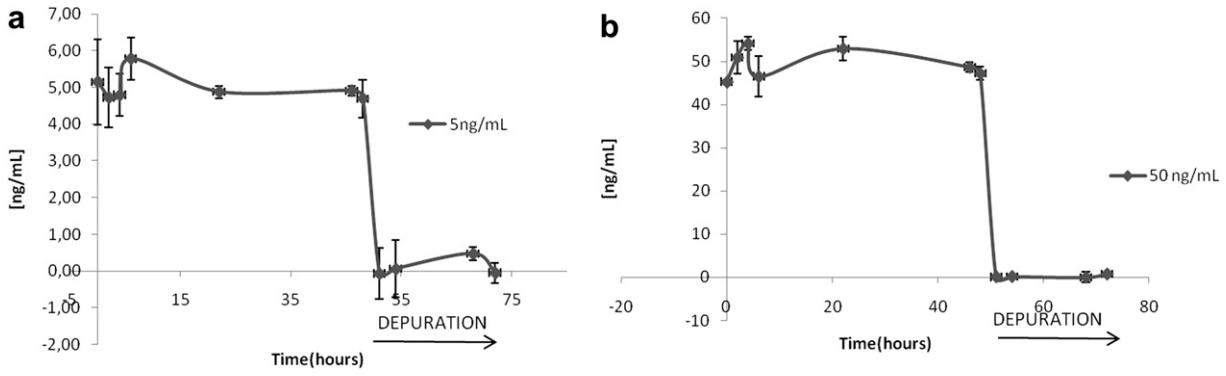

Fig. 1 – Arsenic concentration ($\mu g L^{-1}$) in the exposure solution. (a) nominal content of 5 $\mu g L^{-1}$; (b) nominal content of 50 $\mu g L^{-1}$.

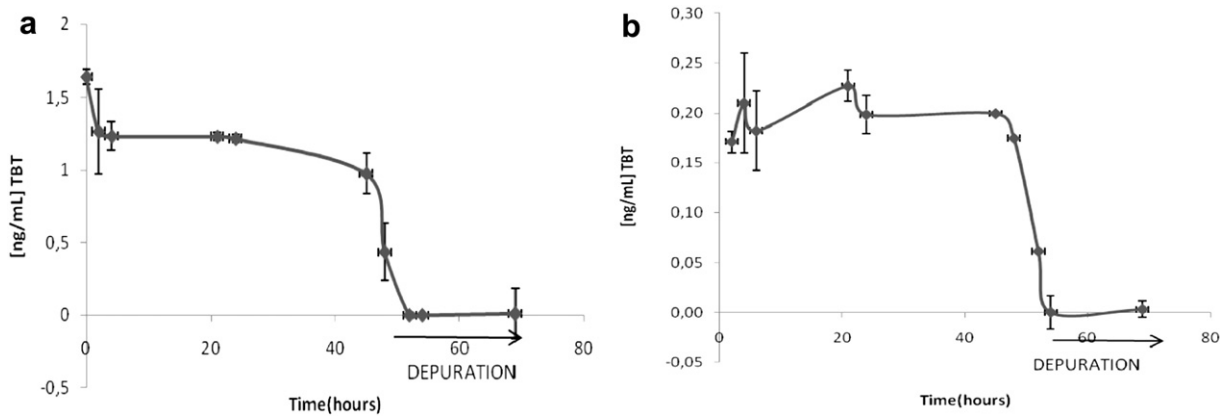

Fig. 2 – Tributyltin concentration ($\mu g\,L^{-1}$) in the exposure solution. (a) Nominal content of 2 $\mu g\,L^{-1}$; (b) nominal content of 0.2 $\mu g\,L^{-1}$.

the two concentration levels tested (Table 2). Measured concentration of TBT in larvae increased with exposure time reaching a maximum value between 48 and 52 h after exposure. After this time, when the larvae were exposed to clean solutions (depuration step), the TBT concentration in the larvae decreased, showing their capability to depurate the analyte as Fent evidenced two decades ago (Fent, 1991). These results have been interpreted in terms of bioconcentration factors.

3.4. *Calculation of bioconcentration factors (BCFs)*

Bioconcentration factors can be calculated as the ratio of concentration of the analyte found in the larvae to the concentration of the analyte in the exposure solution at the steady state (BCF_{ss}) or by fitting these data to a first-order kinetic equation to obtain k_1 and k_2, which allows for BCF_k calculation.

As for TBT, k_1 and k_2 values obtained as described before (Fig. 3) have been summarized in Table 3. Adjustments were carried out using the NONLIN software (Sherrod, 1995) and the proportion of variance explained was 96.9% for the highest concentration tested and 91.7% for the lowest one. BCF_k values obtained from these parameters were 840 for 0.2 $\mu g\,L^{-1}$ and 1280 for the exposure to 2 $\mu g\,L^{-1}$, while the results for the BCF_{exp} were 723 for the 0.2 $\mu g\,L^{-1}$ test and 970 for the high concentration test. The dispersion of the BCF data found in the literature varies enormously, ranging from 0.26 for *Oryzias latines* (American fish) (Nagase et al., 1991) to 21,100 l kg^{-1} for *Platichtys stallatus* (flatfish) (Meador, 1997), but most of them are enclosed within the 500–5000 range. Some investigations carried out under the OECD 305 test (METI-NITE, 2006) established BCFs and the partition coefficient values for the species *Crucian carp* for different organic tin compounds. The value of the BCF found for TBT was not highly bioaccumulative (which means $\log BCF \leq 3$) for the chloride salt and in the range of 2500–9210 for 0.5 $\mu g\,L^{-1}$ and 1830–7510 for 0.05 $\mu g\,L^{-1}$ for the TBT hydroxide. Although it exits a high dispersion in the values, it can be confirmed that values obtained in this work are within the same range of the values previously reported.

Several previous studies have illustrated a direct relationship between the octanol-to-water partition coefficient (K_{ow}) of a substance and its BCF value (Meador, 1997; Petersen and Kristensen, 1998; Parkerton et al., 2008; Arnot and Gobas,

Table 2 – Concentration mean values of three replicates for the tributyltin bioconcentration experiment. Larvae were incubated in (a) 2 $\mu g\,L^{-1}$ TBT; (b) 0.2 $\mu g\,L^{-1}$ TBT. Concentrations are expressed as ng TBT·g^{-1} larvae (wet weight).

Time (hours)	Larvae incubated in 2 $\mu g\,L^{-1}$		Larvae incubated in 0.2 $\mu g\,L^{-1}$	
	[ng g^{-1}] Mean	Std. dev.	[ng g^{-1}] Mean	Std. dev.
2	108.8	66.0	27.7	16.2
4	170.4	72.3	44.2	5.9
6	229.3	47.3	70.3	13.2
21	691.0	81.8	54.2	29.3
24	775.3	100.1	64.9	32.1
45	1114.1	113.2	138.2	2.8
48	1137	28.7	117.9	30.6
52	1101.9	28.7	113.2	43.9
54	1097.4	174.1	110.9	42.2
69	965.1	84.4	53.2	32.4
72	869.3	230.4	93.8	75.3

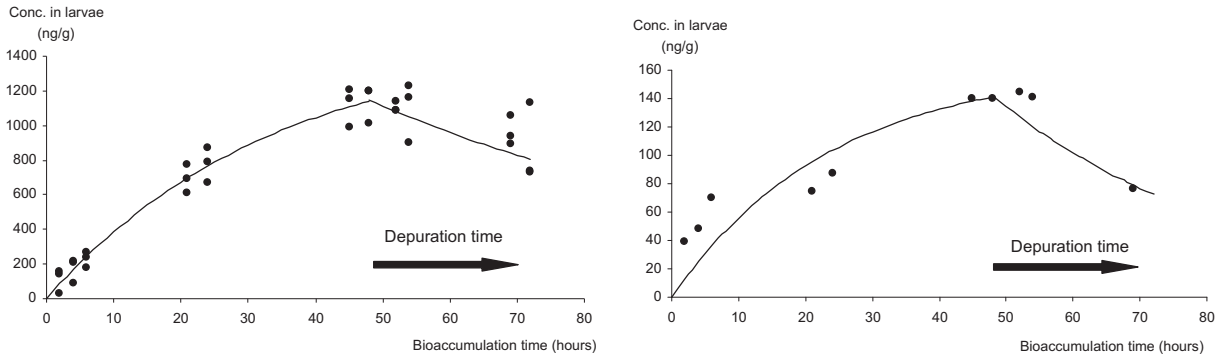

Fig. 3 – TBT bioconcentration factors representation following a first-order toxicokinetics equation for the two concentration studied (50 and 5 microg L-1).

2006; Halfon, 1985). This BCF to K_{ow} relationship results from the link between K_{ow} and cell membrane permeability (Simkiss and Taylor, 1989). Based on that, different regression equations between BCF and K_{ow} have been elaborated (Table 4). Having into account the value of $\log K_{ow} = 3.74$ (Laughlin et al., 1986), and using all equations found in Table 4, a range of values of BCFs between 2.01 and 2.96 were obtained, which is quite similar to the values obtained in the present study ($\log BCF = 2.92$ for 0.2 $\mu g\,L^{-1}$ and 3.10 for 2 $\mu g\,L^{-1}$).

Some authors (Petersen and Kristensen, 1998) have pointed that higher BCF values are expected for larvae compared to BCF values for juvenile/adult fish, because the relatively higher lipid content in larvae. Lipid content of zebrafish larvae at the end of the yolk sac stage has been reported to be near 20% of dry weight, whereas the mean lipid content of juvenile zebrafish has been reported to be 11.0% of dry weight. Also it is underlined that metabolism of the larvae can be lower than adult and juvenile fish, overestimating BCF values (Meador, 1997). Values obtained for the elimination rate constant k_2 from the fitting of the data derived from the depuration period, showed that no significant changes were found respect to the same k_2 (the first-order elimination rate constant) obtained from the fitting derived from the absorption period, thus indicating that there is not a big rate of TBT metabolized. But global BCFs values obtained here show no overestimation, having into account the data in the literature. Even so, future research of tin speciation will be carried out with the purpose of understanding tributyltin metabolism by zebrafish larvae.

As mentioned before, low and dispersive values were obtained for arsenic concentration in larvae, so it was not possible to fit the experimental data to a first-order bioaccumulation model and so, calculate values of absorption and depuration constants and consequently BCF_k. BCF_{ss} values calculated with maximum larvae concentration are 8.6 for 5 $\mu g\,L^{-1}$ and 2.2 for 50 $\mu g\,L^{-1}$. Data published for this compound from Japan METI-NITE, established BCF_{ss} for the species *Crucian carp* of <38 for 5 $\mu g\,L^{-1}$ and <4.0 for 50 $\mu g\,L^{-1}$. Other authors presented BCFs ranging from 15–17 (Santos et al., 2007) for *Corbicula fluminea* or maximum values of 35 for ten freshwater fish species in the Lake Manchar in Pakistan (Qadir-Shah et al., 2009). As no experimental data can be found for octanol-water partition coefficient of arsenic on the literature, the estimation software KOWWIN™ powered by EPI Suite™ of U.S. Environmental Protection Agency (Meylan and Howard, 1995) has been used, providing a value of $K_{ow} = 0.74$. Using this data with linear relation between K_{ow} and BCFs, which can be found in Table 4 for $K_{ow} < 1$, a value of BCF of 1.15 was obtained, close to the one experimentally calculated in this work.

Summarizing, BCFs calculated in the present work are in good agreement with others published previously using adult fishes and also with BCFs values obtained from the octanol-to-water partition coefficients linear relation. A number of recent studies have questioned whether the use of the BCF model is appropriate for describing the relationship between bioaccumulation and the potential effects for naturally occurring inorganic substances such as metals (McGeer et al., 2003, DeForest et al., 2007). Those authors have found an inverse relationship between BCF and exposure concentration for eight metals studied including arsenic but not tin. They indicate that BCF model was designed, developed, and adapted to describe neutral and lipid-soluble organic substances of anthropogenic origin, and its application to metals for the purposes of hazard identification is not supported by the scientific data. Bioaccumulation of metals follows a different paradigm relative to neutral organics. For example, metal uptake occurs via specific mechanisms that can often be modified as a result of exposure. So, for metals and metalloids, unlike organic substances, no one BCF can be used to express bioaccumulation and/or trophic transfer without consideration of the exposure concentration. Data presented here and calculated experimentally found that BCF values are proportional to the exposure concentration, but also that are in agreement to those values calculated with the

Table 3 – Toxicokinetics parameters (k_1 and k_2) values from a concentration-time profile of a tributyltin solution fit a first-order kinetics for both experiments.

	Larvae incubated in 2 $\mu g\,L^{-1}$		Larvae incubated in 0.2 $\mu g\,L^{-1}$	
	Uptake	Depuration	Uptake	Depuration
k_1	37.85		35.12	
k_2	0.029	0.025	0.042	0.028
C_w (ng/mL)	1.178		0.195	

Table 4 – Regression values for estimating BCF from log K_{ow} using a linear relation log BCF = $a + b \log K_{ow}$.

a	b	n	r^2	Life stage	Reference
-0.46 ± 0.46	0.86 ± 0.09	11	0.91	Larvae	Petersen and Kristensen (1998)
-0.23 ± 0.05	0.60 ± 0.01	2393	0.52	Adult fishes	Arnot and Gobas (2006)
0.06 ± 0.11[a]	0.0006 ± 0.05[a]	84	0.00	Adult fishes	Arnot and Gobas (2006)
−1.336	1	71	0.95	Adult fishes	Halfon (1985)

a Only when $K_{ow} < 1$

linear relation between octanol–water partition coefficient and BCFs.

4. Conclusions

A new analytical methodology based on the use of Zeeman graphite furnace atomic absorption, to determine both inorganic arsenic and TBT in the exposure solution and in larvae exposed to a 0.1–1% of their LC_{50} has been developed. The treatment of such small and complex samples presented here represents a relevant analytical advance in terms of rapidity and effectiveness for analyte leaching, low solvent consumption and low hazardous residues production, detection limits achieved, etc.

It has been demonstrated that the larvae exposure procedure is adequate to evaluate the BCF factors for both elements. BCFs values obtained for the two analytes tested are in good agreement with those previously obtained using the OECD 305 guideline. Thus, this should be the beginning of a series of different test to evaluate the proposed model using zebrafish larvae as an alternative to the Bioconcentration OECD 305 test, which requires many adult fish, implies high cost, as well as complex, time-consuming experiments. Actually new experiments are being performed to study other compounds with different accumulation properties (high BCF values) to be zebrafish larvae evaluated as an alternative test for a wide range of compounds. Also, new data is necessary to continue with discussion about the suitability of application of this bioaccumulation model to metals and metalloids and how these compounds behave in natural environments to proper evaluation of hazards of all the chemicals used by humans that contents metals.

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4.2.2. Absorption and Degradation of Tributyltin in Zebrafish Eleutheroembryos (Danio rerio. (Enviado a Biological Trace Element Research)

Los resultados obtenidos de este trabajo fueron presentados como Comunicación en formato Póster en Krakow (Polonia) en 2013 en European Winter Conference on plasma Spectrochemistry.

ABSORPTION AND DEGRADATION OF TRIBUTYLTIN IN ZEBRAFISH ELEUTHEROEMBRYOS (*DANIO RERIO*)

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Keywords: zebrafish eleutheroembryos, organotin compounds, detoxification

Abstract

Organotin compounds are highly versatile group of organometallic chemicals used in industrial and agricultural applications. Their endocrine-disrupting effects are well known and their extensive uses as biocide materials, e.g., in antifouling paints, for many years have led to serious environmental problems. So far, attention has mainly been given to tributyltin pollution in water, sediments, and marine organisms because of its highly toxic effects and high accumulation levels at very low concentrations. In this study we will focus on the conversion of tributyltin after it is absorbed by zebrafish eleutheroembryos, presented here as an alternative model to adult fish for describing bioconcentration. A simplified analytical extraction procedure based on the use of an assisted ultrasonic probe and derivatization by ethylation, followed by Gas Chromatography with a Flame Photometric Detector (GC-FPD) is proposed. This classical methodology for organotin determination has been validated by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Zeeman Graphite Furnace Atomic Absorption Spectrometry (ZGF-AAS) in terms of total tin content. The speciation analysis results show that zebrafish eleutheroembryos absorb high amounts of tributyltin and convert it into monobutyltin and likely in inorganic tin.

Introduction

The toxicological pattern of organotin compounds (OTCs) is very complex. Their biological effects depend on both the nature and the number of the organic groups bound to tin[1].The high toxicity of these compounds towards non-target organisms, particularly in the marine ecosystem, can cause ecological damage and affect human health through the food chain.[2,3]

Thus, strict legislative restrictions have been introduced in 25 Western countries in order to help reduce the accumulation of these compounds in the affected areas.[4] In 2001, the International Maritime Organization (IMO) sponsored the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention). The aim of this Convention is to protect the marine environment and human health from the adverse effects of anti-fouling systems on ships by phasing out the use of harmful OTCs, such as biocides in anti-fouling paints, and establishing mechanisms to prevent the potential future use of other harmful substances.[5] The Convention became effective in September 2008, banning the use of tributyltin (TBT) in anti-fouling paints on ships.[6] Furthermore, the commercialization and use of OTCs within the EU was prohibited as of January 1, 2003 by another Commission Directive.[7] Consequently, TBT, the most widely-used organotin, has been included in the European Union List of Priority Substances and Certain Other Pollutants, in the field of water policy for which environmental quality standards were set in 2008. The last update of the list was done on September 21, 2012.[8] However, it seems that the implementation of the AFS Convention in developing countries[9] appears to be difficult due to lack of analytical resources. Detection level of organotin compounds such as methyl butyltin, dibutyltin, tributyltin, and triphenyltin is low in developed countries because organotin health risks are under control, unlike the developing countries where their use is not strictly regulated.[10] Furthermore, the treatment and disposal of shipyard water contaminated by organotins is a serious concern in those areas. The permanent presence of these contaminants in estuarine sediments and marine organisms is easily explained if the previous factors are considered, as well as the low degradation rate of TBT, particularly in anoxic and/or cold environments.[11, 12]

The fate of TBT in aquatic ecosystems and the ecotoxicological effects directly depend on its persistence and the occurrence of biotic and abiotic degradation mechanisms.[13] TBT degradation involves the sequential

removal of butyl groups, leading to the formation of di- and monobutyltin (DBT and MBT). These dealkylation processes generally result in a reduction of toxicity. Many reports have already evaluated the metabolism in adult fish, demonstrating that TBT is rapidly degraded into the metabolites DBT and MBT that are then transferred to bile and eliminated.[14,15] However, another aspect when controlling the fate and persistence of TBT in aquatic systems is directly linked to the partitioning processes between water, sediments, and biota through biotransformation. Different studies carried out in marine organisms have reported significant bioconcentration of butyltin compounds in benthic macro-organisms, feeding on sediment particles from estuarine and coastal samples, but low degradation by dealkylation processes.[16,17]

On the other hand, early life stages seem to be more sensitive to TBT pollution than adult individuals. Larval bivalve mollusks and juvenile crustaceans appear to be much more sensitive than adults during acute exposures. The 96-hr LC_{50} for the larval Pacific oyster, *Crassostrea gigas*, was 1557 μg L^{-1}, whereas the value for adults was 282 μg L^{-1}.[18] Another test with the fathead minnow, *Pimephales promelas*, caused the death of the whole population after 32 days of exposure to 2 μg L^{-1} TBT.[19]

A previous investigation made by our group[20] was based on the need to study the toxicity, persistence and bioaccumulation of PBTs (Persistence, Bioaccumulation, and Toxic substances), regulated by the European REACH legislation. Zebrafish eleutheroembryos (*Danio rerio*) were chosen as the biological model because they have a series of advantageous features over other vertebrate models, mainly rapid embryonic development and the fact that they can be easily maintained. In such study we analyzed eleutheroembryo bioconcentration capability when exposed to 0.2 and 2 μg L^{-1} of TBT, corresponding to 0.1 and 1% of TBT LC_{50}, as described in the litterature.[21,22] The bioconcentration was assessed by evaluating bioconcentration factors (BCFs) which account the substance concentration ratio measured in the exposure medium and eleutheroembryos tissues. The results revealed that total tin concentration inside zebrafish eleutheroembryos increased with the time of exposure and decreased during the depuration phase.

The aim of this study is to complement the TBT accumulation data observed in

zebrafish eleutheroembryos by measuring the organotin species present in the latest stages of the absorption phase and during depuration. The main analytical challenge was the very small weight of this type of biological samples, around 20 mg for 15-20 eleutheroembryos and their high fat content. For the extraction technique a minimum amount of extractant was used to avoid high analyte dilution. The speciation study was done by Gas Chromatography-Flame Photometric Detector (GC-FPD) after OTCs ethylation with sodium tetraethylborate ($NaEt_4B$). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Zeeman Graphite Furnace Atomic Absorption Spectrometry (ZGFAAS) was employed to validate total tin content by comparing the total amount of species after the chromatographic procedure with the total tin measured using the described techniques.

Experimental

Instrumentation

Organotin speciation was performed in a Gas Chromatograph HP-5890-Series II, equipped with a ZP-5 column (30m×0.25mm). The column temperature was programmed to 1 min at 70ºC, followed to an increase to 250ºC (at 25 ºC min^{-1}), and a *final* temperature of 290ºC held for 2 min. The FPD detector was provided with a 610 nm cut-off *filter*.

Total tin concentration was monitored by a Perkin Elmer 4100 ZL atomic absorption spectrometer with a longitudinal Zeeman background correction, equipped with a transversally heated graphite furnace tube atomizer (THGA) with L'vov platform. Tin concentration was calculated from the integrated absorbance of the atomic absorption signal. A volume of 20 μL was injected manually. The furnace operation was controlled using the Perkin Elmer AA Winlab software, Version 4.1 SSP1. A Perkin Elmer hollow cathode lamp (HCL) with wavelength 286.3 nm and instrument slit width 0.7 nm was used. Alternatively, an ICP-MS HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan), equipped with a Babington nebulizer, Fasssel torch and double pass Scott-type spray chamber cooled by a Peltier system was used. Single ion monitoring at m/z 118 and 120 were used for obtaining the data.

A Vibracell VCx130 ultrasonic processor (Connecticut, USA), equipped with a 2-mm diameter titanium microtip and fitted with a high-frequency generator of 130 Watts at 20 kHz was used to leach OTCs from eleutheroembryos.

Centrifugation was carried out in a centrifuge model type FVL-2400N, (Combi-Spin, Boeco, Germany).

Reagents

Analytical grade chemicals were used for all the experiments in this work. The standards for TBTCl (>97%), $DBTCl_2$ (>97%), $MBTCl_3$ (>95%), and TPrTCl (97%) were obtained from Sigma–Aldrich Quimica S.A. (Madrid, Spain). The organotin stock solutions containing 1000 mg L^{-1} as tin were prepared in pure methanol and stored at 4 ºC in the dark. Glacial acetic acid was purchased from Panreac Química S.A. (Madrid, Spain); sodium acetate, methanol, and hexane were supplied by Scharlab S.L. (Barcelona, Spain). All solutions and samples were prepared using ultrapure water obtained from a Millipore (Bedford, MA, USA) ZMFQ 23004 Milli-Q water system. Sodium tetraethylborate ($NaBEt_4$) was from Sigma–Aldrich Quimica S.A. (Madrid, Spain), the corresponding 1% aqueous solutions (in Milli-Q water) for derivatization were prepared daily under nitrogen atmosphere. The candidate RM oyster tissue T-37 (IRMM, Belgium) was employed for both optimization and validation of the analytical method.

Procedure for zebrafish eleutheroembryos exposure

The Marine and Food Technological Centre of the Basque Country (Azti-Bilbao, Spain) supplied zebrafish eleutheroembryos. The exposure solution had a composition similar to that of fresh river water: 16 mL of the concentrated solution (containing 2.9 g of $CaCl_2$, 17.2 g of NaCl, 0.76 g of KCl, and 4.9 g of $MgSO_4$) were diluted to 1 L with distilled water. Following the OECD guidelines, the conditions of the exposure solution were: 26 ± 2 ºC, dissolved oxygen $\geq$ 60 %, and pH 6-8.5.

To obtain the samples embryos were allowed to develop for 72 hours post fecundation (hpf), at which time the embryos hatched. For the absorption phase, the appropriate number of eleutheroembryos were placed into a tank containing 2 µg L^{-1} of TBT and left for 45 to 48 hours. Next, the eleutheroembryos were moved to a tank with clean exposure solution for the depuration phase analysis. Approximately, groups os 15-25 eleutheroembryos were removed from the tank after 45 and 48 hours (absorption phase), and 60 and 72 hours (depuration phase).

Extraction procedure for OTC speciation

OTC extraction from eleutheroembryos was done by adding 200 µL of acetic

acid and 200 µL of methanol to a vial containing approximately 20 mg of the sample, accounted by estimating the individual eleutheroembryos weight and multiplying it by the individual number contained in each vial. The ultrasonic probe was immersed into the vial for 60 seconds at 50% of its amplitude and the resulting suspension was centrifuged at 4000 rpm for 10 min. Approximately 200 µL of the supernatant was taken and the pH adjusted to 4.8 with acetic acid/acetate buffer. Next, 500 µL of 1% $NaBEt_4$ was added for derivatization lasting 5 minutes and the derivatized analytes were extracted into 200 µL of hexane. The solution was stirred for 15 min and an aliquot of 1 µL injected into the GC-FPD for chromatographic analysis.

Development and validation of the quantification method

OTCs speciation was carried out by GC-FPD as described above. Standard addition calibration curves for each compound were established by spiking increasing concentration of TBT, DBT, and MBT on non-exposed eleutheroembryos. This was the best approach due to the high lipidic content of the samples. Furthermore, the likely errors associated to the derivatization process and the GC-FPD measurements were corrected by spiking 100 µL of 50 µg L^{-1} of tripropyltin as the internal standard after the extraction procedure. The linearity range was comprised between DL and 50 μg L^{-1}. The detection limit was established at 2 μg L^{-1} for the three studied compounds (MBT, DBT, and TBT), being enough for the samples analyzed whose contained a high amount of OTCs due to the high accumulation observed.[20] Validation of the speciation method was performed by comparing the total tin content as the total amount of species of the chromatographic method with the amount of tin measured in the same samples by ZGF-AAS and ICP-MS.

Results and discussion

In our previous work an important accumulation of tin was revealed after 45 hours of exposure of the eleutheroembryos.[21]Such content was evaluated by calculating bioconcentration factors (BCFs) following the current guidelines of OECD 305. BCFs are theoretically defined as the ratio of the analyte´s concentration at the organisms tested and the exposure solution at steady state.[23] Based on the total content of tin found in zebrafish eleutheroembryos, the main objective of this paper was to evaluate the likely transformation and/or degradation of TBT.

Extraction and recovery of study species

The extraction of OTCs from biological samples is a critical step when aiming to achieve quantitative recovery and prevent conversion of the original species. The low stability of OTCs and their strong interaction with the matrix makes their extraction from solid samples a very difficult task. Low extraction efficiency as well as the potential losses in the different analytical steps may lead to underestimation of the concentration of the analytes. Thus, rigorous control of the extraction efficiency is necessary. Combination of an organic solvent, of low to medium polarity, with an acid (e.g., acetic acid or hydrochloric acid) are used in more than 50% of the reported procedures for OTC extraction.[24,25,26] All these studies conclude that acidic conditions enhance the extraction efficiency of these compounds yielding high recoveries.

In our study, the very small sample amount (~ 20 mg) and the high lipid content required a careful analytical optimization of the method in order to achieve high compound extraction recoveries. The lack of reference materials regarding OTC extraction procedures from eleutheroembryos, led us to check the efficiency of our method, performing extractions with oyster tissue (T37, candidate reference material with indicative values for OTCs species).

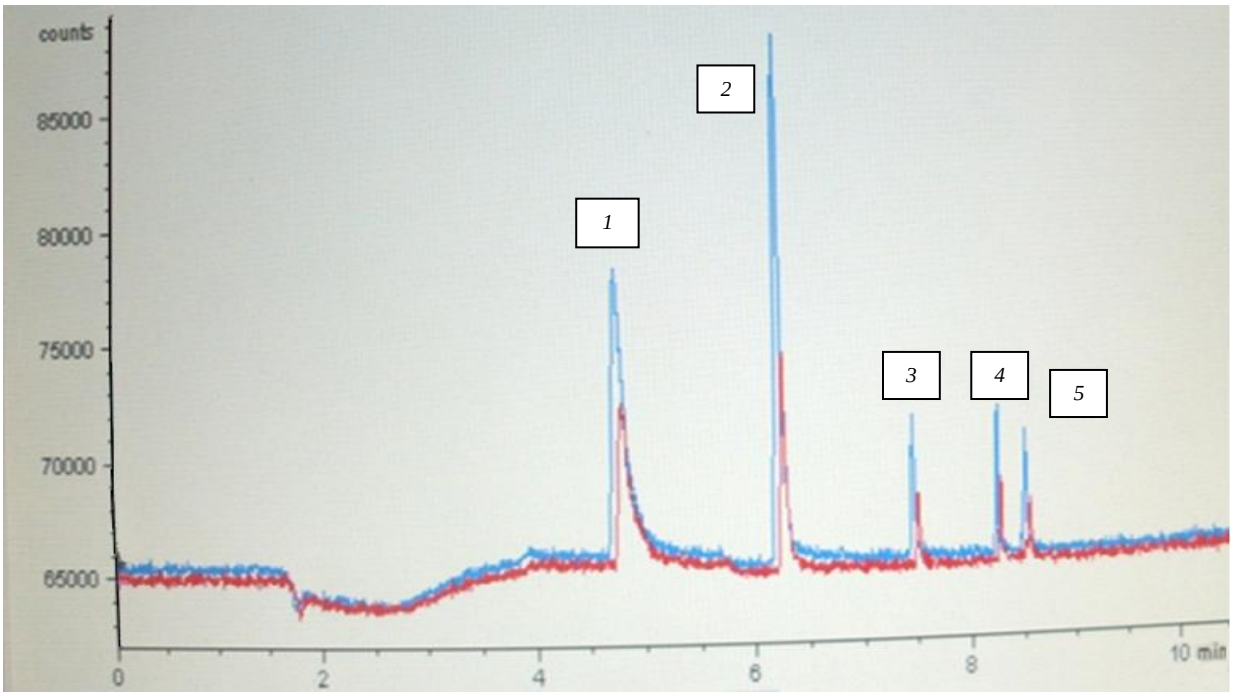

Figure 1: Chromatogram obtained on a ZP-5 column (30m×0.25mm) for the different OTCs from T37 candidate reference material using two extraction mixtures. Compounds: 1) Monobutyltin; 2) Tripropyltin (internal standard); 3) Dibutyltin; 4) Tributyltin; 5) Triphenyltin. Red line: acetic acid/water/methanol (1:1:1); Blue line: acetic acid/methanol Ultrasonic extraction: 60s; 50% amplitude. Derivatization with $NaBEt_4$. Re-extraction into hexane.

Several acetic acid (HAc) and methanol (MeOH) ratios were tested. The extract was then sonicated, derivatized, re-extracted into hexane, and injected into the GC-FPD, following the parameters described in the experimental section. The best extraction efficiency was obtained by mixing HAc:MeOH in a 1:1ratio. The results for two HAc:MeOH ratios are shown in Figure 1. Approximately 80% recovery was obtained for TBT and DBT in a single step; for MBT, the recoveries were much lower. Consequently, two consecutive and identical steps (n=2) were done to achieve quantitative recovery.

The optimized procedure was used with the eleutheroembryos samples. The results of the extractions are shown in Figure 2. Only two species, TBT and MBT, were clearly identified and quantified. A peak corresponding to inorganic tin was also observed, but it was significantly broad, making its quantification impossible. Again, MBT appeared as the most difficult species to extract, with 50% in comparison with the 80% TBT in the first step. It seems MBT is bound more strongly to the biota in comparison to TBT. This is well known and has been reported by several authors; more aggressive operating conditions or using ultrasonic or microwave extraction procedures are required for MBT extraction.[27,28] Other studies have proposed the use of chelating agents and strong acidic conditions for MBT extraction.[29,30]

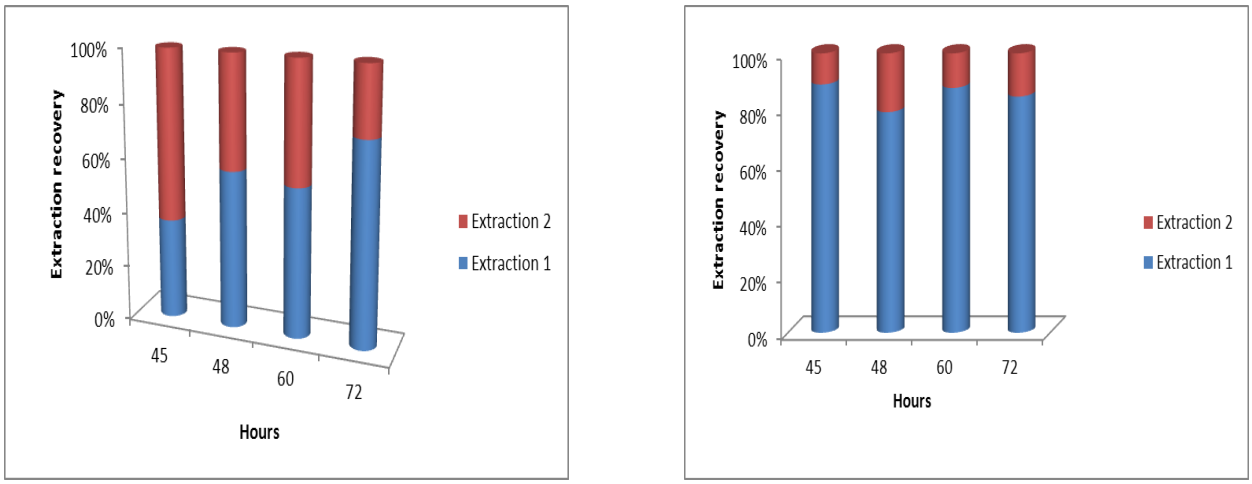

Figure 2: Evaluation and optimization of the extraction efficiency in two steps from zebrafish eleutheroembryos for a) MBT and b) TBT.

Extraction mixture: acetic acid/methanol (1:1). Ultrasonic extraction: 60s; 50% amplitude. Derivatization with $NaBEt_4$. Re-extraction into hexane; X-axis represents the different hours of absorption phase (45 and 48) and depuration phase (60 and 72); N=3

Transformation of TBT by zebrafish eleutheroembryos

We have showed high accumulation of TBT during the absorption phase, reaching a maximum value after 45-48 post- absorption (BCF = 1280), and a 30% decrease of tin concentration during the depuration phase.[20] A set of samples equally exposed to TBT have now been analyzed in order to measure TBT and/or its possible degradation compounds.

Figure 3 represents the comparison of the three analytical techniques mentioned above: GC-FPD (as sum of the species), ZFG-AAS (total tin), and ICP/MS (total tin). Results fitted quite satisfactory within among the three methods compared. The high uncertainty observed in some cases is probably due to the different behavior of living organisms: each replicate is composed of 15-20 eleutheroembryos. A decrease of total tin content was observed over time, either expressed as total tin (GFAAS and ICP/MS) or as the sum of OTCs (GC-FPD). The decrease is more pronounced during the depuration phase (60 and 72 hours), implying a transformation process by dealkylation of the most toxic species, TBT, into the less toxic DBT and/or MBT. The transformation of TBT into DBT and MBT in biota samples has been previously reported.[31] TBT and its main degradation products, DBT and MBT,

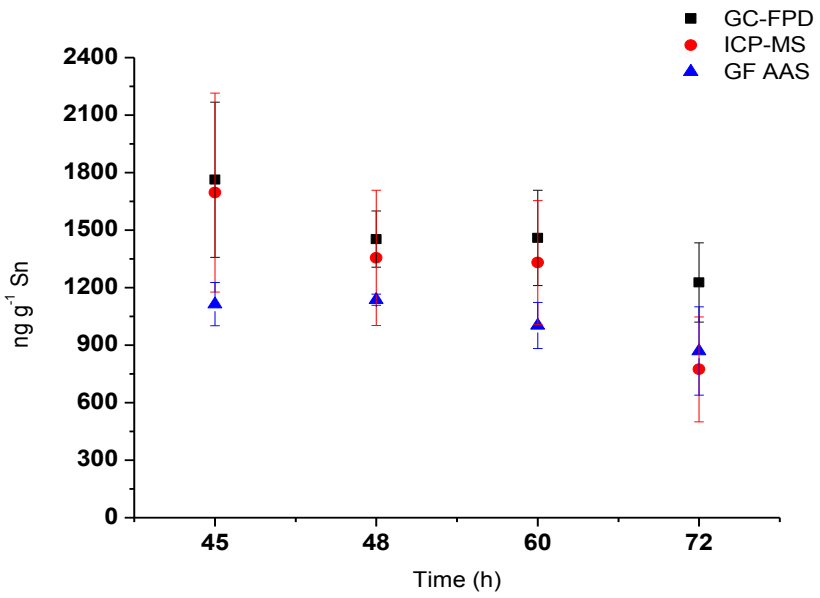

Figure 3: Total tin quantification in zebrafish eleutheroembryos extracts by using: a) GC-FPD (sum of organotin species); b) ICP-MS; c) ZGF-AAS.

X-axis represents the different hours of absorption phase (45 and 48) and depuration phase (60 and 72); N=3

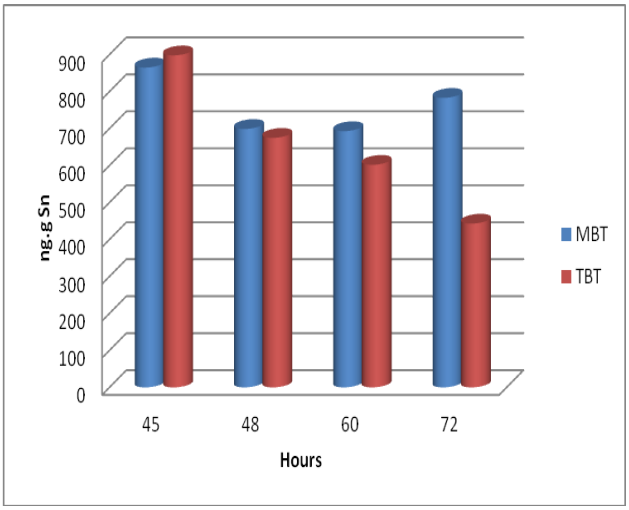

Figure 4: Variation of TBT and MBT concentration inside zebrafish eleutheroembryos. X-axis represents the different hours of absorption phase (45 and 48) and depuration phase (60 and 72); N=3

have been detected in different environmental compartments, both in marine[32,33,34] and terrestrial[35,36] systems. The occurrence of the less toxic compounds in the environment has so far been related to the degradation of TBT caused by microbial activity and/or photochemical reactions, but some evidence for direct input of MBT and DBT has also been found.[3] Some authors have recently determined residue levels of organotin compounds in five species of deep-sea fish, indicating a certain ability of fish to transform TBT, in contrast to triphenyltin, which preferentially accumulates in the liver.[37] TBT is accumulated in large amounts in animals from lower trophic levels, because they have a low capacity to degrade this compound. As an example, Souza *et al.*[38] found a good correlation between TBT and its metabolites in marine mussels, indicating that most of the DBT and MBT in mussel tissues are derived from TBT. Furthermore, this biotransformation can be very fast.[39]

This well documented transformation was also observed in our samples. MBT and TBT concentrations measured in the eleutherioembryos during the absorption and depuration phases are shown in Figure 4 and Table 1. At 45 hours, when a steady-state was reached in the bioconcentration experiment, MBT and TBT concentrations were practically identical. From then MBT starts to increase whereas TBT decreases. It is noteworthy to indicate that TBT is transformed directly into MBT, with no DBT being detected, neither during absorption or the depuration phase. This finding is not very common as DBT is generally found as the first degradation compound of TBT. However, a recent study of accumulation and transformation in *Thais clavigera* supports our results.[40] The study showed that TBT rapidly accumulated in the digestive and reproductive organs, and was rapidly eliminated and biotransformed. MBT was the primary metabolite in all tissues, indicating a significant metabolism of TBT by the whelks. The absence of DBT could indicate a rapid TBT detoxification mechanism in eleutheroembryos. This could be related with the fast

bioaccumulation kinetics of TBT shown in our previous work.[20].On the other hand, the decreasing rate of TBT is not proportional to the increasing rate observed for MBT, which could be due to detoxification and/or conversion to inorganic tin but this fact can only be affirmed qualitatively. Regarding the depuration phase, we have established that after 24 hours of depuration there is a significant fraction of TBT that is not excreted (~40%), which is in agreement with the highly persistent nature of this pollutant.

Conclusions

This work provides further information on zebrafish eleutheroembryos detoxification exposed to TBT. The results suggest a rapid conversion of the high amount of TBT accumulated by these organisms into less toxic species, MBT and likely inorganic tin. The absence of DBT could be attributed to fast biotransformation kinetics. Still, an important amount of TBT remains in zebrafish eleutheroembryos due to its highly persistent character.

Table 1: Mean concentration ± Uncertainty Of TBT and MBT in zebrafish eleutheroembryos

Organotin (ng g^{-1})

Time (h)	MBT	U	TBT	U
45	898	263	865	365
48	699	100	675	248
60	693	150	602	280
72	783	235	443	183

Acknowledgments

The Spanish Ministry of Research and Innovation (Project ATP-Toxbio, grant reference CTQ2011-28328-C02-01) has founded this work and to Interreg "Orque Sudoe Project). The authors also wish to acknowledge the grant received by A. Rocha Borges from the Brazilian Ministry.

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4.3. Estudios de Bioconcentración de Contaminantes Emergentes. Comparación de la bioconcentración de Nanopartículas metálicas (AgNPs y TiO_2 NPs) y sus formas iónicas

Los trabajos de investigación descritos en esta sección surgen, al igual que los anteriores de acuerdo a la normativa REACH para la evaluación de la bioconcentración de contaminantes emergentes. Dentro de esta categoría de contaminantes, nos hemos centrado en un tipo de nuevas sustancias químicas que han gozado de un amplio ámbito de aplicación en las últimas décadas: las nanopartículas. Hoy en día y debido a sus importantes propiedades están siendo explotadas en multitud de campos. Por tanto, su descarga al medio ambiente resulta inevitable y actualmente se requieren trabajos dirigidos a la evaluación y valoración del peligro potencial e impacto asociado a las mismas.

Debido al desconocimiento en el campo de las nanopartículas, antes de llevar a cabo los trabajos de investigación desarrollados en este capítulo con dos de los tipos de nanopartículas más comercializadas en la actualidad, nanopartículas de plata (AgNPs) y de dióxido de titanio (TiO_2NPs), se realizó una amplia revisión bibliográfica de los trabajos más relevantes en este campo. Este trabajo bibliográfico ha dado lugar a la publicación del trabajo presentado en el apartado 4.3.1. para proporcionar al lector una visión global dentro del campo de las nanopartículas y destacar así la importancia de los trabajos de investigación desarrollados a continuación. En él, aparte de revisar las técnicas más empleadas en la caracterización y determinación de las nanopartículas, se incide en los estudios publicados de toxicidad e impacto medioambiental después de su liberación al medio. Dentro de esto último, cabe destacar la amplia divergencia de resultados según los protocolos de análisis empleados. Además, parece evidente la necesidad de estabilizar las nanopartículas evitando su posible degradación y/o agregación para hacer comparables los resultados de los distintos estudios realizados. Otra de las conclusiones de este trabajo fue que son necesarias varias técnicas de caracterización, separación y detección para llegar a un mayor conocimiento de las propiedades de las distintas nanopartículas. Se remarca

por tanto la necesidad de continuar investigando el comportamiento de las nanopartículas, con el fin de comprender y predecir sus mecanismos de acción. Este conocimiento servirá de base para el establecimiento de la legislación que regule los niveles a los cuales esta clase de contaminantes emergentes no impliquen peligro para la salud y el medioambiente.

Los trabajos presentados en 4.3.2. y 4.3.3. han tenido como objetivo principal el estudio de la bioconcentración de AgNPs y TiO_2NPs, respectivamente, en larvas del pez cebra. Dado que muchos de los trabajos publicados referentes al impacto de las nanopartículas no son capaces de discernir entre la toxicidad asociada a las nanopartículas o a su correspondiente forma iónica, se decidió llevar a cabo un estudio comparativo sobre la bioconcentración de las especies iónicas de plata y titanio por larvas del pez cebra. En cada caso se han estudiado las propiedades específicas de cada tipo de nanopartículas y sus problemas de estabilidad en el medio de exposición. Así, en el caso de las AgNPs el problema fundamental fue el de su elevada tendencia a oxidarse a ion plata, mientras que las nanopartículas de TiO_2 (en las dos formas alotrópicas estudiadas, anatasa y rutilo) mostraron una elevada tendencia a la formación de agregados (>100nm). Para evitar este problema, se adicionaron agentes estabilizantes apropiados en cada caso, y más importante, biocompatibles con los organismos vivos, de forma que no originen una elevada mortalidad de las larvas consiguiendo una relativa estabilidad. Se han propuesto unos medios que resultaron adecuados en las condiciones que establece el protocolo OECD 305. Los medios óptimos encontrados fueron: una mezcla de citrato con almidón para las AgNPs, y de ácidos húmicos para las de TiO_2. Paralelamente se procedió a la caracterización de las nanopartículas estudiadas mediante diferentes técnicas lo que nos daba informaciones complementarias, tal y como se vio que era necesario en el trabajo de revisión bibliográfica. Finalmente, los resultados obtenidos en la bioconcentración por las larvas del pez cebra muestran diferencias significativas entre la acumulación de las AgNPs y el ión plata, con BCFs claramente superiores para el ión (BCF~700 frente a 10 para las nanopartículas). Sin embargo, en el caso del titanio tanto para los ensayos con nanopartículas como sus iones en disolución fueron muy similares (se obtuvieron valores de BCF entre 3-76 para el titanio iónico frente a valores entre 3-4.25),

concluyendo que en estas condiciones, presentaban un bajo factor de bioconcentración. Los valores obtenidos son comparables con la literatura publicada, aunque en el caso de las nanopartículas de TiO_2, algunos estudios ya han demostrado una elevada bioconcentración durante tiempos de exposición más prolongados, aumentando significativamente el BCF para t >72 horas (Zhu et al. 2010).

El trabajo presentado en el apartado 4.3.4 tiene un carácter multidisciplinar, y se realizó en colaboración con el Centro de Investigación USGS (United States Geological Survey). Su principal objetivo fue el estudio de la bioacumulación de PVP-Ag NP (AgNPs cubiertas y protegidas con polivinilpirrolidona) por el caracol *Lymnaea stagnalis* tras su exposición a través de la dieta. Se evaluó la influencia de diferentes condiciones medioambientales, la química del agua (expresada como dureza) y la concentración de ácidos húmicos, sustancias naturales que pueden actuar encapsulando e induciendo la formación de AgNPs. El posible efecto de tales condiciones medioambientales se valoró a partir de una serie de parámetros fisiológicos que permiten comparar cuantitativamente la bioacumulación entre diferentes especies metálicas en determinadas condiciones medioambientales. Los parámetros fisiológicos obtenidos de cada experimento pusieron de manifiesto que no existía influencia alguna de las condiciones medioambientales ensayadas y que este organismo asimilaba eficientemente las PVP-AgNPs desde la dieta, independientemente de la composición química del medio acuoso en el que fuesen incubados.

4.3.1. Nanoparticles: A global visión. Characterization, separation and quantifications methods. Potential environment and health impact (Analytical Methods, 2014).

Analytical Methods

CRITICAL REVIEW

Nanoparticles: a global vision. Characterization, separation, and quantification methods. Potential environmental and health impact

Cite this: *Anal. Methods*, 2014, **6**, 38

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Nanotechnology is a multidisciplinary science that includes scientific and technological activities at molecular and atomic scales (1–100 nm). Scientific principles and new properties can be understood and controlled when working at this scale. Nanotechnology is advancing rapidly due to the great progress achieved in various fields including electronics, mechanics, medicine, cosmetics, food, *etc.* This increased use of nano-sized materials leads to the release of a substantial amount of nanoparticles into the environment. To date there is a lack of standardized procedures to assess their safety and impact on the environment. Specific toxicological studies as well as characterization and quantification of nanoparticles are required to establish regulations to control field application of nanoscale materials. A classification of nanoparticles and the techniques employed in their characterization, separation, and quantification are summarized and described in this review. A global perspective on nanoparticle exposure and environmental effect is also discussed.

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www.rsc.org/methods

1. Introduction

Nanomaterials are defined as materials with at least one dimension between 1 and 100 nm. The dimensions of several entities are shown and compared in Fig. 1 to help explain the concept of nanosize. Nanoparticles (NPs) play an important role within the nanotechnology sector and are of great scientific interest.[1] NPs have specific physico-chemical properties that differ from their bulk material. Their high surface : volume ratio causes an exponential increase in the molecule's reactivity. Therefore, the electronic, optical, and chemical properties as well as the mechanical characteristics can greatly differ. The high confinement of electrons causes an increase in the bandgap of semiconductors, optical absorption, and photoluminescence. Furthermore, mechanical properties such as elasticity, hardness, ductility also change. Some NPs can exert high catalytic activity and are used to accelerate oxidation–reduction reactions of certain pollutants. Due to their minute

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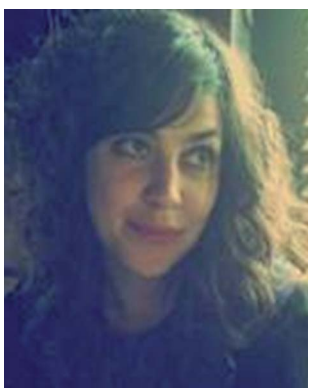

Ana López-Serrano Oliver, a predoctoral student in Analytical Chemistry doing her PhD on bioaccumulation of metals and nanoparticles by aquatic organisms.

Riansares Muñoz Olivas has been a lecturer at the Department of Analytical Chemistry, Universidad Complutense de Madrid since 2008. Her main research topics are sample treatment methodologies as well as analytical method development for trace metal speciation. She has got more than 40 original articles on this topic. Recently, she has been focused on toxicokinetics and ecotoxicity studies in environmental aquatic media.

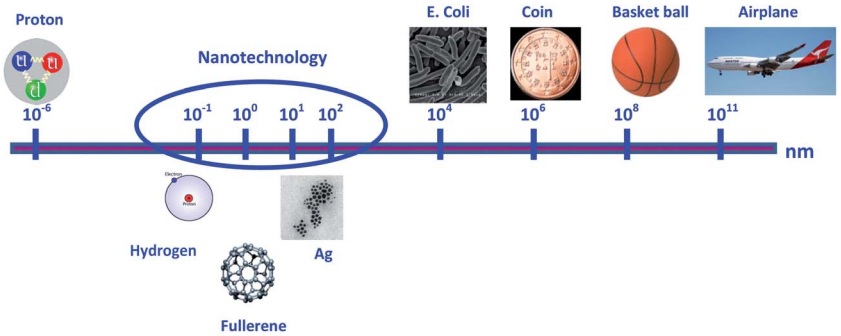

Fig. 1 Comparative sizes of various entities to perceive nanoscale measurements.

size, NPs can improve the antibacterial properties as close contact with bacterial membranes is possible.[2]

Because of above-mentioned extraordinary properties, nanotechnology is one of the fastest growing technological sectors and with high economic impact due to its application in a wide range of areas including cosmetics, biomedicine, analysis, food and food packaging, bioremediation, paints, coatings, electronics, catalysis, and materials sciences.[3] Some nanoscale materials have been used for decades (*e.g.*, in window glass, sunglasses, car bumpers, and paints), whereas others are newly discovered (*e.g.*, in sunscreens and cosmetics, textiles, coatings, sports goods, explosives, propellants, and pyrotechnics) or their applications are currently under development (*e.g.*, in batteries, solar cells, fuel cells, light sources, electronic storage media, display technologies, bioanalysis and biodetectors, drug delivery systems, and medical implants). A spectacular increase in nanomaterial production has occurred in the last few years as illustrated in Fig. 2. It is worth noting that the main area where nanotechnology is being applied is in health and fitness, or in other words to benefit human well-being. However, not much is known about the adverse effects of these new products.

Exhaustive fate and behaviour research studies, as well as studies on their toxicological effects are advisable before increasing and promoting NP production and developing new applications. To date, little is known regarding their safety for humans. Some studies have revealed that the same properties that make NPs so unique, could also be responsible for potential harmful effects on the environment and human health.[4,5] Present and future research is being focused on the interactions of the different types of NPs with aquatic organisms,[6,7] plants,[8,9] and humans.[10,11] Very often, the problem of NPs is their unpredictable behaviour. In this context, NP determination in environmental and biological samples is a major concern. Development of accurate and robust methodologies for the characterization of particle size distribution, chemical composition, shape, *etc.* and quantification of NPs and their forms under real conditions are necessary. Sample treatment methods to ensure NP stability preventing their aggregation and degradation are also mandatory in order to assess their possible risks.[12]

An overview on NP classification, as well as their diverse applications and analytical characterization, separation, and detection methodologies will be presented in this review. Furthermore, and aiming to understand their impact on the environment and health, some NP-related effects on samples obtained from the environment will be discussed. The review also discusses the present knowledge and the future actions needed to prevent irreversible damage. Fig. 3 summarizes the issues addressed in this review.

Jon Sanz Landaluze obtained his doctorate in 2002, at the University of the Basque Country and then joined, as a post-doc, the Department of Analytical Chemistry at the Complutense University of Madrid. He obtained, in January 2013, a position as a lecturer. His main research areas deal with metal speciation, development of analytical methods for organic compound determination an (eco)toxicity studies of these metallic species in the environment.

Carmen Cámara has been a full professor in Analytical Chemistry since 1992 and leader of the research group of "Trace Metals, Speciation and Proteomics" at the Complutense University of Madrid. Her main research interest deals with trace metal speciation, functional food, nanoparticles and organic persistent contaminant determination in food and environmental samples. Recently, she has been focused on toxicokinetics studies by proteomic approaches. She has published more than 250 papers, 3 patents and several books.

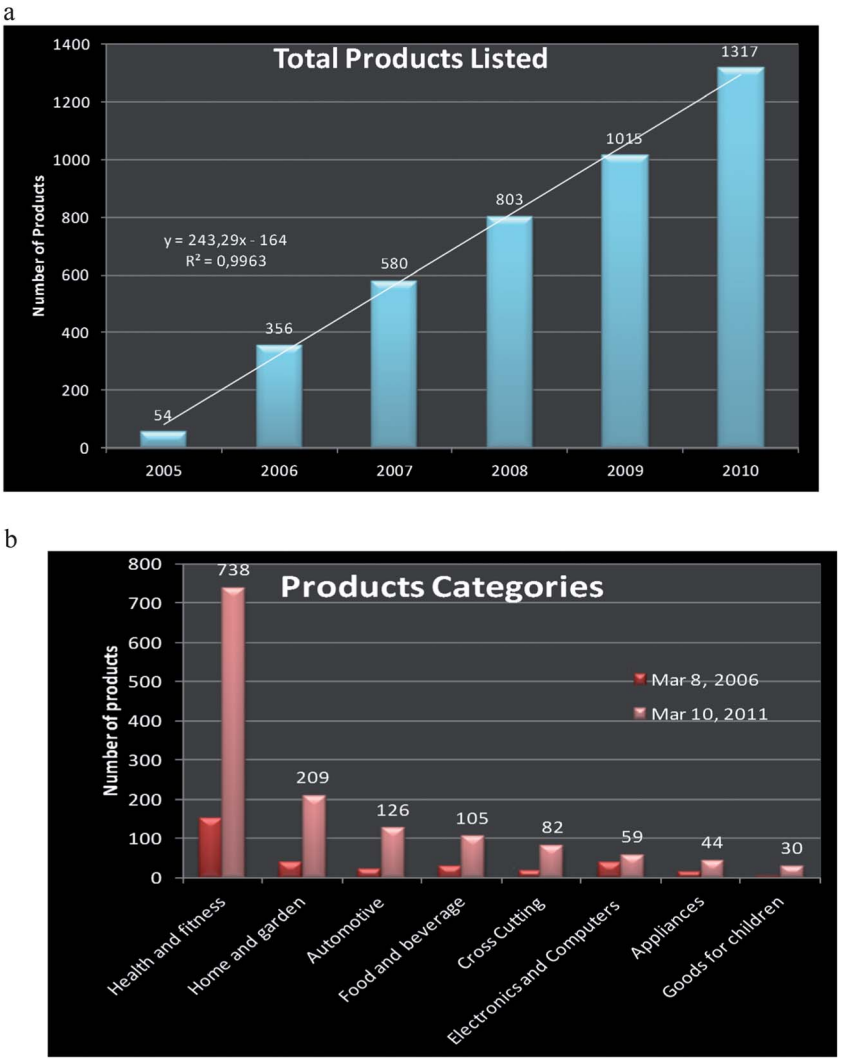

Fig. 2 Production of nanomaterials over the last few years and the potential near future projections. 2a: total products listed; 2b: product categories. Extracted from http://www.nanotechproyect.org/inventories/consumer.

2. Nanoparticle classification and applications

A wide range of NPs has been described. They can be classified based on different parameters, their origin (natural or anthropogenic), chemical composition (organic and inorganic), formation (biogenic, geogenic, anthropogenic, and atmospheric), their size, shape and characteristics, their applications in research and industry, *etc.*

2.1. On the basis of their chemical composition

2.1.1. Carbon-based nanostructures. Carbon-based nanostructures, made up of pure carbon, are classified into two main groups: (a) fullerenes: compounds containing at least 60 carbon atoms and b) carbon nanotubes (CNTs). Fullerene, C_{60}, a ball made up of 60 carbon atoms is the simplest form known as "buckminsterfullerene". C_{60} is a spherical molecule with the carbon atoms arranged at the vertices of a truncated icosahedron structure.[13] There are other less stable forms such as C_{70}, C_{76}, C_{78}, and C_{80} used in a multitude of biological and medical applications. It has been observed that fullerenes have antiviral activity, and are also capable of penetrating and establishing links in the catalytic sites of some enzymes. For instance, complex formation was demonstrated between HIV protease (essential for the survival of the virus) and fullerenes, which inhibited protease activity. Fullerenes lodge the active site residues of the viral protease with strong van der Waals interactions on the enzyme hollow surface.[14]

Carbon nanotubes (CNTs). A large variety of CNTs with different properties are available, which are synthesized by different methods. These nanotubes have good electrical, mechanical, and chemical properties and they are very useful for electronics, polymer industry, energy sector, and medicine.[15] CNTs have many applications such as: elimination of pathogens, natural organic matter, and cyanobacterial toxins from water systems due to their high adsorption capacities. Their

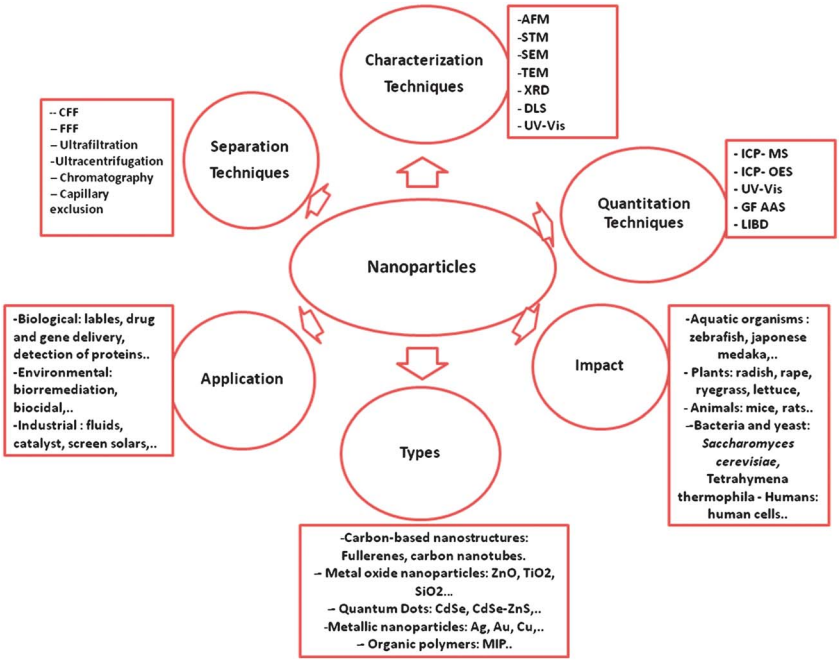

Fig. 3 Categories for nanoparticles.

fibrous shape and large external surface area can be easily accessed by biological contaminants.[16]

2.1.2. Metal oxide NPs. This group includes a variety of transient metal oxides (Fe_2O_3, CuO, TiO_2, ZnO, and CeO), and SiO_2 combining the special properties of these elements with the high reactivity of NPs. They are being employed in an extensive variety of consumer products such as cosmetics and sunscreens, as catalysts, and in biomedicine.[17–19] The engineered Al_2O_3 NPs have commercial applications in catalysis and in heat transfer fluids.[20,21] As an example, the special superparamagnetic properties of iron oxide NPs have been widely used in numerous *in vivo* applications such as magnetic resonance imaging contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, drug delivery, *etc.*[22]

2.1.3. Quantum dots (QDs). Quantum dots (QDs) are autofluorescent semiconductor nanocrystals that have been widely employed for *in vivo* biomedical imaging.[23–26] Due to their quantum confinement, QDs show some unique and fascinating optical properties, such as sharp and symmetrical emission spectra, high quantum yield, special chemical properties and high photo-stability. The simplest chemical constituents of QDs are binary metal complexes, CdSe, CdS, CdZn, *etc.* being the most widely used for biological labelling in a variety of animal cells. Other QDs can be formed by combination such as CdSe–ZnS core–shell nanocrystals and are employed as bioactive fluorescent probes in sensing, imaging, immunoassays, and other diagnostic applications.

2.1.4. Elemental metallic NPs. This group involves some inorganic NPs mainly composed of noble elements (Au, Ag), but also some transition metals (Fe, Zn) with many applications: transport, catalysis, optical sensors, bioremediation, solar panels, surgery, detection of biomolecules, *etc.* Silver NPs have unique antimicrobial activity due to the close contact between the Ag nucleus and the cell walls causing their disruption.[27–30] Their applications have become silver nanoparticles in the largest and fastest growing class of nanomaterials. Nanoscale zero-valent iron remediation has been used for contaminated groundwater and soils because of the high adsorption capacity and reactivity.[31] Gold NPs are normally employed in optical sensors.[32,33] They have recently become an important goal as elemental tags in proteomics because gold NPs can be conjugated with biomolecules through their functional groups and serve as chemical anchors and elemental markers.[34]

2.1.5. Organic polymers. There are organic polymeric NPs that can be highly stable in contact with biological fluids. Their polymeric nature permits the attainment of desired properties such as controlled and sustained drug release. It has been shown that biodegradable polymeric NPs with appropriate surface modifications can deliver drugs of interest beyond the blood–brain barrier for diagnostic and therapeutic applications in neurological disorders.[35] Their use as modifiable and/or imprinted polymers has shown a high specificity for binding to the recognition site for given chemical compounds. Therefore, they are widely used in other areas like solid phase extraction, catalysis, medicine, clinical analysis, environmental monitoring, and sensors. For instance, molecularly imprinted polymer NPs synthesized for human rhinovirus immunoglobulins result in artificial antibodies with improved selectivity and sensitivity.[36]

2.2. On the basis of their application area

The classification and applications of NPs are summarized in Table 1.

2.2.1. Biological applications. This is one of the areas with the highest NP production and application. NPs may be used as

Table 1 Classification and main applications of nanoparticles

Nanoparticle	Chemical composition	Applications	Principle of application	References
Carbon nanotubes (CNTs)	Pure carbon	Environmental	Adsorption of pollutants from contaminated sites	17, 47
			Increasing growing rate of plants	16, 48
		Energetic	Alternative energy storage media	16
		Construction	Increase the strengths of materials to build spacecrafts, space elevators, artificial muscles, and land and sea vehicles	56
		Medical	Selective reactivity with certain biomolecules	16
Fullerenes	Pure carbon	Medical	Selective reactivity for antiviral activity	14, 15
nAPU	Polyurethane	Environmental	Remediation of soils contaminated with PAHs	46
Metal oxides	TiO_2 and ZnO	Cosmetics	Filter UV radiation	52
	TiO_2	Construction building	Photocatalytic capacity of semiconductor materials	54
	Silica	Constructing building	Increase the strength of cementitious composites	55
	TiO_2	Paint industry	Whitening pigments	18
	TiO_2	Environmental	Positive effects on strength and growth of plants	49
	CeO	Energetic	Catalysts that increase fuel efficiency	19
	CuO and Al_2O_3	Energetic	Improvement of thermal conductivity	50
	CuO	Energetic	Catalysts	20
	Iron oxides	Biomedical	Superparamagnetic properties	21
			Sensors for microbial detection	45
Quantum dots (QDs)	CdSe	Medical diagnosis	Luminescence properties for labelling bacteria	36
	CdS	Biomedical imaging	Biomarkers	22–25
	CdSe/ZnS		Bioactive fluorescence; immunoassay applications	36, 37
Metallic nanoparticles	Ag	Consumer products	Antimicrobial properties	26–29
		Sensors	Localized surface plasmon resonance	26–29
		Biomedical	Antibacterial activity	40
	Au	Sensors	Surface plasmon resonance	31, 43, 44
	Au	Proteomic studies	Conjugation with biomolecules	32, 33
	Fe	Environmental	Detoxification of organic pollutants	30
Polymers	Alginate/chitosan	Biomedical	Slow drug delivery	35
		Agroindustrial	Encapsulated, adsorbed or dispersed bioactive compounds maintaining their structure, activity and releasing over a longer time	51

(a) biological labels, because of their size (generally within the same size range as proteins) and fluorescent properties, particularly quantum dots. NPs form the core of nano-biomaterials and interact with the biological target through non-covalent interactions. The approaches used to construct these nano-biomaterials are based on their biocompatibility, antigen detection, shape recognition, and fluorescent signalling. They can also be used for bacterial detection.[25] Also, CdS NP tracers can be conjugated with specific bacteria; this combination allows the detection of DNA hybridization for immunological assays;[37] (b) drug and gene delivery; the inclusion of drugs or genes into NPs protects them from degradation and improves

cellular uptake.[38,39] Silver NPs entrapped in montmorillonite were administered to mice, proving that they can offer novel advantages over current agents used as drug carriers;[40] (c) protein ultrasensitive electrochemical detection, by employing nano-sized biosensors with high versatility and inherent electrochemical advantages.[41] Quantum dots have been used for rapid and sensitive detection of prostate-specific antigen (PSA) in human serum,[42] achieving detection limits of 20 pg mL^{-1}; (d) determining DNA structure, by forming DNA conjugates and exploiting their unique optical and electronic properties. This way, gold NPs are able to distinguish between target-free and target-bound oligonucleotides *via* surface plasmon resonance[43] or can detect target-response structural variations of DNA;[44] (e) microbial monitoring and detection, offering an attractive alternative for *in vitro* and *in vivo* identification of molecular targets. Iron oxide nanosensors have been shown to be very sensitive for the quantification of biomolecular targets in cell lysates and tissue extracts.[45]

2.2.2. Environmental applications. This is one of the most attractive and promising applications within the nanotechnology sector. (a) Bioremediation, since NPs have the particular property of having a high adsorption capacity. For example, amphiphilic polyurethane (APU) NPs have been used to remediate soils contaminated with PAHs. These NPs have been proved to have high affinity for phenanthrene and hydrophilic surfaces promoting particle mobility in soils.[46] Furthermore, carbon nanotubes serve as nanosorbents with exceptional adsorption properties and used for removing heavy metals, organic pollutants, and biological impurities;[47] (b) acceleration of growth in some plants; it has been demonstrated that carbon nanotubes can significantly accelerate germination and growth of tomato seeds.[48] Nanostructures were found to penetrate tomato seeds and affect their germination and growth rates. The germination was found to be drastically increased in the presence of CNTs. Another interesting study reported the positive effects of nano-TiO_2 (rutile) on spinach seed strength and growth, suggesting their use in agricultural applications.[49] The strength came from the fact that nano-TiO_2 facilitates chlorophyll formation, and enhances rubisco activity and the photosynthetic rate during the growth stages of spinach. (c) Agroindustrial applications: NPs are being used to encapsulate, adsorb or disperse bioactive compounds through the particle matrix. NP-based release systems are able to maintain the structures and activities of the associated substances, and release the substances over a longer period. For example alginate/chitosan NPs are used to encapsulate paraquat, a non-selective contact herbicide with important secondary effects on the photosynthesis of plants. Alginate/chitosan NPs have been prepared as carrier systems for the herbicide paraquat resulting in more effective reduction of its negative impacts. The formulation of herbicides with NPs offers an increased effect of the chemical on specific targets, while reducing problems of environmental toxicity.[50]

2.2.3. Industrial applications. NPs have been used in different industrial branches over the last two decades. (a) Heat transfer: NPs exhibit high thermal conductivity that can be used in heat transfer. Thus, industrial heat transfer uses NPs in fluids such as water, ethylene glycol, or engine oil to produce a new class of engineered fluids;[51] (b) food industry and nutrients, *e.g.*, lipid-, protein- or polysaccharide based particles that are able to disperse hydrophobic β-carotenes in beverages, to entrap enzymes for cheese production, and to fortify dairy products with vitamins increasing their nutritional quality and aiding their digestion process;[52] (c) general personal care consumer products such as sunscreens, cosmetics including deodorants, soaps, toothpastes, shampoos, hair conditioners, anti-wrinkle creams, moisturizers, face powders, lipsticks, blush, eye shadows, nail polishes, perfumes, and after-shave lotions in which NPs are already being used.[4] (d) Construction and building materials: the most relevant application of nanomaterials in the construction industry relates to the photocatalytic capacity of semiconductor materials. Titanium dioxide (TiO_2) NPs are applied onto surfaces and through their volume incorporation in the conventional asphalt mixtures with tailored photocatalytic properties. The TiO_2 NPs reduce building facade maintenance costs and also the pollution effects, particularly important in metropolis with high population density, by their photocatalytic and self-cleaning effects.[53] Moreover, NPs are being used to increase the strength and durability of cementation composites. Silica NPs increase the compression strength of cement pastes since they contribute to denser microstructures.[54] Materials containing carbon nanotubes may be also strong enough to build spacecrafts, space elevators, artificial muscles, and land and sea vehicles.[55]

3. Nanoparticle analysis

Chemical composition, structure, size, and shape play a major role in the particular and unique properties that characterize NPs and their environmental and health impact. Several studies have tried to establish which characteristics of the NPs are required for hazard identification. The authors have emphasised the need to carry out NP characterization in a reliable and robust way.[56–58] So far, there are no validated analytical methods for this purpose. In order to elucidate the mechanisms of toxicity of NPs, to underpin the processes of their environmental fate and behaviour, and to be able to extrapolate results between NPs, it is essential to characterise the materials used in the different studies as far as possible. Many of the analytical techniques that have been used in NP characterization are reviewed here.

3.1. Size determination

3.1.1. Microscopic techniques. Microscopic techniques are the most employed characterization tools as they create surface images using a physical probe that scans the specimen. Some of the used microscopic techniques are: (i) Atomic Force Microscopy (AFM), which provides qualitative and quantitative information on many physical properties including size, morphology, surface texture, and roughness. This technique overestimates the dimensions of the NPs when the geometry of the tip is larger than the NPs themselves;[59] (ii) Scanning Tunneling Microscopy (STM) allows the chemical identification of the atoms and molecules that make up the NPs. STM can operate in a ultra-high vacuum allowing characterization in liquid and gaseous

suspensions;[60] (iii) Scanning and Transmission Electron Microscopy (SEM and TEM) provide information about the sample's surface, crystal structure, elemental composition, size, shape, and other properties such as electrical conductivity.[61] However, most of these imaging techniques can lead to imaging artefacts, due to previous treatment of the sample (coating, drying, staining, freezing, and embedding) before measuring and also due to the vacuum conditions in the sample chamber;[62] (iv) Environmental scanning electron microscopy (ESEM) is an approximation; currently the imaging of non-modified samples is still not possible;[63] (v) WetSEM technology, in which stainless steel capsules are used and equipped with an electron-transparent membrane so that wet samples can be placed into the capsules and imaged in a standard SEM. However, there are still drawbacks to this technique.[59]

3.1.2. Light scattering techniques. Light scattering techniques, such as Dynamic Light Scattering (DLS), are commonly used to determine particle size. Furthermore it is a nonintrusive, sensitive, and powerful analytical tool that is commonly employed for the characterization of macromolecules and colloids in solution.[64,65]

3.1.3. X-ray-based methods. X-ray-based methods such as X-ray absorption (XAS), X-ray fluorescence (XRF), X-ray photoelectron spectroscopy (XPS), and X-ray diffraction (XRD) are in general highly surface-specific and can provide information on surface properties and coatings, crystallographic structure or elemental composition. X-ray spectroscopy is often combined with SEM and TEM for the assessment of the elemental composition and quantitative analysis.[66,67]

3.1.4. Spectroscopic techniques. Spectroscopic techniques, such as UV-visible, are commonly used in NPs with surface plasmon resonance by collective oscillations of their conduction band electrons in response to electromagnetic waves. This allows obtaining information on NP size, aggregation, structure, stabilization, and surface chemistry.[68] Metal NPs display specific absorbance bands in their spectra when the incident light enters into resonance with the conduction band electrons on their surface. For example, Ag-NPs exhibit a characteristic absorbance band, in the UV region, due to the excitation mode of their surface plasmons, which is dependent on NP size.[69]

3.1.5. Nanoparticle tracking analysis (NTA). Nanoparticle tracking analysis (NTA) is an innovative system for sizing particles from about 30 to 1000 nm, with the lower detection limit being dependent on the refractive index of the NPs. This technique allows direct visualisation of a liquid NP suspension. The technology comprises a metalized optical element illuminated by a laser beam at the surface and a conventional optical microscope fitted with a low cost camera and a dedicated analytical software package. It is employed to control release or the precise delivery to a specific target of drugs encapsulated with NPs.[70]

3.1.6. Hyperspectral imaging. Hyperspectral imaging works by scattering the obliquely incident visible and near-infrared light in an enhanced darkfield background. The sample information is shown in terms of the spatial distribution as well as spectral characteristics unique to each nanoparticle type, at a sensitivity of a single nanoparticle (size <10 nm). Hyperspectral imaging with enhanced darkfield microscopy is employed for detection and characterization of engineered NPs in environmental systems, facilitating studies on fate and transformation of these particles in water samples. This includes the ability to map the presence and location of nanomaterials in a wide range of environments.[71] In addition, hyperspectral imaging is used to characterize unique surface chemistry and functional groups added to nanomaterials. One of their most common uses is imaging of NPs inside cells.[72]

3.2. Extraction, separation, and fractionation

Instability of NPs is well known. They can be very reactive depending on the composition of the surrounding media or physico-chemical parameters. NPs can aggregate, change their initial chemical form, *etc.* Furthermore, these properties can change during sample manipulation, storage, residence within the biological system, among other reasons, and these changes may greatly influence nanoparticle uptake and/or behaviour. Several techniques are used for a suitable extraction, separation, and fractionation of NPs during pre-quantification. Some of the most employed techniques are briefly explained below.

3.2.1. Cloud Point Extraction (CPE). Cloud point extraction (CPE) used for extracting NPs is a critical step in their analytical processing. There are several methods for this purpose, although many of them still need to be tuned up. Besides the matrix effects in environmental samples, the low concentration of NPs usually requires an enrichment step prior to their analytical determination. One approach for NP quantification is CPE, which consists in adding a surfactant to the sample at a concentration that exceeds the critical micelle concentration. At a temperature higher than that for a specific cloud point, the surfactant forms micelles in which non-polar substances are encapsulated. Since the density of the micelles is higher than that of water, they settle after some time, a process that is usually accelerated by centrifugation. Up to now, this methodology has been applied for determining silver NPs in water, allowing their separation from ionic silver.[73,74]

3.2.2. Cross-flow filtration (CFF). NPs are fractionated based on their particle size according to their diffusion coefficients through a very thin open channel. Recirculation of the sample prevents clogging, concentration polarization, and other artefacts caused by traditional dead-end filtration.[75] As an illustrative example, cross-flow filtration of a nano-sized nickel catalyst suspension was used in combination with ceramic membranes achieving 100% separation of the NPs and no adverse effects were observed during the catalysis.

3.2.3. Field-flow fractionation (FFF). Field-flow fractionation (FFF) is an emergent analytical technique for size separation of natural and inorganic NPs. The two main principles are sedimentation (SedFFF) and asymmetric flow field-flow fractionation (AF4). Separation of high-molecular mass compounds, from macromolecules (low nm range) to NPs and μm-size particles, is achieved by the interaction of sample components with an external generated field that is applied perpendicularly to the direction of the mobile phase flow. It is fast and non-destructive although some NP aggregation within the channel may occur. In addition, it can be coupled with a

second flow stream resulting in flow field-flow fractionation (F4), where a greater separation is accomplished.[76,77] Elution time under identical processing conditions is solely related to particle size and follows a linear correlation. The combination of AF4 techniques with UV and ICP-MS detectors allows the determination of particle-size distribution and quantification of metallic NPs.[78] Due to their nature, NPs can change under environmental conditions and sample treatment; sampling and sample preparation have to be optimised to prevent these changes. Stabilization of NPs and ionization efficiency in the ICP can be improved by adding surfactants or methanol to the carrier solution.[79] AF4 requires optimization of a relatively large set of parameters such as cross-flow rate, injection/focusing time, equilibration-cleaning of the membrane surface, sample pH, *etc.*, to achieve an efficient particle size separation. A further advantage of F4 is the possibility to couple the separation channel with several in-line detectors. Some recently published studies investigated the possibilities of AF4 with a multidetector approach coupled with UV-Vis, light scattering, and ICP-MS, for size fractionation chemical analysis of gold and silver NPs.[80] Although very versatile, F4 is hampered by the need to optimize a relatively large set of parameters to provide ideal running conditions for all particles present in the sample. This fractionation technique was tested as a representative example with a mixture of polystyrene colloids in three different nominal sizes, 19, 50, and 102 nm, obtaining quantitative data for trace particle concentration by Laser-Induced Breakdown Spectroscopy (LIBS).[81]

3.2.4. Ultrafiltration (UF). Using membranes with pore sizes in the nm range allows separation of NPs of different sizes without much perturbation of the sample. This technique is fast and requires little sample preparation.[60] Some studies carried out with silver NPs proved that the ultrafiltration method was more efficient and effective, providing greater size control, concentration, and aggregation state in comparison with conventional isolation methods such as ultracentrifugation.[82] The disadvantages of using ultrafiltration are the possible interactions with the ultrafiltration membrane as well as poor size resolution. A particular ultrafiltration procedure is tangential flow ultrafiltration that uses a series of membrane models with pores ranging from 1 nm to 100 μm in a single-step procedure.[83]

3.2.5. Ultracentrifugation (UC). Ultracentrifugation (UC) is a very versatile and powerful tool for NP characterization and fractionation. It may be applied to a very wide range of molecular masses (25 kDa to 1.5 MDa). The sample is subjected to a vacuum at a controlled speed and temperature while its concentration distribution is recorded at set time intervals. This technique allows analysis of the different fractions and obtaining information about their size and behaviour in a specific medium. Sample perturbation is low but, similar to UF, low-size resolution is achieved.[84,85]

3.2.6. Chromatographic techniques

Size exclusion chromatography (SEC). Size exclusion chromatography (SEC) has been historically used to separate different compounds on the basis of size and shape, but in the case of NPs some inherent problems such as degradation or losses by irreversible adsorption can be very important. Addition of surfactants in the mobile phase may reduce the mentioned adsorption problems but this new agent can result in a lack of separation resolution.[86]

Hydrodynamic chromatography (HDC). The separation takes place in the inter-particle channels of narrow open packed capillaries, or in wider capillaries with non-porous packing materials, that create capillary routes. Larger components elute sooner than smaller ones. Sample components are separated in the cartridge according to the different eluent velocities experienced by the different particles, as a consequence of the velocity gradient created in the inter-particle spaces of the packed bed of the effective capillaries.[87]

Reversed-phase high-performance liquid chromatography (RP-HPLC). A method using this technique has been developed for the speciation of engineered Ag-containing NPs, allowing the complete simultaneous and precise determination of Ag bound NPs and ionic silver species.[88]

3.2.7. Capillary electrophoresis (CE). Capillary electrophoresis (CE) measures the electrophoretic mobility of NPs based on their charge and size distribution in the sample, when an external electric field is applied. Ions move towards the electrode of opposite charge. The separation would be achieved by the mobility of the species depending not only on the solvent medium, but also on the charges, sizes, and shapes of the NPs. The ability of capillary electrophoresis as a separating tool for mixed NPs according to their sizes as well as the nature of materials has been demonstrated. However, as the amount of the sample used is rather low a high concentration of NPs is needed.[89]

3.3. Quantification

NP quantification is the third but decisive step to understand the wide range of processes in which they may be involved. The main bottleneck for NP quantification is the lack of validated analytical methods and standards. Currently, research focuses on the characterization of NPs, while information on quantification is very scarce. Furthermore, the size of NPs is far from being uniform and generally coupling of separation with quantification analytical techniques is required to obtain a fraction within a desirable size range, and also to distinguish between NPs and other species that can reach the detector. The most frequently used analytical techniques are summarized below and are all based on elemental determination coupled with one of the previously described fractionation techniques.

3.3.1. Inductively coupled plasma mass spectrometry (ICP-MS). Inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive analytical technique widely used for ultra-trace metal determination in a wide range of samples. It is being used for the determination of NPs and their corresponding ionic forms in biological samples exposed with the aim to evaluate the different toxicity of both compounds. However, the quantification of NPs by ICP-MS is usually related to solution chemistry since NPs have to be digested into a soluble speciated state. ICP-MS cannot differentiate NPs or dissolved forms unless a physical separation is previously done. Only in a few

cases, and very dependent on NP size and shape, the nebulization rate of ICP-MS can avoid the need to dissolve the nanoparticles. Some authors[90] have used ICP-MS to determine quantum dots in mice, as a primary quantification method, by monitoring Cd isotopes. Single-walled carbon nanotubes (SWCNTs) can also be quantified by ICP-MS using CNT-associated nickel as the probe.[91] Other NPs quantified by ICP-MS are gold[92] and nickel NPs.[93] Several improvements have been proposed over the last few years for enhancing NP analysis using ICP-MS. One clear example is the introduction of single particle (sp)-ICP-MS for size distribution determination of NPs in colloidal suspensions.[94] In single particle analysis, the analyte is spatially concentrated, in comparison to a solution of a soluble form of the same analyte. When one particle is introduced into the ICP, the atoms of the analyte produce a flash of gaseous ions in the plasma, which are measured as a single pulse by the detector. Meanwhile, the technique has been improved, *e.g.*, by using micro-droplet sample introduction devices,[95] to achieve better detection limits. The principle of sp-ICP-MS is based on transient signal spike counting statistics of highly diluted samples, which requires a fast data acquisition. The major advantage of sp-ICP-MS is the relatively low instrumental effort, which makes the technique easily accessible in every ICP-MS laboratory. However, sp-ICP-MS analysis is limited by the time resolution of the mass spectrometer used.[96] Another recent analytical strategy is the detection by ICP-MS using isotopically modified NPs. This procedure allows low detection limits and an adequate monitoring of their fate and transformation in the sample of interest.[97] In addition, recently new instrumentation has been developed for the analysis of gold NPs, using a customized electrospray-differential mobility analyzer (ES-DMA) to achieve real-time upstream size discrimination before ICP-MS detection.[98] DMA, also known as scanning ion mobility spectrometry, is a high-resolution size classification technique relevant to the analysis of discrete (dispersed) nano-scale species such as NPs, viruses, proteins, and DNA.

3.3.2. Inductively coupled plasma optical emission spectroscopy (ICP-OES). Inductively coupled plasma optical emission spectroscopy (ICP-OES) is also a powerful analytical technique that is being employed in NP analysis.[99,100] Concentrations at the $\mu g\ L^{-1}$ are perfectly detected with this technique. An analytical method to simultaneously detect three varieties of NPs using an ICP-OES (CdSe/ZnS, Au, and Fe_3O_4) has been developed.[101] ICP-OES has been widely used as a tool for assessing *in vitro* nanotoxicity. Uptake of transferrin-coated gold NPs of different shapes and sizes in mammalian cells has been studied. Finally, a mathematical equation to predict the relationship of AuNP exocytosis and size was developed. These models would have implications in nanotoxicity studies.[102]

3.3.3. Liquid chromatography-mass spectrometry (LC-MS). The separation of nanomaterials from the sample is based on the rates at which they elute from a stationary phase typically over a mobile phase gradient. Differing affinities of the mixture's components for the stationary and mobile phases lead to their separation. This technique has drastically improved in sensitivity, specificity and reliability. It is one of the most common techniques employed for the analysis of fullerenes in environmental matrices. First, they have to be extracted from the sample to facilitate their determination by LC/MS since the water solubility of unmodified C_{60} is low. The extraction of fullerenes has been achieved by exploiting their solubility in toluene. nC_{60} concentrations as low as 300 ng L^{-1} in water were quantified using the solid-phase extraction (SPE) separation method.[103] High-performance liquid chromatography (HPLC) coupled with mass spectrometry at a negatively charged m/z of 720 is also used to quantify C_{60}–toluene extracts providing specificity to differentiate fullerenes based on the m/z of charged molecules.[104] Recently, the quantification of fullerenes is carried out with approximation of this analytical methodology using more sensitive approaches such as: liquid chromatography-tandem mass spectrometry (LC-MS/MS)[105] and liquid chromatography-electrospray ionisation coupled to mass spectrometry (LC-ESI-MS) in the negative ion mode.[106]

3.3.4. Laser-induced breakdown spectroscopy (LIBS). Laser-induced breakdown spectroscopy (LIBS) uses the light emitted from laser-generated microplasma, which is analyzed to determine the elemental composition of a material.[107] When a solid nanoparticle passes through the focal volume of a pulsed laser, the power density required to induce breakdown of the dielectric properties of the solution is lower than that for pure water. If the laser energy is correctly tuned, plasma formation will only occur when a nanoparticle passes through the focal volume of the optical cell. LIBS provides the possibility of multi-element microanalysis of bulk and residue samples at the parts-per million-range with little or no sample preparation. LIBS is a non-destructive technique and is extremely sensitive to small NPs with detection limits within the ng dm^{-3} range.[108] LIBS is so sensitive that most samples have to be previously diluted. However, LIBS cannot discriminate between different sizes of NPs and even, different nanoparticle compositions have different breakdown probabilities (instrument responses). Therefore it is not possible to use one set of calibration standards for different types of NPs This drawback implies the need for using quite homogeneous dispersions. A good example of this technique for NP quantification is the work published by Thang *et al.* to determine colloidal NPs of different sizes, 19, 50, and 102 nm after FFFF.[109]

3.3.5. Graphite furnace atomic absorption spectroscopy (GF-AAS). Graphite furnace atomic absorption spectroscopy (GF-AAS) allows direct injection of NPs into the graphite tube with minimum manipulation of the sample. Silver ion release r from NPs by GF-AAS and UV-Vis spectroscopy was evaluated.[110] The UV-Vis spectra obtained with 300–700 nm allowed detection of NPs modified in size, morphology, and stability over time and GF-AAS was employed to develop kinetic models on the behaviour of AgNPs in natural waters. New advances in this technique point towards the use of high-resolution continuum source GF-AAS. It offers enhanced features for the detection and correction of spectral interferences, as well as for expanding the linear range. This last characteristic becomes significant when direct solid sampling is attempted because it is not feasible to dilute the sample if the analyte content exceeds the upper limit of the linear range.[111] Moreover, carbon-based nanomaterials in

Table 2 Nanoparticle characterization, separation, and quantification techniques

	Characterization technique	Information provided
Microscopic techniques	AFM	Size, morphology, surface texture, electrical and mechanical properties
	STM	Elemental and molecular composition
	SEM	Surface, size, shape morphology, crystallographic composition,
	TEM	elemental composition, electrical conductivity
X-ray based techniques	XAS, XRF, XPS, XRD	Surface, crystallographic, and elemental composition
Light scattering	DLS	Particle size
Spectroscopy techniques	UV-visible	Size, aggregation, structure, surface chemistry
	Fractionation technique	**Principle of separation**
Filtration techniques	CFF	Diffusion coefficients through an open channel
	FFF	Interaction with an external and perpendicular field
	UF	Diffusion through a membrane
	UC	Deposition of particles at controlled speed and under vacuum
	CE	Electrophoretic mobility under an external electrical field
Chromatographic techniques	SEC	Interaction with the stationary phase
	HDC	Routes formed by open capillaries packed with non-porous materials
	Quantification technique	**Analytical performances**
Plasma techniques	ICP-MS	Low detection limits; isotopic analysis; multi-element analysis
	ICP-OES	Simultaneous NP analysis; relative low detection limits
	LIBS	Multi-element microanalysis; little or no sample preparation
	GF-AAS	Direct NP injection; little or no sample preparation

biological samples are commonly determined with this technique by measuring the low content of the cobalt catalyst that remains bound to CNT after purification.[112,113]

Characterization, fractionation, and quantification techniques of the most used NPs are summarized in Table 2. After reviewing the techniques mostly used for NP analysis, we believe that several techniques must be simultaneously used in order to get a complete picture of these complex analytes.

4. Difficulties associated with the instability of nanoparticles

NPs differ from conventional dissolved chemicals in their heterogeneous distribution in size, shape, surface charge, composition, degree of dispersion, persistence, mobility, bioavailability, *etc.* Before analyzing the potential effects of NPs, their chemical behaviour, *e.g.*, their ability to oxidize, reduce, and dissolve in biological media, leading to the release of toxic ions, must be evaluated. Because the toxicity of NPs is not well understood yet and their initial chemical form can be affected by different external factors, it is essential to consider their stability, or more precisely, their lack of stability.[114,115] In order to predict the potential risk of exposure to NPs, a complete understanding of several additional aspects such as their release into air, water and soil as well as their persistence and mobility among compartments is necessary.[116]

A key parameter to assess would be the coexistence of NPs and their ionic forms, most probably with different fate and transport characteristics, and independent or synergistic toxicity pathways. Parameters such as the release rate of ionic forms, their aggregation, solubility, and stabilization in different media are frequently evaluated.[117] Similarly, sample preparation may be critical and should ideally be optimized to find the right balance between reducing the complexity of sample handling and maintaining its representativeness. NPs are usually stabilized by adding capping agents or adjusting certain parameters such as pH, temperature, and UV light.[118] Some relevant examples are described below.

Liu *et al.* investigated the effects of dissolved oxygen, pH, temperature, salt content, and natural organic matter on ion release kinetics and particle persistence in aqueous nanosilver colloids.[119] The study led to an interesting finding: a simple nAg colloid consisted of three silver forms, Ag^0 NPs, free Ag^+ (including any soluble complexes), and surface-adsorbed Ag^+. It was also predicted that AgNPs would not persist in environmental compartments containing significant dissolved oxygen. On the contrary, it would be oxidized to ionic silver. However, this redox process occurs slowly; therefore, NPs may persist long enough to open new pathways for silver partitioning and transport. Furthermore, the presence of natural ligands and ionic strength in the environment plays an important role in NP toxicity because they can interact with ligands forming different types of complexes. The presence of ligands such as sulphide in wastewater treatment may affect NP toxicity to nitrifying organisms, influencing the oxygen uptake rate measurements.[120] Addition of some ligands such as S^{-2}, Cl^-, PO_4^{-3}, and EDTA reduced nanosilver toxicity, due to a decrease of their bioavailability, more effectively than that caused by Ag^+.

The effect of adding stabilizing agents to avoid the release of both ions and NP agglomeration has been evaluated by different authors. A study on AgNP stability and persistence in natural freshwaters and synthetic aquatic media of citrate-capped AgNPs showed that a significant fraction of the initially singly dispersed AgNPs was agglomerated and decanted in the sediments, while a small fraction remained in solution.[121] This

finding showed the potential risk of AgNPs due to the high transport rate into estuarine waters and high availability to aquatic organisms. Surfactants, as stabilizing agents, mainly prevent the agglomeration[122] of various types of metallic NPs, which remain stable for more than three months when NPs are synthesized in the presence of apiin (apigenin-7-apiosyl-glucoside, a green biologically compatible agent). Poly(vinylpyrrolidone) (PVP) has been reported to be a good stabilizing agent for selenium NPs in aqueous solution,[123] also leading to highly regular morphologies and small diameters in comparison to those synthesized without PVP.

TiO_2 NPs tend to aggregate and settle in an aqueous medium.[124] Studies on the effects of environmental conditions such pH, ionic strength, organic matter, *etc.*, are a predominant research topic with these highly unstable NPs in order to clarify their mobility and stability in complex aqueous matrixes. The use of different coating agents is being applied for NPs that tend to agglomerate easily. Humic and fulvic acids,[125] polyethylene glycol (PEG),[126] mono- and binary-ion systems naturally present in natural waters[111,127] are among the tested stabilizing agents. However, in many cases, the results do not fit the expectations.

5. Exposure to nanoparticles: health and environmental impact

5.1. Exposure to nanoparticles

The increasing production and uses of NPs have raised concern about the release of engineered nanomaterials to the air, water, and soils due to their potential toxicity impact. To be able to minimize their negative effects, research on this matter is urgently needed, *e.g.*, assessment of NP fate, behaviour, and biological effects.[128]

There are two main types of exposure: (i) external exposure, bioavailability of NPs in the surrounding environment of an organism; and (ii) internal exposure, NPs that have been taken by the organism and can be metabolized, and transferred to different organs and tissues. In both cases, it is important to characterize the concentration of the expected NPs that may be present in the air, water, and soil, and how the human population can be directly or indirectly exposed by consuming food that has accumulated NPs.[129] To assess the external exposure risk, accurate measurements of environmental concentrations, speciation, and mobility are required; studies on internal exposure need to quantify uptake, metabolism and NP excretion.[130]

When NPs are present in the air, the primary route of human exposure is by inhalation. The common sources of anthropogenic NP inhalation are probably motor vehicle emissions in an urban environment or fossil fuel combustion. NP exposure, uptake, distribution, and degradation from the environment in different sectors have been recently discussed.[13] Occupational exposure must be also considered, as well as some industrial activities in which exposure to NPs may occur. The following fields can be representative examples: nanotechnology sector, chemical and pharmaceutical companies, powder-handling processes including paint, pigment, and cement manufacture, and other processes where NPs are by-products. Furthermore, cutaneous exposure is an important entry route because nanomaterials have been used in cosmetics and pharmaceutical compounds for many years. To date, knowledge on NP impact is very poor. Currently, most research is focused on NP skin penetration using different formulations of cosmetics. Finally yet importantly, ingestion exposure should also been considered. NPs can translocate from the lumen of the intestinal tract *via* aggregation of intestinal lymphatic tissue. Most of the studies focused on ingestion exposure concluded that the smaller the NPs the greater the danger due to their high permeation.[131]

5.2. Nanoparticle-related health and environmental impact

Once the NPs are released into the environment, assessment of their potential harm in the different organisms must be considered. The impact of NPs will be assessed by evaluating their toxicity and bioaccumulation in living organisms. There are several parameters that provide risk assessment for any toxic compound: LC_{50} (lethal concentration of a contaminant that will kill half of the sample population), EC_{50} (effective concentration of the toxin inducing a response between the baseline and maximum after some specified exposure time), NOEC (not-observed-effect-concentrations), BCF (bioconcentration factor defined as the ratio between the toxicant concentration in the biological tissue and the surrounding medium). Some examples of recent studies performed with NPs are summarized in Table 3 and discussed below.

5.2.1. Bacteria and yeast. They are commonly employed as indicators to assess the toxicity of several emergent chemicals. Choi *et al.* used the nitrifying bacteria present in wastewater to evaluate AgNP toxicity by measuring the oxygen uptake rate.[120] They noted that some ligands, *e.g.*, sulphide, effectively reduce nanosilver toxicity as a consequence of a decrease of their bioavailability by forming a chemical silver complex. Zheng *et al.* showed that toxicity of AuNP and carbon nanotubes in luminous bacteria was due to their ability to enter and aggregate into cells, the toxicity decreasing with the size of NPs.[132] Speciation studies on the protozoan *Tetrahymena thermophila* exposed to ZnO and CuO NPs and their respective soluble oxides revealed in all cases a similar or moderately higher toxicity of NPs than that for oxide forms. The toxic effect of both NPs was attributed to their solubilization fraction (the toxicity of ZnO NPs being significantly higher than that of CuO NPs).[133] Similar results were obtained in another study with the unicellular eukaryotic yeast *Saccharomyces cerevisiae*.[134]

5.2.2. Invertebrates. There are a few toxicity studies within this category of organisms, but they are interesting because they may act as a pollution source to higher organisms. *Drosophila melanogaster* is the currently investigated model in relation to human health, particularly because it is specifically relevant to model oxidative stress response.[3] Different effects were found in a study performed by Posgai *et al.*[135] Reproductive effort and viability were influenced by NP size, coatings, and antioxidant matter; nanosilver biochemical toxicity was higher than that of titanium dioxide NPs; both NPs had comparable effects on

Table 3 *In vivo* toxicity studies using different types of nanoparticles

Organism tested	Type of NPs	Conclusion	Reference
Nitrifying bacteria	nAg	Sulphide ligands reduce their toxicity	107
Luminous bacteria	nAu CNTs	Toxicity decreases with increasing nanoparticle size	132
Tetrahymena thermophila (protozoan)	nZnO	Toxic effects are caused by their solubilization fractions	133
Sacharomyces cerevisiae	nCuO		134
Drosophila melanogaster	nAg $nTiO_2$	Impact on glutathione levels Decreased superoxide dismutase levels	135
Lumbriculus variegatus	Fullerenes	Decreased feeding rate and depuration	136
Pseudokirchneriella subcapitata (microalgae)	nZnO $nTiO_2$ nCuO	CuO > ZnO > TiO_2 (toxicity)Toxicity is directly related to nanoparticle solubility	8
Red algae	nCu	Bioaccumulation increases with nanoparticle concentration	138
Radish, rape, ryegrass, lettuce, corn, cucumber	nAl, nAl_2O_3, nZn, nZnO, MWCNT	Growth inhibition with ZnO on ryegrass and corn	137
Allium cepa	nZnO nCoO	Elongation decrease of A. cepa roots by both the cobalt and the zinc oxide NPs	139
Hydra vulgaris	MWCNTs	MWCNTs could cause chromosomal aberrations, DNA fragmentation and apoptosis in Allium root cells	140
	Inorganic and organic	EC-50 ranged from 0.1 to 1 mg to inorganic NPs and 1–10 mg L^{-1} for carbon nanotubes in hydra organisms	142
Zebrafish embryos	QDs	QDs caused changes of cell morphology, leading to cell and animal death	143
	nAg	Several toxicity parameters (mortality, hatching delay…) highly dependent on NP concentration	66, 144
Medaka fish	Coated nAg	Dysmorphology and embryo viability dependent on nanoparticle shape and size	86, 145
	nNi	Dysmorphology and embryo viability dependent on nanoparticle shape and size	86,145,146
	TiO_2	Reduction in embryo viability	
	nAg/Ag^+	Cellular DNA damage, carcinogenic agents	93
Fathead minnows	nAg/Ag^+	Larvae abnormalities induced by both species. Higher mortality with Ag^+	147, 148
Daphnia magna	nAg, nCu, nNi, $nTiO_2$	Toxic effects except for TiO_2. Lower LC_{50} for nAg	117
Arenicola marina	$nTiO_2$	Genotoxic due to generation of radical species	150
	CNTs	Not accumulated or excreted	
Mice	nAu	Body weight, hematocrit decrease with nAu concentration Accumulation in brain under intraperitoneal administration Elevated liver levels under intravenous administration	35,151,152
	Superparamagnetic nFe_2O_3	Nanoparticles entered and were accumulated in the breast cancer cells as a function of particle size and exposure time	153

Table 3 (*Contd.*)

Organism tested	Type of NPs	Conclusion	Reference
	CNTs	Production of ROS species and DNA damage	154
	Polymeric	Autophagy, apoptosis, oxidative stress	155, 156
	Al_2O_3	Pathological alteration and inflammatory diseases	157
Humans	nAg	Increase of ROS species with depletion of ATP	158,159
	Buckyballs	Oxidative stress by depletion of GSH Death cell, (HDF) and (HepG2) cells, exposed to nanoC_{60}, caused by the generation of oxygen radicals.	160
	QD's	Skin permeability depending on QD physicochemical properties	162
	CdTe	Damage to the mitochondria membrane and nucleus mediated by ROS	163
	CeO	Reduces cancer cell viability; alternatives for cancer chemotherapy	164

superoxide dismutase levels, while nanosilver had a much greater impact on glutathione, GSH, levels; nanosilver toxicity was partially or completely solved by applying vitamin C to the fly. *Lumbriculus variegates* is a worm that has been used to study the extent to which fullerene-spiked sediments may cause adverse effects.[136] In this report, sediments were spiked with 10 and 50 mg fullerenes per kg sediment dry mass and toxicity assessed using the following endpoints: mass change, reproduction, number of organisms, feeding rates, and morphological changes using transmission electron microscopy (TEM). Fullerenes did not cause any impact on the survival or reproduction regarding the control group but their feeding rate and depuration were reduced for worms exposed to themselves. It is noteworthy that fullerenes accumulated in the digestive tract but they were not absorbed by gut epithelial cells.

5.2.3. Plants. This group of living organisms is highly affected by NP release and consequently can provide unique information regarding transport pathways. Similarly, they can be an important bioaccumulation pathway into the food chain. The main studies carried out with plants have reported the effects caused on seed germination, and root and plant growth. A study about these considerations in several types of plants (radish, rape, ryegrass, lettuce, corn, and cucumber) revealed that none of the used NPs (multi-walled carbon nanotube, aluminum, alumina, zinc, and zinc oxide) caused any effect on seed germination except in the case of Zn and ZnO NPs which inhibited ryegrass and corn species respectively.[137] Another study using Cu NPs showed their bioavailability and toxicity.[138] It was also shown that bioaccumulation increased with increasing concentration of copper NPs. An interesting work to discuss the mode of entry, formation, transport, and effects of different NPs in plants, as well as their ultimate effects as a consequence of their intensive use nowadays has been already published.[9] *Pseudokirchneriella subcapitata* microalgae have been the target organisms to carry out the studies on ZnO, TiO_2 and, CuO NP toxicity in comparison to bulk formulations of metal oxides such as $ZnSO_4$ and $CuSO_4$.[8] From the results it was concluded that zinc oxides were equally toxic in the bulk and in nano formulations and TiO_2 and CuO NPs themselves were significantly more toxic to algae. The highest toxicity was exhibited by nano-ZnO followed by nano-CuO and nano-TiO_2. The fact that CuO NPs are also more soluble and more toxic than bulk CuO demonstrates that the toxic effect was solely due to copper ions. In contrast, nano-TiO_2 formed aggregates that were entrapped by algae cells. Thus, solubility proved to be a key issue in the toxicity of metal-containing nanoparticles in plants. An indicator organism commonly used in phytotoxicity studies of NPs is *Allium cepa* that has a well-developed root system in a hydroponic culture. Ghodake *et al.* noted several adverse effects when *A. cepa* was exposed to fresh solutions of zinc and cobalt oxide NPs.[139] The nano-sized cobalt and zinc oxide particles are able to permeate *A. cepa* roots and affect the roots' elongation, metabolism and genetic materials. The phytotoxicity of the oxide NPs was evident and increased with the increasing concentrations. Genotoxicity of multi-walled carbon nanotubes, MWCNTs, was also studied on *A. Cepa*. The internalization of MWCNTs within the plant cells was confirmed by the presence of a large number of black dots distributed throughout the cytoplasm. MWCNTs caused chromosomal aberrations, DNA fragmentation and apoptosis in Allium root cells. These findings also indicate the importance to consider the plants as an important component of the ecosystem when evaluating toxicity of engineered nanomaterials. Another organism model in nanoscience is the freshwater *Cnidaria Hydrozoa*. Hydra is sensitive to a range of pollutants and has been used as a biological indicator of water pollution.[140] Hydra is one of the most sensitive species among the aquatic model organisms. Blaise *et al.* examined the toxicity of 11 NPs (copper zinc iron oxide,

nickel zinc iron oxide, yttrium iron oxide, titanium dioxide, strontium ferrite, indium tin oxide, samarium oxide, erbium oxide, holmium oxide, fullerene-C_{60} and single-walled carbon nanotube or SWCNT) in a test battery of aquatic biotests.[141] They found values of EC_{50} in the 0.1–1 mg L^{-1} range for inorganic NPs: copper and zinc iron oxides, indium tin oxides and holmium oxide NPs; and EC_{50} in the 1–10 mg L^{-1} range for single-wall carbon nanotubes. Tino *et al.* studied the toxicological effects of fluorescent CdTe QDs, presenting different coating, thioglycolic acid and glutathione, on *Hydra vulgaris*.[142] The effects on animal behaviour and morphology were investigated over different incubation times and LC_{50} values were quantitatively estimated. They demonstrated that the interaction of both types of QDs with *Hydra* induced progressive changes in cell morphology, leading finally to cell and animal death after 72 hours of exposure. This cytotoxicity was associated with QD exposure time and concentration and with the surface chemistry and coating of the QD.

5.2.4. Aquatic organisms. The toxicity and impact studies carried out with NPs have been performed mostly in different aquatic organisms. Several models to predict acute toxicity to aquatic vertebrates have been employed.

Due to its high homology with the human genome, the freshwater Zebrafish is commonly employed as a vertebrate model to evaluate nanotoxicity. Studies on AgNP transport and effects carried out at the early embryonic stage revealed that biocompatibility and toxicity of AgNPs were highly dependent on their concentration or dose.[143] A similar study, in which AgNPs[66] were used, showed the direct dependence of some deleterious effects in zebrafish embryos (by means of mortality, hatching delay, pericardial oedema and heart rate) on NP concentration. NP size and shape have been reported as two parameters affecting toxicity. A study with nickel NPs showed that the toxicity by NPs of different sizes, *i.e.*, dendritic aggregates larger than 60 nm and soluble nickel, was very similar. However, they noted that nickel NP shape had greater influence than size on toxicity.[86] Another well-established parameter associated with toxicity is the addition of capping agents employed in NP stabilization. Powers *et al.* compared the effects of ionic silver, citrate-coated AgNPs, and polyvinylpyrrolidone-coated Ag-NPs in zebrafish embryos and larvae.[144] The dysmorphology and loss of viability in the embryos were less remarkable with the exposure to AgNPs in comparison with exposure to Ag^{+}; however, AgNPs led to neurodevelopment impairment highly dependent on particle coating and size. Similar experiments were performed by testing sub-lethal effects of TiO_2 bulk and TiO_2 NPs in the same fish species.[145] Results showed a noticeable increase of Ti concentration for both chemical forms of titanium. Interestingly, concentrations returned to control levels by the end of the experiment. Furthermore, limited oxidative stress and organic pathology were observed for an exposure time of 14 days, but both chemical forms led to a decreased embryo viability.

Medaka fish is a species very similar to zebrafish that is also extensively used in toxicity studies. The toxic behaviour and environmental impact of AgNPs were evaluated with Medaka fish.[93] Changes in the expression of stress-related genes after exposure to different concentrations and various silver species (AgNPs and $AgNO_3$) were evaluated. Both ionic and AgNPs caused cellular and DNA damage, being carcinogenic agents and responsible for oxidative stress. AgNPs induced certain genes related to metal detoxification/metabolism regulation and radical scavenging action, while ionic silver induced an inflammatory response and a metallic detoxification process in the liver of the fish.

The fathead minnow is another living organism used for testing the effects of AgNPs in comparison to dissolved ions.[146] No relation between NP size and mortality rates was found. Interestingly, the evaluation of two different procedures for NP preparation (sonication and stirring) showed significant increase in embryo mortality when exposed to sonicated NPs with respect to nanoparticles that had been previously stirred.[147] Furthermore, the same authors noticed higher toxicity when the silver species was $AgNO_3$ in comparison with AgNPs, which induced concentration-dependent larval abnormalities, mostly oedema.

Daphnia species (magna, duplex) are an ecologically and relevant group of organisms, as an important part of the food chain.[148] These aquatic organisms have been chosen to cover a range of trophic and taxonomic levels in many reports. For instance, the exposure to several NPs (silver, copper, nickel, and titanium dioxide) caused toxicity in all cases, with AgNPs being the most toxic in terms of LC_{50}. On the other hand, titanium dioxide NPs did not cause toxicity in any of the organisms tested.[117] Considering LC_{50}, filter-feeding invertebrate organisms showed higher susceptibility to nanometals than larger organisms. The authors conclude that the toxicity of nanometals does not appear to be a generic response to nanosize particle exposure, but rather, each particular nanometal has an intrinsic property conferring toxicity. Thus, chemical composition of NPs seems to be a relevant parameter that should be taken into consideration when assessing toxicity.

Arenicola marina (lugworm) is a particular organism used for evaluating its exposure to the NPs present in the sediments. The lugworm ingests large amounts of sediments; for this reason significant effects should be expected. Galloway *et al.* evaluated the adverse effects from sediments containing high amounts of nano-titanium dioxide and carbon nanotubes for *A. marina*.[149] A significant impact was observed in organisms exposed to TiO_2 NPs; however, nanotubes were not found in the gut lumen after a 24 hour starving period, suggesting that these particles either remain in the sediment or pass through the gut and are excreted. In contrast, TiO_2 NPs, with a high tendency to form aggregates, were genotoxic due to the generation of free radical species that can react with most DNA components.

5.2.5. Mice. The mouse has become the model experimental animal par excellence, and there is a huge amount of literature available on it. Furthermore, the increasing use of NPs in biomedicine has promoted more studies on toxic effects in the last few decades. The effects of gold nanoparticles, AuNPs, have been studied in these animals through the assessment of animal survival, weight, haematology, morphology, and organ index.[35] Results have showed that low concentrations of AuNPs do not cause any obvious decrease in body weight or induce an

appreciable toxicity; but higher concentrations (500–2000 μg kg^{-1}) induced loss in body weight and a decrease in red blood cells. Intraperitoneal administration of different AuNP concentrations revealed an increase in gold concentration in organs, while it remained constant in blood.[150] Thus, the AuNPs administered intraperitoneally were absorbed into systemic circulation and distributed into tissues, indicating that AuNPs have a high capacity to accumulate in tissues. The brain was the organ with the highest accumulation capacity, suggesting a non-saturable uptake of AuNPs from the blood to the brain. Additionally, an efficient clearance of AuNPs from the body occurred at elevated doses. However, intravenous administration of AuNPs revealed accumulation in several organs such as the kidneys, lungs, brain, liver, and spleen, but especially liver.[151] Authors noted that PEG-coated AuNPs induced acute inflammatory response and apoptosis in the liver of mice.

The sub-cellular accumulation of superparamagnetic iron oxide NPs has been studied in breast tumours and peripheral organs.[152] Cluster formation of high-density NPs in cancer cells and their accumulation *via* conjugation to receptors was observed. These results showed that iron NP conjugates have potential applications in breast cancer detection and treatment.

Carbon nanotube toxicity has also been assessed in mice.[153] The cytotoxicity and genotoxicity were among the parameters studied, as well as the inflammatory response and intracellular production of ROS. An intracellular production of ROS and DNA damage were detected and attributed to oxidative DNA damage. Carbon nanotubes caused necrosis and oxidative stress due to incomplete phagocytosis or mechanical damage of the plasma membrane. The results obtained by TEM evidenced that macrophages were able to phagocytize and internalize the nanotubes in phagolysosomes. Therefore, it was shown that genotoxicity of CNT's was greater than that of carbon black particles, causing double- or single-strand damage. Other experiments performed with organic polymeric NPs, carried out using rat macrophage cell lines, evidenced uptake upon incubation for 2 h at the μg L^{-1} concentration level,[154] as well as signs of autophagy, apoptosis, and oxidative stress.[155] These results indicate that polymeric NPs are able to cross the mitochondrial membranes and enter the organelles inducing a cascade of events such as the unbalance of the oxidant/antioxidant homeostasis and the modification of the gene and protein expression.

Aluminum oxide NPs have been studied at the nanoscale level due to their easy access to the central nervous system. Effects of these NPs have been evaluated in rat brains after peripheral exposure.[156] Li *et al.* analyzed the activation of microglia and astrocytes (dominant and major immune cells in the CNS considered a key factor in many pathological alterations and inflammatory diseases) after exposure to aluminium oxide NPs. The authors observed that nanoscale aluminum oxide could induce the activation of these cells in various regions in the brains of rats. Furthermore, NPs were able to produce significant inflammatory effects in the brain of the rat, while both nanoscale and non-nanoscale aluminum oxide NPs had almost the same inflammatory effect on the liver and kidney.

5.2.6. Humans. This is the final link in the food chain for toxicity and bioavailability tests. Despite the fact that there are not many research works, some conclusions are related to oxidative stress and apoptosis caused by some types of NPs when the exposure occurs at elevated concentration levels. Some studies carried out with silver NPs in human cells resulted in: (i) an increase of intracellular ROS and mitochondrial damage associated with ATP[157] depletion, and (ii) oxidative stress as a consequence of the depletion of glutathione (GSH), which led to the increase of lipid peroxidation.[158] Carbon based nanomaterials also present ability to generate oxygen radicals responsible for cell death. Sayes *et al.* demonstrated the formation of superoxide species in human cells treated with several water soluble fullerene species. It was the first cytotoxicity study of buckballs in human cells.[159] Several water-soluble fullerenes (sublimed C_{60} and $C_{60}(OH)_{24}$, C_3 and $Na^+_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]_{(2-3)}$) were tested in two different human cell lines, human dermal fibroblasts (HDF) and human liver carcinoma (HepG2) cells. For cells exposed to nano-C_{60} cell death occurred because of lipid oxidation caused by the generation of oxygen radicals; more highly derivatized C_{60} systems were not as facile at generating these species and thus had lower cellular toxicity. Their findings contributed to the strategy for enhancing the toxicity of fullerenes for certain applications such as cancer therapeutics, as well as remediation for the possible unwarranted effects of pristine fullerenes.

On the other hand, quantum dots have been the focus of interest for different human cell line studies due to the increased use of these NPs for diagnostic and biomedical purposes.[160] Ryman-Rasmussen *et al.* studied the ability of quantum dots to penetrate skin.[161] Different coatings and structural characteristics were evaluated. The level of penetration and kinetics were highly dependent on the nature of the NP coatings. They speculated with a passive diffusion mechanism of entry, most likely *via* an intercellular route of passage, meaning that the skin is permeable to nanomaterials depending on diverse physicochemical properties. Cytotoxicity of CdTe QDs has been investigated in a human breast cancer cell line (MCF-7).[162] Several biological responses were determined by assessing cell apoptosis: plasma membrane stability, mitochondrial and nuclear damage, generation of ROS, release of cytochrome C in mitochondria. The final observations revealed damage to the mitochondria membrane and nucleus mediated by ROS (that could be prevented by treatments with some antioxidants). Another study performed in lung cancer cells after exposure to cerium oxide NPs showed decreased cell viability depending on NP dose and exposure time.[163]

An interesting biomedical research work on the selectivity and toxicity of ZnO NPs towards Gram-positive bacteria and cancer cells was proposed by Premanathan *et al.*[164] Their findings support the fact that ZnO induced toxicity in a specific cell and their proliferation were dependent on NP characteristics. The obtained results may be of relevant clinical interest as novel alternatives for cancer chemotherapy. The wide variability of observed NP effects confirms that unexpected and harmful responses in biological organisms exposed to NPs may occur.

Thus, further thorough studies on their wide range of effects are needed before continuing with their extensive production and uses.

6. Conclusions

The available information on toxicity and NP accumulation by living organisms is still confusing and the available results are difficult to compare. Here we have included several studies assessing the potential risk of NPs, as well as the controversial effects found indicating that many important factors affecting the toxicity of NPs are not yet well understood. Despite many studies being assessed to evaluate the impact from NPs, there are no clear conclusions on their associated risks. Further studies need to be developed in order to understand and predict their possible impact and hazards to health and environment.

Development of analytical methodologies for nanomaterial characterization and quantification as well as their monitoring in different environmental and biological samples are mandatory. The techniques that provide the most relevant information are: TEM, which provides the most direct information on size distribution and shape of particles but can alter NP properties; DLS, which allows obtaining measurement rapidly, but has several pitfalls because dust particles or small amounts of large aggregates may cause interferences; ultrafiltration and ultracentrifugation, which give sufficient information regarding NP stability in the media; ICP-MS is a good quantification technique when coupled to AF4 or other separation methods, *etc.* The scientific community agrees that using only one technique is not enough to get a complete picture of the difficulties caused by NPs. Another key subject is the need for the implementation of prediction models on their fate and behaviour within the environment. Further environmental risks by these particles will have to be tested under regulatory schemes such as REACH, to set regulations fixing the limits at which nanomaterials can be found in the environment.

To avoid irreversible consequences on health and on the environment, studies on the impact of NPs must be carried out before looking for other applications of these novel materials. Furthermore, the elaboration of an appropriate legislation, as well as establishing laws regulating their release, in order to avoid further episodes of possible negative consequences on humans and other organisms, is mandatory.

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4.3.2. Stability of silver nanoparticles in aqueous medium compatible with aquatic organisms: comparison of bioconcentration of ionic silver and silver nanoparticles in zebrafish eleutheroembryos (enviado a Environmental Pollution 2013, Ref. ENVPOL-D-13-00821)

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Stability of silver nanoparticles in aqueous medium compatible with aquatic organisms: comparison of bioconcentration of ionic silver and silver nanoparticles in zebrafish eleutheroembryos

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Abstract

The production of silver nanoparticles has reached nowadays a very high level. These particles have a wide spectrum of applications and their manufacturing cost is very low, which will inevitably lead to their presence in air, water, soils, and organisms. Bioconcentration studies, information on persistence, and toxicity are fundamental to assess the global risk of a chemical compound and thus necessary to establish legislations regarding their use. Previous studies on silver nanoparticle toxicity have determined a clear correlation between their chemical stability and toxicity. In this report, the conditions able to keep silver nanoparticles (AgNPs) at the nanometer scale (<100 nm) without ionic silver release have been optimized. Once the stability conditions were established, silver bioconcentrations post-exposure of zebrafish (*Danio rerio*) eleutheroembryos to ionic silver and to AgNPs were compared. A protocol alternative to the OECD 305 technical guideline was used. To determine the silver concentration in both the eleutheroembryos and the exposure media, an analytical method consisting in ultrasound assisted extraction, followed by inductively coupled plasma mass spectrometry (ICP-MS) and graphite furnace atomic absorption spectrometry (GF-AAS) detection, was developed. Once the concentrations at the selected accumulation and depuration times were determined, two different methods to estimate bioconcentration factors (BCFs) were used. The results revealed that ionic silver was more accumulative for zebrafish eleutheroembryos than nanoparticles at the levels tested.

Keywords: silver nanoparticles, stability, aggregation, bioaccumulation.

1. Introduction

Nanotechnology is a new and fast-emerging field that involves the manufacture, processing, and application of structures, devices, and systems by controlling shape and size at the nanometer scale. Nanomaterials possess unique and specific physicochemical and surface properties related with their size, i.e., mechanical resistance, electronic properties, thermal conductivity and chemical reactivity.[1] AgNPs are among the most widely used NPs, basically due to their important anti-bactericidal properties.[2] AgNPs have distinctive physicochemical properties, including high electrical and thermal conductivity, surface enhanced Raman scattering, chemical stability, catalytic activity, and non-linear optical behavior. These properties make them interesting to be used in inks, microelectronics, and medical imaging.[3,4] Consequently, the natural environment becomes a key area of concern regarding the high volume of Ag NP production. Different biological levels could be affected by AgNPs, such as cellular, subcellular, and molecular,[5,6] as well as complex organism.[7,8]

Although silver has long been used by the food industry as an antimicrobial agent, dye, and additive in foodstuffs and beverages,[9,10] it is well known that ionic silver is highly persistent and quite toxic to prokaryotes and many freshwater and marine invertebrates and fish (LC_{50} 0.8 $\mu g \cdot L^{-1}$ for freshwater fish species).[11,12] Ionic silver has a pronounced tendency to accumulate in organisms. The chemical properties of ionic silver allow its uptake via cell membrane ion transporters, a process similar to that regulating Na^+ and Cu^+ ions transport into the cells.[13] Thus, silver is listed in the 1977 US EPA priority pollutant list and in the EEC 1976 Dangerous Substance Directive List II (76/464/EEC) and its discharge into the environment has been regulated.

On the other hand, Ag NP waste regulations are hampered by a big knowledge gap regarding their environmental behavior, biological effects, and uptake routes of AgNPs by different organisms.[14,15] Parameters such as ecotoxicity, mobility, persistence, bioaccumulation, and degradation have to be considered to correctly assess the impact of AgNPs on human health and environment. Bioaccumulation in sensitive organisms is often a good integrative indicator of the chemical exposure to organisms in polluted ecosystems, including uptake of a substance via body surface (bioconcentration) and food (biomagnification).[16]

Toxicity of silver highly depends on its chemical species; different ligands able to complex silver can be found in natural waters. In freshwater for example, free ionic silver is present in traceable amounts and is one of the most toxic commonly occurring metals. However, stable silver chloride complexes prevail in marine ecosystems, leading to moderate toxicity. The effects of silver as NPs are still not well understood and its toxicity is a matter of

ongoing debate among scientists. It is not yet clear if the toxic effects are due to the size and shape of the particles,[17,18] to the release of ionic silver,[19, 20] or both.[21] In addition, Ag NP surface coatings (capping agents) can affect their chemical behavior, controlling their transport in surface water and groundwater.[22] Based on the current knowledge regarding the potential problems of AgNPs for the environment and human health, there is a clear need to characterize them considering a number of important factors such as: size, chemical composition, shape, surface structure, surface charge, aggregation and solubility, and the presence or absence of other chemical functional groups. Many of these characteristics and thus, behavior, bioavailability, and toxicity are affected and modified by surrounding media properties, including pH, ionic concentration, presence of natural organic materials, colloids, etc.[15,23,24] Handling and sample preparation, as well as storage conditions, might be critical. Changes in the chemical media occurring during sample preparation or analysis can result in significant changes of the samples.

Several standard tests are currently employed to evaluate the bioconcentration factor (BCF) of a compound: the OECD Bioconcentration Test 305, the ASTM E1022-94 from the American Society for Testing and Materials, and the OPPTS 850.1730 from US EPA.[25] All these guidelines propose measuring the chemical content in fish tissue and the exposure solution, at increasing exposure time until a steady-state is reached under well established conditions. A whole bioconcentration experiment requires the use of more than 100 adult fishes, which implies an extensive analytical work, high cost (about 100000 Euros for each test/compound), and important ethical issues. Any alternative to this expensive experiments or reduction in animal use is definitively desirable. Zebrafish embryos and eleutheroembryos, corresponding to the embryos from hatching until the phase of free swimming and active feeding, are not considered *in vivo* systems from a legal point of view and they can provide the same type of information as *in vivo* systems.[26] Consequently, they could be used in bioconcentration studies as an alternative to those involving living laboratory animals. Zebrafish, a small tropical fish, is an animal of great scientific interest due to many unique advantageous features over other vertebrate model systems: low husbandry cost, small size, rapid generation of a large number of transparent embryos, and fast embryonic development.[27] Some ecotoxicological tests involving zebrafish embryos and eleutheroembryos have previously been described[28,29] and an official protocol using fish eggs under short-term exposure has already replaced the fish assay for acute wastewater toxicity.[30]

Bioconcentration of ionic silver is well established,[31,32] but the accumulation of AgNPs has only been studied over the last few years and none of the studies has been carried out

following officially established bioconcentration or bioaccumulation guidelines.[7,20,22,33,34] In this paper we first focused on the dynamics of exposure concentration changes (possible release of ionic silver from nanoparticles, stability over time, aggregation/dispersion) as a consequence of any alteration in physicochemical properties of AgNPs. Next, we evaluated the possibility of developing a bioaccumulation test with zebrafish eleutheroembryos. Finally, AgNPs bioconcentration in zebrafish eleutheroembryos was assessed after their exposure to a previously optimized aquatic medium that assured their stabilization while being compatible with the life of the aquatic organisms. Silver ion bioconcentration was also assessed in order to compare the effects caused by both chemical forms.

2. Methods

2.1. Preparation and characterization of silver nanoparticles

The AgNPs for our experiments were provided by Polytech & Net (Germany), assuring that 80% of nanoparticles had a size of 20 ± 10 nm with polyhedral shape. Initial characterization

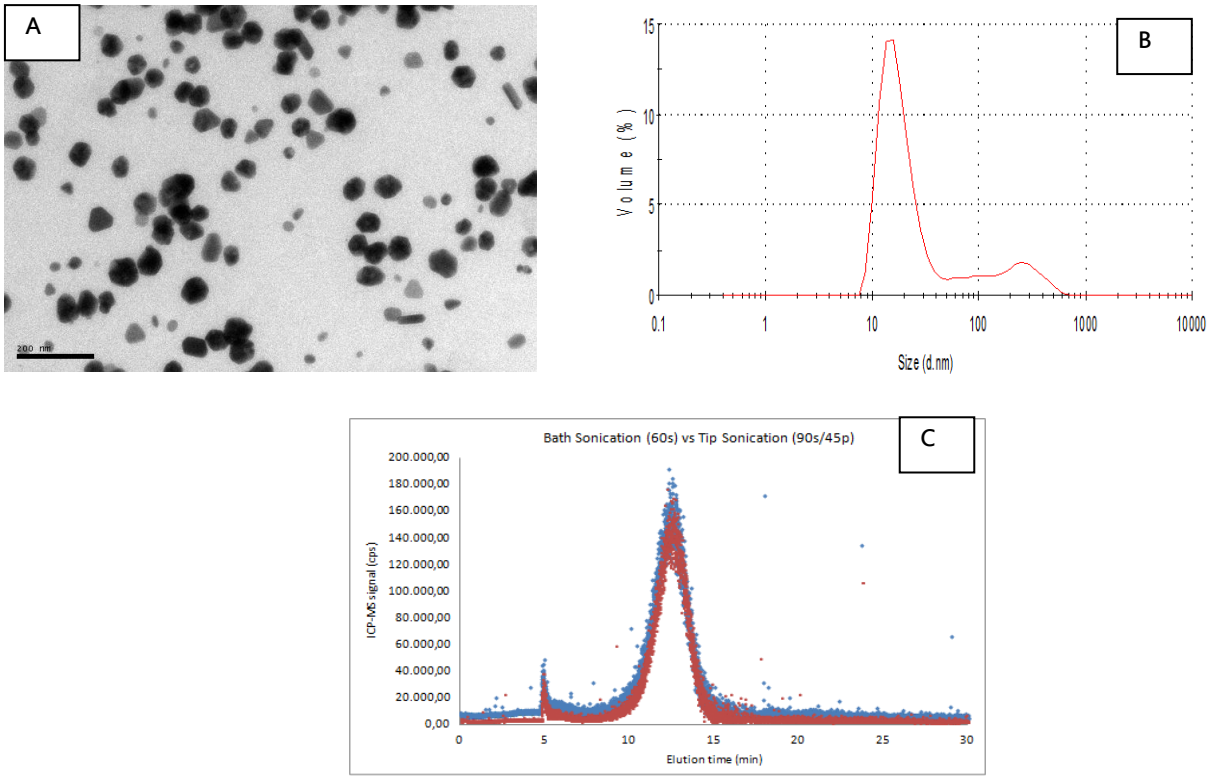

Figure 1. Size and shape characterization of silver nanoparticles using several techniques: a) TEM; b) Light scattering; c) AF4-UV.

(Figure 1) was performed by transmission electron microscopy (TEM) using a JEOL JSM JEM 2000FX (Japan) instrument equipped with microanalysis (Oxford instruments) and by dynamic light scattering technique (Zetasizer nano, Malvern Instruments, UK). TEM results revealed that the particles had a polyhedral shape (Figure 1a). The hydrodynamic diameter determined by DLS technique was between 10-30 nm in 80% of the particles (Figure 1b). Finally, Figure 1c shows a fractogramme obtained by Flow Field Flow Fractionation (AF4) (AF2000 MultiFlow FFF, Postnova, Berlin, Germany) showing sizes comprised between 21-32 nm and thus corroborating the results obtained with the other techniques. The results obtained confirmed that the size of the particles was consistent with the specifications of the manufacturer.

Dehydrated trisodium citrate (CAS: 68-04-2 (99% purity) from Sigma Aldrich (Germany)) and soluble starch GR (provided from Merck KgaA (Germany)) were employed as stabilizers for AgNPs. All suspensions tested at different concentration levels were dilutions from 10 g L^{-1} stock suspension with the zebrafish growing media (a mixture of salts to simulate freshwater, containing 294 mg·L^{-1} of $CaCl_2 \cdot 2H_2O$, 123.3 mg·L^{-1} of $MgSO_4 \cdot 7H_2O$, 63 mg·L^{-1} of $NaHCO_3$ and 5.5 mg·L^{-1} of KCl in deionizater water). Next, the suspensions were filtered through a 0.45 µm filter andsonicated for 15 min in an ultrasonic bath (Ultrasons-HD 10 Litres, Selecta, Barcelona, Spain) to avoid possible nanoparticles aggregation. Different mixtures of citrate and soluble starch were tested as green stabilizers based on their compatibility with eleutheroembryos survival.

2.2. Characterization and analysis of Ag NP stability

To evaluate the role of citrate and soluble starch on the stability and behavior of the AgNPs in the exposure media, total silver content was determined in all the suspensions and fractions obtained after ultracentrifugation and ultrafiltration experiments by GF-AAS with a longitudinal Zeeman background correction and by ICP-MS in flow injection operation mode . Specific sample treatment and instrumental conditions are described in SI (Suportting Information), Table S-1. For silver determination, 2% nitric acid was added to the obtained suspensions and filtrate to eliminate matrix effects.

To determine which additive better stabilizes AgNPs in the exposure media for further bioconcentration studies, the oxidation rate and particle size were analytically characterized by ultracentrifugation and ultrafiltration. Ultracentrifugation was employed to evaluate the release of ionic silver from AgNPs over time.[36,23] An ultracentrifugation speed of 16 g for 10 min was enough for Ag NP deposition, while ionic silver remained in the supernatant. Ultrafiltration was used to evaluate the size distribution of AgNPs. Membrane and cellulose filters with different pore sizes were used to classify the state of AgNPs in solution: membrane filter Durapore (100 and 50 nm) and Amicon Ultra 3K cellulose filters (1.3 nm) (Millipore, Ireland). Figure 2 indicates the filtration procedure: 10 mL solutions of AgNPs at concentrations of 50 and 1000 $\mu g \cdot L^{-1}$ were poured over the different membrane filter mentioned above getting the suspensions 1, 2 and 3 respectively. Total silver concentrations were measured in the original suspension prepared, and at each one of the suspensions filtrated. Silver recovery was determined from a mass balance of the amount of Ag at each suspension regarding the initial suspensions prepared. Silver content in each suspension was determined by ZGF-AAS in triplicate. Silver concentration measured from solutions filtered through Amicon Ultra 3K (≈1.3 nm) filters was considered to be ionic silver. Validation was also performed by FI-ICP-MS.

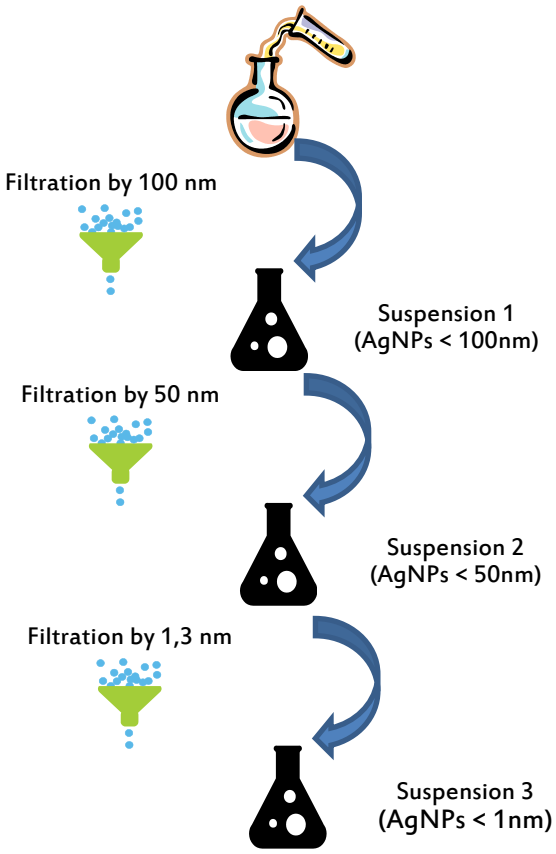

Figure 2. Ultrafiltration design for evaluating AgNPs size distribution in the exposure media

AgNPs size measurements obtained by the ultrafiltration experiments were validated by TEM. AgNPs were dissolved in butanol and sonicated for half an hour ; two drops of different filtrates suspensions obtained after ultrafiltration were deposited over a copper grid and left to dry at room temperature for 24 hours. Imaging was taken with the electronic microscope described in section 2.1.

The effect of the temperature and recipient containers on Ag NP stability of over time was also analyzed. Temperatures of 4 and 25ºC, simulating refrigeration and room temperature, were tested. Preliminary studies at -20ºC were

also conducted. However, at this temperature properties of starch were lost causing aggregation and deposition of the AgNPs. The two container walls tested were made of glass and polyethylene (PE), as they are the most widely employed for sample storage. Both parameters were evaluated at two Ag NP concentrations: 50 and 1000 $\mu g \cdot L^{-1}$.

2.3. Exposure of the eleutheroembryos

Eleutheroembryos were obtained from wild-type adult zebrafish bred and maintained in the AZTI Zebrafish Facility (EU-10-BI) under standard conditions. All experimental procedures were approved by the Regional Animal Ethics Committee. Two concentrations were tested for each chemical form. The OECD technical guidance[37] was used as a reference for determining the nominal concentrations for the exposure; the highest concentration should be approximately 1% of the compound LC_{50} value and the second concentration should differ by a factor of ten. For ionic silver, data found in the literature indicated markedly different 96-h LC_{50} values, but most of them are between 15 and 35 $\mu g \cdot L^{-1}$ for fish embryos,[38,39] so the chosen nominal concentrations were 0.05 and 0.01 $\mu g \cdot L^{-1}$. For AgNPs, the concentrations tested were 1000 and 100 $\mu g \cdot L^{-1}$ (silver content) as in most of the studies dealing with fish embryos, LC_{50} values ranged between 1 and 15 $mg \cdot L^{-1}$.[40,41,42]

An appropriate number of 72 hpf eleutheroembryos were transferred to 1 L tanks filled with the previously mentioned zebrafish growing media spiked with the selected concentrations of ionic silver and AgNPs (1 container per concentration) or simply to embryo water in the case of the control. AgNPs were previously treated with citrate and soluble starch for stabilization purposes as described above. Exposures were performed in 1 L plastic tanks, incubated at 27°C with a photoperiod of 12 h light and 12 h darkness. The exposure test consisted of two phases: a) absorption: 48 h exposure to a contaminated exposure medium; and b) depuration: 24 h exposure to a clean exposure medium that in this case was embryo water. About 25 eleutheroembryos were taken at different times (t_0; t_{3h}; t_{6h}; t_{21h}; t_{45h} and t_{48h}) from the tanks to determine the concentration of the analyte. According to the OECD 305 technical guidance,[37] the loading rate of eleutheroembryos at the beginning of the experiments ranged between 0.7-0.8 $g \cdot L^{-1}$ (10 individuals for each 20 ml of exposure solution) and the mortality of eleutheroembryos was lower than 20% at the end of the test.

Specific analytical methods were developed to assess the analyte bioconcentration factors in the exposure media and eleutheroembryo samples. Total silver quantification by ICP-MS (from ionic silver and AgNPs) in the exposure media was done after the addition of 2% nitric acid to eliminate matrix effects. A focused ultrasonic probe (USP) was employed for

leaching the analytes from the eleutheroembryos (described in SI). The aqueous suspension formed was injected into the ZGF-AAS with no matrix effects observed for silver determination. Zebrafish eleutheroembryos exposed to AgNPs were also analyzed by TEM (preparation procedure at SI).

2.3.Toxicokinetics and statistics

Two different procedures were used to determine bioconcentration factors using the experimental data: a) the procedure defined in the OECD 305 technical guidance,[37] which defines the BCF as the ratio between the concentration of the compound studied in the target organism at the maximum time of the uptake phase (48 h) and the media of the silver concentration in the exposure medium (BCF_{48h}); and b) using a first order two-compartment (water and aquatic organism) model[43,44] which describes the uptake and depuration process with exponential equations representing the variation of analyte concentration in eleutheroembryos versus uptake time thoroughly described in SI. Fitting the experimental data obtained previously to the model, which was done using the software NONLIN 3.0 (fit in terms of squared deviations)[45] the uptake and depuration constants were obtained, allowing to calculate BCF_k values.

3. Results

3.1. Ag NP characterization in the exposure media

Ultracentrifugation experiments showed that when the citrate concentration was increased to 0.2% w/V the released silver ion to the zebrafish exposure media decreased from 21 to 1%. Similar behavior was observed at two different levels of concentration (50 and 1000 $\mu g \cdot L^{-1}$), and after 48 hours of exposure. However, this citrate concentration was no compatible with zebrafish early stages life and additional studies to find the appropriate stabilizer green agent were done. Several percentages of starch (0.1% and 0.2% w/V) were employed as stabilizing agent based on the results reported in the literature[14]. Ultrafiltration was used to quantify the size distribution and stability of AgNPs. Figure 3 illustrates the AgNPs recovery at each suspensions obtained after ultrafiltration

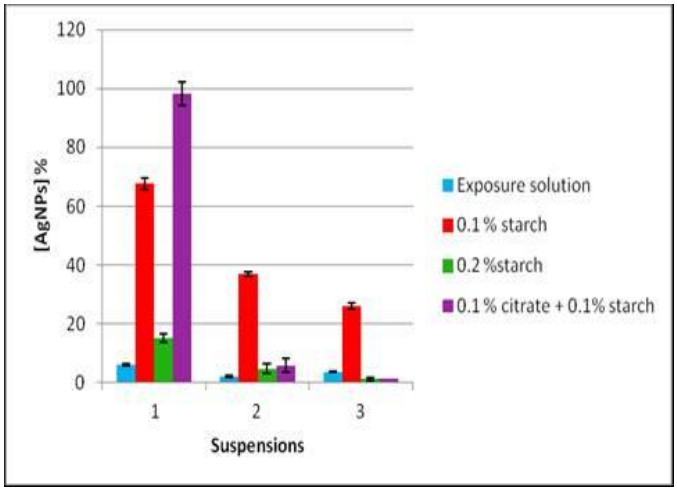

Figure 3. Results of ultrafiltration experiments to set nanoparticle size distribution. Addition of different stabilizers. Suspension number got from Figure 2. [Nominal AgNPs] = 50 $\mu g.L^{-1}$.

(already explained above) for all the exposure media assayed. Results showed aggregation of AgNPs forming aggregates larger than 100 nm. When starch was added, up to 60% of total silver crossed a 100 nm pore size membrane. However, after ultrafiltration by 1.3 nm, silver concentration in the filtrate obtained was approximately 20% of total silver, demonstrating that starch did no prevent the oxidation from Ag^0 to Ag^+. The combination of citrate as reducing compound plus starch as the stabilizing agent (0.1% starch + 0.1% citrate) was also tested. Under this combined condition, the AgNPs maintained an adequate size distribution (ranged from 50 to 100 nm) according to specifications given by nanoparticles providers, while ionic silver release from AgNPs was negligible. Therefore, this composition for the exposure media was chosen for the bioconcentration test. Furthermore a study over time and in presence of eleutheroembryos was performed in the selected exposition media demonstrating that AgNPs also stated as so (SI-Figure S-1) AgNPs with a range of size comprised between 50-100 nm (Figure S-1a and S-1c) were more abundant than nanoparticles lower than 50 nm (Figure S-1b). These images also corroborated the stability of AgNPs for at least 24 hours, as not significant differences in the appearance and size of corresponding samples at 0 or 24 hours after preparation were detected (Figure S-1a and S-1c).

The effect of the temperature and the container over time on AgNPs stability was also evaluated. Results are illustrated in SI, Figure S-2. This figure gives the ratios of the mean values of total silver concentration (X_T) of three replicates prepared at different times (24, 48, 72 hours) and the mean value of total silver concentration (X_{T0}), from 3 determinations made at the beginning of the stability study (Eq 1).

($R_T = X_T / X_{T0}$)

(Eq. 1)

The uncertainty U_T can be defined attending the Eq 2 at each point tested.

$$U_T = \frac{R_T \sqrt{RSD_T^2 + RSD_{T0}^2}}{100}$$

(Eq. 2)

In the case of ideal stability, the ratios R_T should be 1. In practice, however, there are some random variations due to errors in the measurements. Thus the stability can be established if value 1 lies between $R_T + U_T$ and $R_T - U_T$.

The results indicate a clear dependence on the nanoparticles concentration tested. In fact, AgNPs at 50 $\mu g \cdot L^{-1}$ showed good stability in almost all cases (no significant loss of silver concentration), being better the storage at 25 ºC, independently of the container employed. At this temperature, the expression $R_T \pm U_T$ fitted in all cases with $R_T = 1$. On the contrary, the highest concentration, 1000 $\mu g.L^{-1}$ showed instability (loss of silver concentration) after 72 hours in all cases, but silver losses already

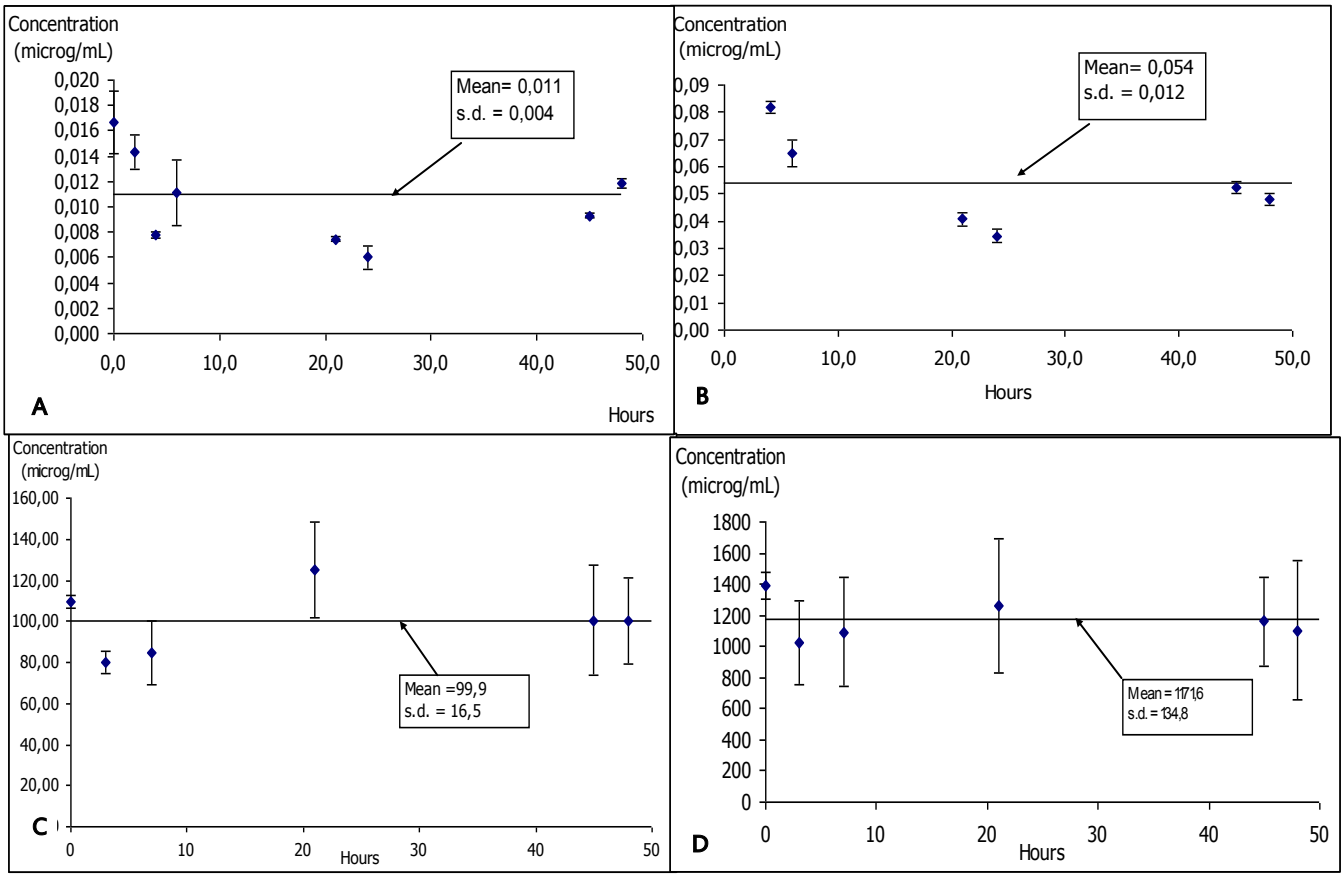

Figure 4. Total silver content in exposure medium where eleutheroembryos were incubated in the ionic and nanoparticles silver bioaccumulation experiments. (A) Ag^{+} nominal concentration 0.01 µg·L^{-1}. (B) Ag^{+} Nominal concentration 0.05 µg·L^{-1}. (C) AgNPs nominal concentration of 100 µg·L^{-1}. (D) AgNPs Nominal concentration of 1000 µg·L^{-1}.

started at t= 24 h. Again, the best storage temperature was 25 ºC, which could be related to a lower efficiency of starch provoking AgNPs aggregation when temperature decreases, similarly to what occurs when freezing the suspensions. Additionally, polyethylene seemed to be a better container material than glass despite of total stability of both concentration tested was not achieved. We must point out that measurements of these latter samples were performed after a 1:10 dilution, in order to achieve similar uncertainty for both concentrations. The lack of stability at the 1000 µg·L^{-1} could be explained by adsorption which would increase with NP concentration and/or aggregation which is generally more pronounced at higher concentrations.

3.2. Bioconcentration experiments

3.2.1 Concentration in the exposure medium and in eleutheroembryos

The uptake of ionic silver by eleutheroembryos increased with the exposure time until reaching a steady state of around 7-11 ng·g^{-1} silver

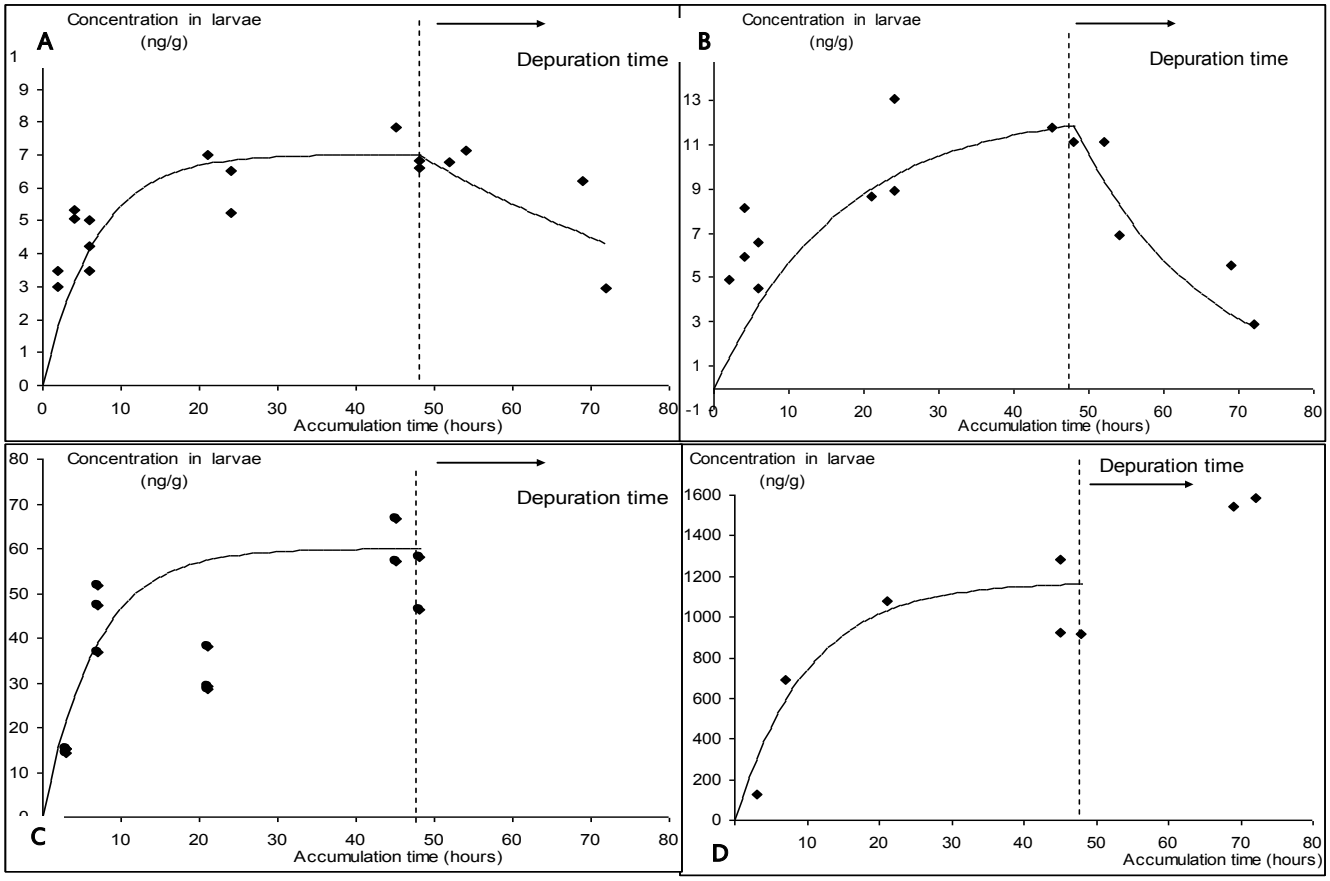

Figure 5. Profile of Ag+ and AgNP accumulation in eleutheroembryos in the experiments carried out at (A) Ag^{+} nominal concentration 0.01 µg·L^{-1}. (B) Ag^{+} Nominal concentration 0.05 µg·L^{-1}. (C) AgNPs nominal concentration of 100 µg·L^{-1}. (D) AgNPs Nominal concentration of 1000 µg·L^{-1}.

concentration (wet weight), when the averaged concentrations in the exposure solutions were 0.011±0.004 and 0.054±0.012 µg·L^{-1} silver concentration, respectively (Figure 4 and 5, A and B). A 50% decrease in the concentration of silver in eleutheroembryos during depuration in less than 24 h was observed. The concentrations of silver accumulated by eleutheroembryos also increased over time when they were exposed to AgNPs, reaching values up to 60 and 1080 ng·g^{-1} of silver for the tested concentrations of 1171±135 and 100±17 µg·L^{-1}, respectively (Figure 4 and 5, C and D). During the depuration phase, silver concentration accumulated by eleutheroembryos was higher than the concentrations accumulated during uptake phase, revealing no elimination of AgNPs occurred. The lower exposure concentration (100 µg·L^{-1}) could not be fully represented in Figure 5 C because data for 68 and 72 h were out of scale (silver concentration achieved in larvae were 119.48±74.23 and 268.23±169.18 ng g^{-1} respectively). Silver concentration in the exposure medium during the depuration phase was negligible for both bioconcentration tests.

3.2.2. Bioconcentration Factors

Table 1 shows the toxicokinetic values obtained for ionic silver and AgNPs with both methods, as well as the values for concentrations in the exposure medium (C_w), in eleutheroembryos (C_f), the uptake and depuration rate constants (k_1, k_2) and the bioconcentration factors (BCF_{48h} and BCF_k). Bioconcentration factors obtained for all the experiments highlight an appreciable ionic silver bioaccumulation by zebrafish eleutheroembryos (BCF_k 233-666). On the other hand, the low BCF values obtained for the bioaccumulation of AgNPs (BCF_k 0.6-1) indicate that the silver accumulated in this form was negligible. TEM study performed in order to have a direct observation of the AgNPs inside the eleutheroembryos showed the formation of NP aggregates of around 500 nm (Figure 6) corresponding to a brain section

Table 1. Toxicokinetic parameters and bioconcentration factors (BCF48h, BCFk) obtained from experimental data adjustments

	Ionic Ag		AgNPs	
C_w (µg·mL^{-1})	0.011	0.059	99.9	1171.6
C_f (ng·g^{-1})	7.01	11.84	59.9	1162.0
k_1 (mL·ng·h^{-1})	100	14	0.09	0.1
$k_{2\ (acum)}$ (ng·mL^{-1})	0.15	0.06	0.15	0.1
$k_{2(dep)}$ (ng·mL^{-1})	0.02	0.06	-	-
BCF_{48h}	665.3	220.2	0.599	0.991
BCF_k	666	233.3	0.6	1

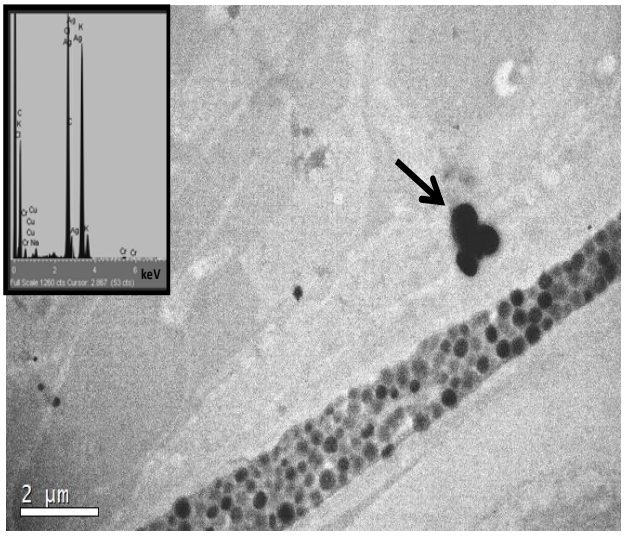

Figure 6. TEM low magnification image corresponding to a brain section. The inset shows EDS spectra corresponding to the arrowed cluster. The silver signal is outlined

4. Discussion

4.1. Characterization of AgNPs stability in the exposure media

Ultracentrifugation results revealed high release of ionic silver from AgNPs and a clear tendency to aggregate or interact between them or with other surrounding particles in aqueous media. The oxidation of silver is thermodynamically favored at room temperature (ΔG^0_{298}= -11.25 kJ·mol^{-1}).[21,46] Regarding AgNPs, there is an interesting controversy about the role of the nanoparticles and the ionic forms on toxicity and if they have independent or synergistic toxicity pathways. The nature of the nanoparticles can be modified by adsorption since surface charge D ys a dominant role.[47,48] Here, AgNPs stability over time was shown, with minimum aggregation and/or ionic silver release in a zebrafish exposure media. While ultrafiltration results showed that both types of AgNPs were in the exposure media as aggregates larger than 100 nm, in line with other reports.[46,49] The addition of 0.1% citrate plus 0.1% starch (w/v) to AgNPs prepared suspensions guaranteed their stability for 48 hours in a range of size between 50-100 nm, with a lower release of ionic silver. It is also important to underline that the pH of such suspension was between 7-7.5, which has been reported as the best pH value to prevent aggregation.[50]

On the other hand, nothing has been reported concerning temperature and concentration in terms of AgNPs stability in aqueous media over time. Here, it has been reported the appropriate conditions of for AgNPs storage (25ºC and polethylene containers) to guarantee their stabilization since it is well known that careful consideration should be given to the selection of type of vessel used to storage nanoparticles[51,52].

4.2 Silver bioaccumulation by eleutheroembryos zebrafish

The significant variation of nominal concentration of silver in the exposure media when the bioaccumulation of ionic silver was studied may be due to the ability of this ion to form complexes with some of the salts present in the exposure media. These complexes may precipitate and sink, being no longer bioavailable to zebrafish eleutheroembryos, resulting in less toxicity. It is also well known that the content of ionic silver in solution decreases with the increasing salinity, because ionic silver tend to react with chloride ligands in solution.[53] According the OECD technique guide 305, a maximum variation of 20% of the nominal concentration was established.[37] Here, to evaluate Ag NP bioaccumulation, a variation of 17% and 12% for nominal concentration of 100 µg·L^{-1} and 1000 µg·L^{-1}, respectively was detected, while for ionic silver, the detected values were higher, with variations of 36% and 22% for 0.01 µg·L^{-1} and 0.05 µg·L^{-1}, respectively. This could be due to the very low studied concentration investigated, which is already close to the quantification limit of the method.

In this study, the obtained BCFs are similar to those reported in adult fishes (Table 2). Furthermore, in this study an inverse relationship between the BCFs and the exposure concentration has been determined with higher bioconcentration rates for the lower concentration level tested. Similar tendencies have been observed by McGeer *et al.*, who compiled BCF data for a variety of aquatic organisms exposed to different metals.[54] Accumulation mechanisms are dependent on the nature of the tested compound. While passive diffusion across the lipid bilayer of biological membranes, as predicted by Fick´s Law, is the main process for neutral organic substances, the lipophilicity of metals is usually low; hence, they accumulate in the biota via different ways compared to neutral organic molecules.[55] The uptake of metals shows complex internal dynamics specific channels in the cell membrane, active transport or endocytosis.[56] Other attributes are also different, e.g., storage in detoxified forms, such as inorganic granules or binding to metallothionein-like proteins, active elimination, etc. Other authors highlighted the importance of the surrounding media composition (salt content, pH, etc.) for metal accumulation.[54,57] Bioconcentration factors do not recognize essential mineral nutrients, normal metal background concentration, animal capabilities to vary uptake and elimination depending on exposure concentrations, or the specific ability to isolate, detoxify and store the internalized metal. Therefore, data evaluation and comparison might become troublesome explaining why the BCF values found here depend on the concentration of the exposure media. Moreover, a remarkable and fast depuration occurred when eleutheroembryos were placed in the exposure media without silver after their exposure to ionic silver at both concentrations tested. This is also in agreement with other publications on silver bioaccumulation by aquatic organisms, highlighting that more than 30% of the accumulated silver could later be eliminated.[58]

On the contrary, the low values of BCFs obtained indicates there was no significant bioaccumulation of AgNPs by eleutheroembryos for both tested concentrations. Very few studies have been carried out to determine Ag NP bioconcentrations and none of the studies has been done following officially established bioconcentration tests,[7,20,33,34] with bioconcentration factors showing remarkable variability (Table 2). As previously mentioned, most of those studies did not take into consideration the concentration evolution and size distribution of the NPs along the experiments, which may explain these variations. Therefore, to obtain reliable ecotoxicity data, it is imperative to establish new protocols considering those relevant parameters.[51]

Regarding AgNPs depuration, zebrafish eleutheroembryos did not eliminate the bioaccumulated silver. This lack of depuration of AgNPs is consistent with a study carried out with TiO_2 NPs in *Daphnia magna*,[59] where it was established that titanium needs a rather longer timespan of about 72 h to accumulate. The values of silver concentration found in eleutheroembryos in this study are much lower than those found for titanium. Nevertheless, based on the results obtained in the present study and considering the information found in the literature, we can conclude that the bioaccumulation potential of AgNPs is relatively low compared to ionic silver.[60] The precise pathways for NPs uptake and excretion are still not well understood. Olasagasti *et al*. ,2011 found through Environmental Scanning Electron Microscope studies that NPs were retained on the embryo chorion surface in unhatched individuals when exposed to AgNPs, whereas NPs are found inside the digestive tract in hatched eleutheroembryos and larvae.[61] In this work, the TEM image revealed some aggregates in the brain tissue of the zebrafish, but no evidence has been obtained in other analyzed sections. Other authors have also raised the possibility of an association

Table 2. BCFs values reported in literature for for ionic silver and AgNPs in aquatic organisms

Biological species	Compound	BCF	Reference
Adult Trout (*Oncorhynchus mykiss*)	$NaAgS_2O_3$	335	22
Adult Carp (*Cyprinus carpio*)	$AgNO_3$	250-460	40
Larvae zebrafish (*Danio rerio*)	$AgNO_3$	233- 666	This work
Adult Trout *(Oncorhynchus mykiss*)	AgNPs (10-35 nm)	7.07-42	41
Adult zebrafish (*Danio rerio*)	AgNPs (26.6 nm)	1.02-0.0096	42
Adult Carp (*Cyprinus carpio*)	AgNPs (35 nm)	590-997 (organ specific)	43
Embryo zebrafish (*Danio rerio*)	AgNPs (26.6 nm)	14-24	26
Eleutheroembryos zebrafish (*Danio rerio*)	AgNPs (60 nm)	0.6-1	This work

.

between AgNPs and the membrane surface, the oxidation of Ag^0 to Ag^+ within the superficial microlayer of the membrane could create nano-environments of very high ionic silver concentration, conducive for rapid uptake of the ion.[8,16] Other authors pointed to endocytosis as the mechanism responsible for NPs uptake into the cells.[60]

It could be argued that such situation is unprecedented in the evolutionary exposure of organisms to trace metals and would also create circumstances outside the predictive power of the models that currently describe dissolved trace metal bioavailability. In such case, the hazard may indeed be from the metal ion, but the NPs may create novel pathways for delivery to the cell leading to enhanced ionic uptake. Up to date, none of these potential mechanisms could be unequivocally proved. Regarding AgNPs, this is partly due to the technical challenges of both imaging and quantifying AgNPs at or within the membrane of an organism.[51,60]

5. Conclusions

Addition of 0.1% citrate plus 0.1% starch stabilizes AgNPs in the exposure media. It has been shown that the alternative test of bioconcentration using eleutheroembryos zebrafish is adequate to evaluate silver bioconcentration. Therefore, this model is a good alternative to the Bioconcentration Test OECD 305, which requires a large number of adult fishes, implying high costs as well as complex, time-consuming experiments. The BCFs values obtained for ionic silver are in line with those previously established using OECD 305 guidelines. On the other hand, the results obtained in the present study show a lower bioconcentration of AgNPs by zebrafish eleutheroembryos, also in agreement with most data on NPs bioaccumulation published to date. However, the available information on NP accumulation is still confusing and the results of the different studies are quite difficult to compare, which evidences that many important factors affecting the toxicity of NPs are still not well understood. Additionally, new methods for rapid detection from tissue and for the evaluation of NP characteristics related with these ecotoxicological tests should be developed. Once this is established, further studies should be carried out in order to understand and predict the real impact and hazards of NPs to the health and environment. To begin with, new standardized ecological accumulation test protocols should be designed, taking into account the physical-chemical characteristics of NPs.

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Supporting Information

Stability of silver nanoparticles in aqueous medium compatible with aquatic organisms: comparison of bioconcentration of ionic silver and silver nanoparticles in zebrafish eleutheroembryos.

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Analytical methodology employed

A Perkin-Elmer 4100 ZL atomic absorption spectrometer with a longitudinal Zeeman background correction (ZGF-AAS), equipped with a transversely heated graphite tube atomizer (THGA) with L´vov platform was used for silver determination. A Perkin Elmer silver hollow cathode lamp (HCL) with a wavelength of 328.1 nm and a slit width 0.7 nm was used. Alternatively, an inductively coupled plasma mass spectrometer (ICP-MS) HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan) equipped with a Babington nebulizer, Fassel torch and double pass Scott-type spray chamber cooled by a Peltier system was employed. Single ion monitoring at m/z 109 and 107 was selected for data collection. NPs were introduced into the plasma employing a Flow Injection device.

Ultracentrifugation assays were carried out in a centrifuge model type: FVL-2400N, Combi-Spin, Boeco (Germany) and in a centrifuge model Epppendorf 5415 R.

Ultrasonic probe: A Vibra cell VCx130 ultrasonic processor (Connecticut, USA) equipped with a titanium 2-mm-diameter microtip and fitted with a high-frequency generator of 130 W at 20 KHz was used for the leaching of the analytes from eleutheroembryos. About 250-400 μL of 2.5% HNO_3 solution was employed to promote the leaching of silver from zebrafish eleutheroembryos.

All solutions and samples were prepared using high-purity water with a resistivity of 18.0 MΩ•cm, obtained from a Millipore ZMFQ 23004 Milli-Q water system (Bedford, MA, USA). Working solutions were prepared daily in deionized water. Calibration standards were prepared daily from a 100 $mg.L^{-1}$ of $AgNO_3$ (CAS: 7761-88-8, from Sigma Aldrich (Germany) stock solution. Analytical grade chemicals were used for all experiments. $AgNO_3$, was used as calibrant for further determination of total silver content.

Matrix effects of the salted components of the zebrafish exposure media were eliminated by adding 2% HNO_3 when total silver was determined by ICP-MS. This HNO_3 was also acting as matrix modifier for total silver determination by ZGF-AAS. The exposure media of ionic silver was directly analyzed but in the case of AgNPs a 20-fold

dilution was needed to match the linear range of ZGF-AAS and ICP-MS instruments. Final conditions of both quantification techniques are summarized in Table S-1.

The ultracentrifugation experiments were performed in triplicate and determination of ionic silver concentration in the supernatant was performed by FIA-ICP-MS. Silver content in each suspension obtained after ultrafiltration was determined by ZGF-AAS in triplicate. Validation was also performed by FI-ICP-MS.

Table S-1. Optimized conditions for the direct determination of total silver by ZGF-AAS and FI-ICP/MS : a) ZGF-AAS temperature programme; b) FIA-ICP/MS operating parameters

a)

STEP	Temperature (°C)	Ramp time (s)	Hold time (s)	Ar flow rate (mL)
Dry 1	110	1	15	250
Dry 2	300	5	10	250
Pyrolysis	600	5	15	250
Atomization	1800	0	3	0
Cleaning	2000	1	3	250

b)

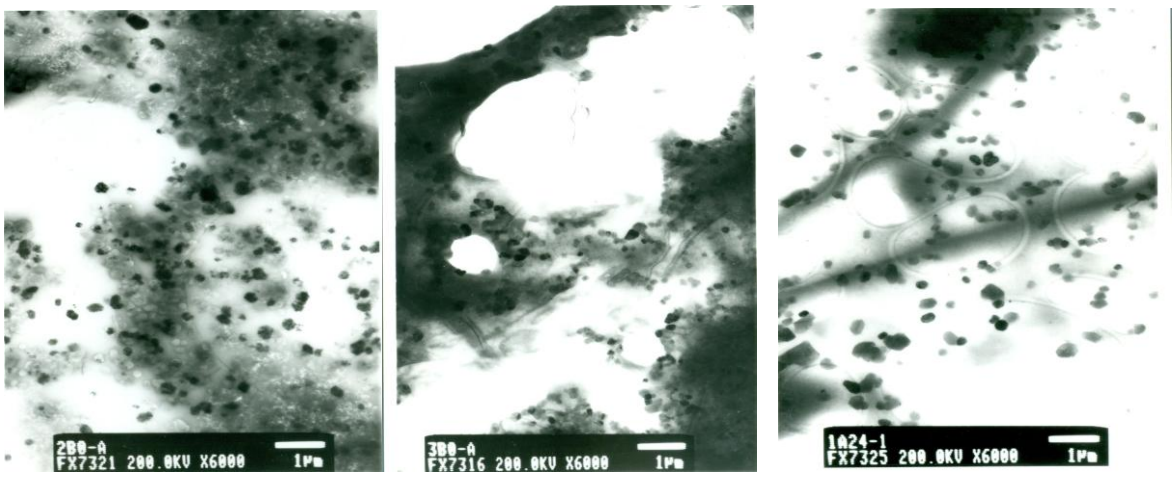
RF power	1550 W
Argon flow rate	Plasma gas: 15 L min^{-1} Nebulizer: 1 L min^{-1}
Isotope monitored	107 Ag and 108 Ag
Dwell time	100 ms
Introduction mode	Flow Injection (50 µL)

TEM Imaging of filtrates suspensions after ultrafiltration

Samples for imaging were prepared by deposition of two drops of filtrates suspensions in the appropriate medium obtained after ultrafiltration on a copper grid for Transmission Electron Microscopy (TEM) analysis. Then, samples were dried at room temperature for 24 hours prior to imaging. Measurements were performed the following day.

Figure S-1. TEM imaging of filtrates suspensions obtained by ultrafiltration. AgNPs with a range of size comprised between 50-100 nm (Figure S-1a and S-1c) and AgNPs lower than 50 nm (Figure S-1b).

a) b) c)



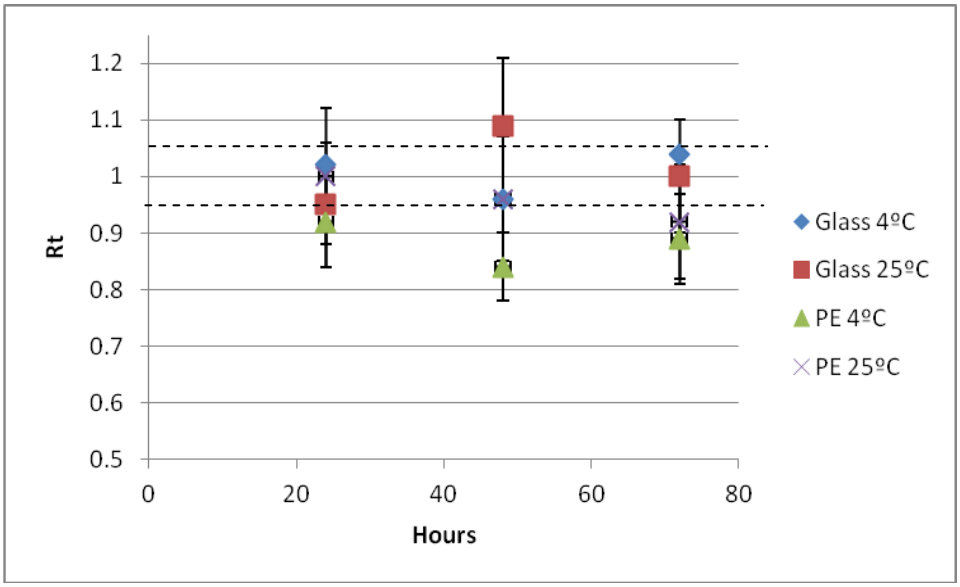
Imaging of AgNPs in zebrafish eleutheroembryos

Analysis of zebrafish eleutheroembryos exposed to AgNPs was performed by TEM, by fixing each individual eleutheroembryo in 2.5% gluteraldehyde and dehydrated. The eleutheroembryos were embedded in resin (Spurru´s resin) and sectioning was done using a Reichart Jung ULTRACUT Stereo Ster instrument. Ultrathin sections of tissues of interest (mainly eyes and brain) were selected using a microscope 2.8X Reichart FORMAT 1 1762.

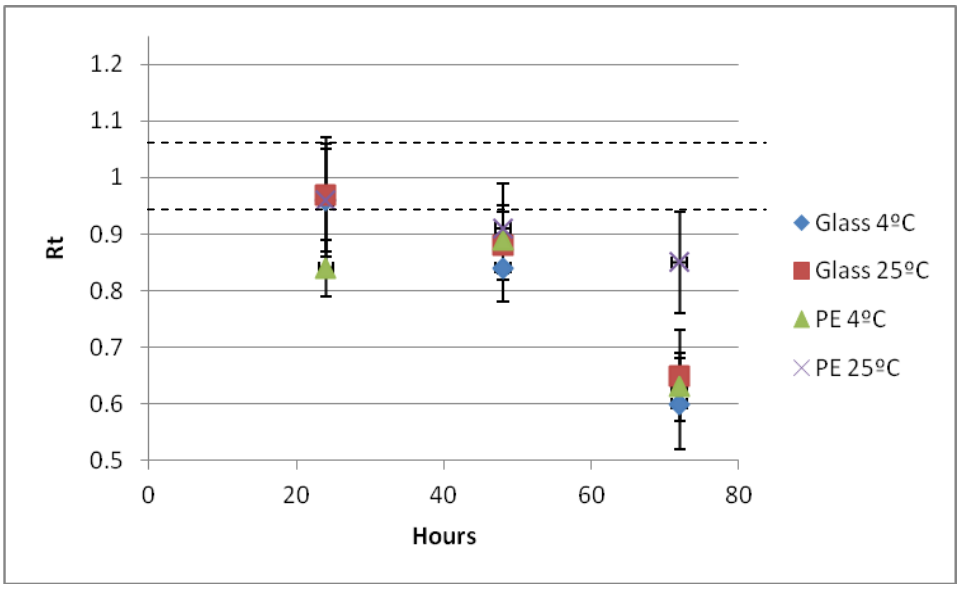
Stability of AgNPs over time depending on the container material and storage temperature

Figure S-2. Effect of the nature of the containers walls, polyethylene (PE) and glass on the stability study over time for AgNPs. *Stabilizingagent: 0.1 starch + 0.1 citrate*. a) 50 $\mu g.L^{-1}$; b) 1000 $\mu g.L^{-1}$.

a)



b)



Biodynamic model equations

When a steady state is not reached, BCF values can also be calculated from a first order two-compartment (water and aquatic organism) model which describe the uptake and depuration process as Eq. 1,

$\frac{dCf}{dt} = k_1 \cdot C_w - k_2 \cdot Cf$ (uptake) $\frac{dC_f}{dt} = -k_2 \cdot C_f$ (depuration) Equation S1

where C_f is the concentration in fish (in ng/g), t is the exposure time (h), k_1 is the first-order uptake constant (litre per kilogram dry weight per hour), C_w is the concentration of the compound in the exposure media (ng/mL) and k_2 is the first-order elimination rate constant (per hour). Assuming that at t0, the concentration of the test substance in fish is negligible and the concentration of the tested compound in exposure media is constant, Eq. 2 is obtained:

$C_f = \frac{k_1}{k_2} \cdot C_w(1 - e^{-k_2 t})$ (uptake) $C_f = C_{f,0} \cdot e^{-k_2 t}$ (depuration) Equation S2

Where $C_{f,0}$ denotes the analyte concentration in the organism when the depuration phase begins. k_1 and k_2 values can be obtained if the experimentally determined concentration values in the bioconcentration test fit to this equation. When the equilibrium is reached (steady-state), equation 2 may be reduced to equation 3.

$C_f/C_w = BCF_k = k_1/k_2$ Equation S3

The software NONLIN 3.0. was used for kinetic calculations.

4.3.3. Bioaccumulation of titanium disolved and titanium dioxide nanoparticles in a vertebrate aquatic model: Zebrafish eleutheroembryos (enviado a Water Research 2013 , Ref:WR25272,).

Bioconcentration of ionic titanium and titanium dioxide nanoparticles in an aquatic vertebrate model: zebrafish eleutheroembryos.

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Abstract

The production of titanium dioxide nanoparticles (TiO_2NPs) for commercial applications has increased extraordinarily over the last years and so its risk for human health. Information on the behavior of these nanoparticles in the environment and their potential toxicity to the aquatic organisms is very scarce, and there is greater concern regarding their release into the environment. Bioconcentration serves as a good integrator to assess chemical exposure in aquatic systems and is dependent of factors such as the exposure routes, diet, and aqueous medium. Here, we calculated the experimental bioaccumulation of ionic titanium and TiO_2NPs by zebrafish (*Danio rerio*) eleutheroembryos through bioconcentration factors (BCFs), after 48 and 72 hours of exposure. The stability of both chemical forms in a normal aquatic system was fully characterized for further bioconcentration studies. Several coating agents (humic acid, soluble starch, polyethylene glycol, $Na_4P_2O_7$, and Na_2HPO_4) for anatase, rutile, and a combination of both, were evaluated to check the evolution of the 48-72 hour aggregation process in aquatic medium. A high percentage of TiO_2NPs remained disaggregated under simulated environmental conditions and 50 $mg{\cdot}L^{-1}$ of humic acids. However, the presence of eleutheroembryos in the exposure medium increased TiO_2NP aggregation during experimental tests. BCFs values below 100 were obtained indicating there was low bioaccumulation of both chemical forms (ionic titanium and TiO_2NPs) in early life stages of zebrafish at both tested concentrations (0.1 and 1% of their LC_{50}).

Keywords: titanium dioxide nanoparticles, stability, aggregation, bioaccumulation, zebrafish eleutheroembryos

Introduction

Nanotechnology is a fast growing sector. The properties of nanomaterials make them potentially attractive for different fields. Titanium dioxide nanoparticles (TiO_2NPs), have gained much importance due to their ability to act as a shield against light, and/or its high photocatalytic activity (Gao and Zhang, 2001). Annual production of TiO_2NPs was estimated to be 5000 metric tons in 2006-2010, 10000 metrics tons in 2011-2014, and around 2.5 million metrics tons by 2025 (Menard *et al.*, 2011). They are widely used in sunscreens, cosmetics, paints, surface coatings, photocatalysts, ceramic membranes (French *et al.* 2009; Serpone *et al.*, 2007) and even as a tool for soil, water, and air decontamination (Herrmann, 2005). Topical application may lead to direct or indirect release of TiO_2NPs into the environment (air, soil, and water) increasing the risk derived from their presence.

The physical and chemical properties of the ionic and the NP form of an element or compound are different. Thus, the toxicity of TiO_2NPs and ionic TiO_2 is also expected to be different and needs to be assessed. The bulk form of ordinary TiO_2 powder has been classified by the American Conference of Governmental Industrial Hygienist as "nuisance" dust with a Threshold Limit Value of 10 $mg{\cdot}m^{-3}$ (Ferin and Oberdörster, 1985). Toxicity of bulk TiO_2 is low in mammals (Warheit *et al.*, 1997). TiO_2 does not produce significant toxic effects and has been used as an inert marker to quantitatively estimate food intake in fish (Short *et al.*, 1996), as well as in implants (Brunette *et al.*, 2001). However, some studies have revealed that the hemolytic activity of anatase TiO_2 is correlated with cytotoxicity (Zitting and Skytta, 1979; Ferin and Oberdörster, 1985). It is difficult to compare the toxicity of NPs with similar composition but different form and so, some authors have showed that TiO_2 toxicity varies if present as bulk, ionic form, or NPs, with controversial conclusions: some authors have found that TiO_2NPs toxicity is higher than that of bulk TiO_2 (Xiong *et al.*, 2011), while other authors indicate that the adverse effect of the TiO_2NPs and the bulk form are similar (Ramsden *et al.*, 2013).

Besides classic measurements of toxicity, bioaccumulation is another indicative parameter in risk assessment. Over the last years, national and international environmental agencies (US EPA, Environment Canada, German UBA, European ECHA, OECD, etc.) have used this parameter for environmental hazard identification, determining the potential for adverse effects to biota (Phillips and Rainbow, 1994). To our knowledge, studies on the biological impact of TiO_2NPs are scarce and the information on bioaccumulation by aquatic organisms is still inconclusive. Toxicity studies suggest that TiO_2NPs exert low acute toxicity to fish, since the 96-h LC_{50} has been reported to be 124.5 $mg{\cdot}L^{-1}$ for zebrafish (*D. rerio*) (Xiong *et al.*, 2011) and >100 $mg{\cdot}L^{-1}$ for trout (*Oncorhynchus mykiss*) (Warheit *et al.*,

2007), far above the ng·L^{-1} concentrations of TiO_2NPs predicted in water surface (Zhu *et al.*, 2008; Gottschalk *et al.*, 2009). However, Zhu *et al.* showed bioconcentration factors of up to 118062 L·g^{-1} for TiO_2NPs in *Daphnia magna* when exposed to 1 mg·L^{-1} of TiO_2NP for 72 hours (Zhu *et al.*, 2010a). No accumulation of nano-TiO_2 was detected in rainbow trout (*O. mykiss*) exposed to concentrations of up to 1 mg L^{-1} of TiO_2NPs for 14 days (Federici *et al.*, 2007). Additional studies are required to properly understand the toxicity of these NPs and establish the corresponding regulations on TiO_2NP release limits into the environment.

Understanding the toxic effects of TiO_2NPs to aquatic organisms is a complicated task. Quantifying their environmental fate, transport, behavior, and stability in aquatic media needs to be previously studied. A number of studies have shown the aggregation effect of TiO_2NPs in aqueous environments (French *et al.*, 2009; Von de Kammer *et al.*, 2010). NPs aggregate rapidly once released to the aquatic environment, forming larger particles (>100 nm). These larger TiO_2NPs particles can later settle or deposit in sediments or be transported as stable particles for long distances in the aquatic medium. There are many environmental factors such as pH, ionic strength, presence of ligands in natural waters, etc. that can affect their mobility, persistence, bioavailability, and reactivity. For example, natural organic matter, ubiquitous in natural waters, has been shown to significantly affect the stability of some types of NPs in aquatic environment (Li and Sun, 2011; Gao *et al.*, 2012). Humic acids act protecting and inhibiting the aggregation of TiO_2NPs by electrostatic repulsion, modifying their surface properties (e.g., electric charge, size, or chemical nature of the exposed surface sites) (Domingos *et al.*, 2009). Therefore, additional studies should be designed to clarify TiO_2NP toxicity and the underlying mechanism under realistic environmental conditions.

One of the main requirements of all standard ecotoxicological tests is to keep a constant concentration of the study compound. Regarding NPs, particularly TiO_2NPs, their concentration can change in water as they tend to agglomerate easily and precipitate under certain conditions. It is important to establish the conditions at which TiO_2NPs are stable. Capping agents are usually employed to stabilize TiO_2NPs, to prevent their aggregation as much as possible. Several capping agents have been used for TiO_2NPs, but those that are compatible with aquatic life are the following: polyethylene glycol (PEG), used for coating TiO_2NPs in biomedical applications increasing their biocompatibility and efficiency (Devanand Venkatasubbu *et al.*, 2013); soluble starch, as a green capping agent for NPs (Vasileva *et al.*, 2011); $Na_4P_2O_7$ and Na_2HPO_4 as electrolytes to increase the electrostatic repulsion between NPs or between NPs and surface particles, reducing their aggregation (Von de Kammer *et al.*, 2010); humic substances that inhibit the aggregation of

TiO_2NPs in aqueous environments by electrostatic repulsion (Domingos *et al.*, 2009).

In this work, we first separately tested the aggregation of two different allotropic structures for TiO_2NPs (anatase and rutile) in an exposure media using different capping agents compatible with aquatic life. Both, anatase and rutile, have different surface properties, reactivity, and turn different toxicity. Rutile has lipophilic properties, whilst anatase has hydrophilic properties. Thus, considering the definition of nanoparticles, we have studied them separately trying to maintain a size range below 100 nm. Furthermore, the aggregation rate of a 75% anatase and 25 % rutile mixture for TiO_2NPs, commonly used the toxicological assays, was also evaluated. An alternative assay to the bioconcentration Test 305 (OECD, 1996), validated in previous works with other chemicals (López-Serrano *et al.*, 2011; El-Amrani *et al.*, 2012) was used to assess the bioaccumulation of TiO_2NP by zebrafish eleutheroembryos as a vertebrate model system, calculating BCFs from a biodynamic model previously proposed (Spacie *et al.*, 1985). Bioaccumulation of ionic titanium was also evaluated to understand the differences in behavior between ionic titanium and TiO_2NPs.

2. METHODS

2.1. Reagents

Analytical grade chemicals were used for all experiments. Titanium elemental solution (1000 $mg \cdot L^{-1}$ Ti, TraceCert®, Sigma-Aldrich, Germany) ICP was used to prepare the standard solution. Diluted solutions for bioaccumulation tests were prepared from a stock solution using Milli-Q Element ultrapure water (Millipore, Billerica, MA, USA). Nitric acid (HNO_3. 65%), hydrofluoric acid (HF, 47-51%) (Merck, Darmsted, Germany), hydrogen peroxide (H_2O_2,. 35%), and boric acid (H_3BO_3.) (Panreac, Barcelona, Spain) were used for digesting TiO_2NPs from the eleutheroembryos. Exposure ISO solution (ISOwater) of similar composition to fresh river water was prepared as follows: 294 mg of $CaCl_2*2H_2O$; 123.3 mg of $MgSO_4*7H_2O$, 63 mg of $NaHCO_3$ and 5.5 mg of KCl were diluted to 1 L with distilled water (ISO, 1996).

Standard dispersions were prepared using rutile and anatase TiO_2NPs purchased from Sigma Aldrich (Germany). Titanium (IV) oxide, anatase, nanopowder, < 25 nm particle size, 99.7% trace metals (CAS: 1317-70-0), and rutile, nanopowder, < 100 nm particle size, 99.5% trace metals (CAS: 1317-80-2). The tested capping agents were: soluble starch GR (Merck); polyethylene glycol, PEG, (Aldrich); Humic Acids (Sigma Aldrich), $Na_4P_2O_7$ (Sigma Aldrich), and $NaHPO_3$ (Panreack).

2.2. Instruments and apparatus

A sonication bath (Ultrasons-HD 10 Litres, Selecta, Barcelona, Spain) was used to prepare the assayed TiO_2NP dispersions. A Vibra cell VCx130 focused ultrasonic probe (USP) (Connecticut, USA) equipped with a 3 mm in diameter titanium microtip and fitted with a

high-frequency generator of 130 W at a frequency of 20 KHz was used for sample treatment. A centrifuge model FVL-2400 N from Combi-Spin (Boeco, Germany) was used for sample centrifugation during experimentation. Inductively coupled plasma mass spectrometer (ICP-MS) HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan) equipped with a Babington nebulizer, Fassel torch and double pass Scott-type spray chamber cooled by a Peltier system was employed to determine total titanium content. Single ion monitoring at m/z 48 was selected for data collection. Introduction of NPs into the plasma was done with the aid of a Flow Injection device. Electron microscopy studies to evaluate the state of TiO2NPs in the exposure media were performed using a JEOL JEM 3000FX (Japan) Transmission Electron Microscope (TEM) equipped with a microanalysis system (Oxford instruments) and using the light scattering technique (Zetasizer nano, Malvern Instruments, UK).

2.3. Anatase/Rutile NP preparation

Stock dispersions of 1000 $mg \cdot L^{-1}$ for anatase, rutile, and the 75%anatase/25% rutile mixture in Milli Q water were prepared by adding the TiO_2 powder into deionized water, followed by ultrasonication in a sonication bath for 30 min. All tested dispersions were diluted from 1000 mg L^{-1} TiO_2NPs with the ISOwater media after adding the corresponding capping agent at different concentrations. Sonication was next performed for 30 min with the ultrasonic bath to avoid possible NP aggregation.

2.4. Characterization of TiO2 NP stability in aqueous media

To determine the capping agent that best maintains TiO_2NPs as NPs in the exposure media, several dispersions at 2 $mg \cdot L^{-1}$ of TiO_2NPs were prepared for each capping agent. The assayed concentrations were (Table 1): 0.1-0.2% w/v of soluble starch; 0.1% w/v of chitosan; 0.1% w/v of PEG; 2.5-100 mg L^{-1} of humic acid; 1-5mM of $Na_4P_2O_7$, and 0.3% w/v of NaH_3PO_4. All suspensions were analytically characterized by ultrafiltration as previously described (López-Serrano *et al.*, 2013). Briefly, a dispersion of 100 mL (fraction 0) was prepared, from which two aliquots of 2 mL were taken and analyzed at different times. Next, 2 mL of that suspension were filtered through a 100 nm pore size membrane, obtaining fraction 1; two 500 µL aliquots of this filtrate were collected for analysis. The same procedure was repeated using 50 and 25 nm pore size membranes, obtaining fractions 2 and 3, respectively. Titanium content in each fraction was determined by a flow injection system coupled to an ICP-MS detector; each determination was performed in triplicate. Ultrafiltration experiments were performed in duplicate and determination of total titanium concentration in the supernatant was performed by FIA-ICP-MS.

TiO_2NP size measurement results obtained by the ultrafiltration experiments were also

validated through TEM. Homogeneity of TiO_2NP suspensions dissolved in the ISOwater and 50 $mg{\cdot}L^{-1}$ of HA, at the desired concentration, was achieved by sonicating for half an hour with a) focused ultrasonic probe, b) ultrasonic bath, and c) ultrasonic bath and posterior freezing of sample. Next, two drops were deposited over a cupper grid and measurements performed.

Table 1. Ultrafiltration results to establish particle size distribution. Optimization of the capping agent to maintain TiO2NPs in an ultrafine form, <100 nm, in the standard OCDE media.

	Anatase			Concentration Tested ($mg{\cdot}L^{-1}$)	Rutile		
	% Recovery				%Recovery		
Capping agent	100nm>NP>50nn Fraction 1	50nm>NP>25nm Fraction 2	25nm>NP Fraction 3		100nm>NP>50nm Fraction 1	50nm>NP>25nn Fraction 2	25nm>NP Fraction 3
None	2.20±0.01	2.97±0.01	6.90±0.01	2	7.15±0.20	0	0
0.1% starch	16.40±0.20	0	0	2	8.00±0.30	7.20±0.15	0
0.2% w/v starch	11.30±0.52	12.00±1.81	1.80±0.10	2	15.70±1.20	10.30±1.65	0
0.1% w/v chitosan	ND	ND	ND	2	ND	ND	ND
0.1% w/v PEG	2.95±1.00	2.00±0.90	2.70±0.50	2	5.30±1.03	5.70±1.20	3.50±0.60
2.5 $mg{\cdot}L^{-1}$ HA	3.15±1.50	1.90±0.05	1.86±0.03	2	2.20±0.50	3.30±1.10	2.90±0.02
20 $mg{\cdot}L^{-1}$HA	12.40±1.0	1.10±1.00	0.03±0.01	2	79.80±5.35	25.80±1.50	1.60±1.15
50 $mg{\cdot}L^{-1}$ HA	64.00±3.80	15.00±1.20	7.40±3.00	2	44.10±0.08	6.20±0.60	5.50±0.08
100 $mg{\cdot}L^{-1}$ HA	44.10±0.0	6.20±0.20	4.90±0.10	2	18.00±0.01	4.90±1.65	4.60±0.60
1 mM $Na_4P_2O_7$	32.5±3.10	2.70±0.90	1.70±0.10	2	1.70±0.45	0.40±0.25	ND
2.5 mM $Na_4P_2O_7$	40.10±5.00	6.50±0.25	2.10±0.80	2	21.50±3.60	3.10±0.50	1.40±0.20
5 mM $Na_4P_2O_7$	110.00±15.70	3.80±2.84	4.50±0.10	2	92.30±3.20	7.20±1.50	3.50±0.60
0.3 % w/v NaH_3PO_4	56.00±0.60	0.13±0.01	ND	2	6.10±0.20	ND	0.30±0.05
	75% Anatasa/25% Rutilo						
50 $mg{\cdot}L^{-1}$ HA	38.90±0.50	9.00±0.20	4.60±0.50	0.2			
50 $mg{\cdot}L^{-1}$HA	23.40±1.00	1.90±0.03	1.50±0.60	2			

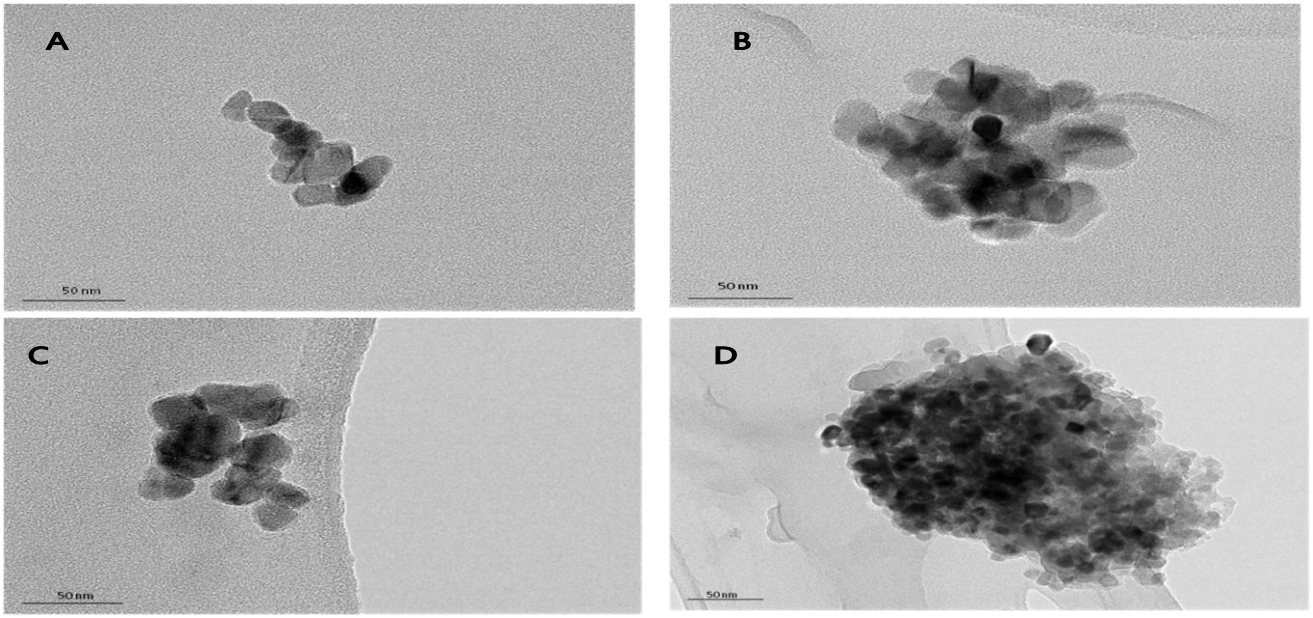

Figure 1. Micrographs obtained by TEM applied to TiO_2NPs (Anatase 75%/Rutile25%). A and B samples were treated in ultrasonic bath; C sample treated with an ultrasonic focused probe; D sample stored at -20ºC and thawed at the time of the analysis.

2.5. Exposure of eleutheroembryos

Eleutheroembryos were obtained from wild type adult zebrafish bred and maintained in the AZTI Zebrafish Facility (EU-10-BI) under standard conditions. All experimental procedures were approved by the Regional Animal Ethics Committee. Bioconcentration studies were carried out at two different concentrations of titanium ions and TiO_2NPs. The nominal concentrations used for the exposure were selected on the basis of Test OECD 305 (OECD, 1996). According to this test, the highest concentration to be tested should be around 1% of the LC_{50} value of the compound and the second concentration should differ by a factor of ten. For bulk TiO_2, data in the literature indicate a 96-h LC_{50} value above 500 $\mu g \cdot L^{-1}$ for *Daphnia magna* (Heinlaan *et al.*, 2008) and a 48-h LC_{50} >20$mg \cdot L^{-1}$ for TiO_2 for the rice fish (*Oryzias latipes*) (METI-NITE, 2006); the nominal concentrations chosen were 0.1 and 1 $\mu g \cdot L^{-1}$. The information on TiO_2NPs in the literature is scarce and the concentrations tested were 2 and 10 $mg \cdot L^{1}$ because in most of studies dealing with fish the reported LC_{50} were 124.5 $mg \cdot L^{-1}$ for fish embryos (Xiong *et al.*, 2011) and >100 $mg \cdot L^{-1}$ for trout (*O. mykiss*) (Warheit *et al.*, 2007).

To characterize the bioconcentration of both chemical forms in the exposure media and compare BCF in each case, an appropriate number of 72 hpf eleutheroembryos (10 individuals for every 20 ml of exposure solution) were transferred to 1 liter tanks filled with ISOwater spiked with two different concentrations of ionic titanium and TiO_2NPs (1 container per concentration) or simply to ISOwater (control). TiO_2NPs were previously treated with 50 $mg \cdot L^{-1}$ of humic acid (HA) to avoid their aggregation in the standard aqueous media, as described above.

Exposures were performed in 1-liter plastic tanks, incubated at 27°C with a 12-hour photoperiod (12 h of light and 12 h of darkness). The exposure test consisted of two phases: a) absorption (48 h in a contaminated exposure medium), and b) depuration (24 h in a clean exposure medium that in this case was embryo water). About 25 eleutheroembryos were removed at different times from the tanks for determination of analyte concentration. According to the OECD Test 305, eleutheroembryo loading rate at the beginning of the experiments should range between 0.7-0.8 $g{\cdot}L^{1}$ (wet weight) and the observed mortality of eleutheroembryos should be lower than 20% at the end of the test. Sampling times were as follows: t0, t3h, t6h, t21h, t45h, and t48h. Additional larvae were left up to 72 hours in the uptake phase and up to 96 hours in the depuration phase for both chemical forms, each at two different concentrations. The results of this extended uptake study was consistent with previous results found in the literature (Zhu *et al.*, 2010b). A significant increase of accumulated titanium concentration after 72 hours of exposure to TiO_2NPs suspended in water was reported by the authors.

2.6. Sample preparation and analysis

To evaluate the role of the capping agents assayed for TiO_2NP aggregation in the ISOwater, total titanium was determined in all dispersions and fractions obtained in the ultrafiltration experiments by FIA-ICP-MS. Ionic titanium in the exposure contaminated media was acidified up to 10% nitric acid to eliminate matrix effects and concentration of the element was determined by ICP-MS. However, TiO_2NP quantification in the exposure media at 50 $mg{\cdot}L^{-1}$ of HA concentration was directly done by FIA-ICP-MS using a standard calibrations suspension of TiO_2NPs. The USP was employed for leaching the analytes from the eleutheroembryos after the incubation period in the exposure media spiked with ionic titanium. Eleutheroembryos incubated in the exposure media spiked with TiO_2NPs were digested with 0.3 µL of HNO_3 (65%), 0.15 µL of concentrated HF (47-51%), 0.15 µL of H_3BO_3 (0.1% w/v), and 0.05 µL of H_2O_2 (35%).

2.7. Toxicokinetics and statistics

To assess bioaccumulation by zebrafish larvae of both chemical forms, bioconcentration factors were determined according to the OECD guideline 305 (OCDE, 1996). Total titanium concentration in both eleutheroembryos and exposure solution at 48 h of accumulation (BCF_{48h}) was quantified according the BCF classic definition (Feijtel *et al.*, 1997). However, when a steady state is not reached, BCF values can also be calculated from a first order two-compartment (water and aquatic organism) model (Spacie *et al.*, 1985) (Gobas and Zhang, 1992), which describes the uptake and depuration process through Eq. 1

$$\frac{dC_f}{dt} = k_1 \cdot C_w - k_2 \cdot C_f$$

(uptake)

$$\frac{dC_f}{dt} = -k_2 \cdot C_f$$

(depuration)

Equation 1

where C_f is the concentration in fish (in ng·g^{-1}), t is the exposure time (h), k_1 is the first-order uptake constant (litre per kilogram dry weight per hour), C_w is the concentration of the compound in the exposure media (ng·mL^{-1}), and k_2 is the first-order elimination rate constant (per hour). Assuming that at t_0, the concentration of the test substance in fish is negligible and the concentration of the tested compound in exposure media is constant, Eq. 2 is obtained:

$$C_f = \frac{k_1}{k_2} \cdot C_w (1 - e^{-k_2 t})$$

(uptake)

$$C_f = C_{f,0} \cdot e^{-k_2 t}$$

(depuration)

Equation 2

Where C_{f0} denotes the analyte concentration in the organism when the depuration phase begins. k_1 and k_2 values can be obtained if the experimentally determined concentration values in the bioconcentration test fit to this equation. When the equilibrium is reached (steady-state), equation 2 may be reduced to equation 3.

$C_f/C_w = BCF_k = k_1/k_2$

Equation 3

The software NONLIN 5.1 was used for kinetic calculations (Sherrod, 1995). Bioconcentration factors were calculated applying the two different procedures to the experimental data, obtaining BCF_{48h} and BCF_k.

3. Results

3.1. TiO_2NP characterization in the exposure media

The capping agents tested for each allotropic structure to avoid NPs aggregation in the ISOwater media used for the bioaccumulation tests are shown in Table 1. Ultrafiltration results revealed that total titanium in fraction 1 was below 20% with most assayed capping agents; i.e., anatase and rutile TiO_2NPs remained in the exposure media as larger particles (>100 nm). Only the $Na_2P_4O_7$ was successful in protecting from TiO_2NP aggregation in both allotropic structures, anatase and rutile. $Na_2P_4O_7$ concentration was optimized to be compatible with larval life. Changing from 1 to 5 mM of $Na_2P_4O_7$increased the fraction of TiO_2NPs as NPs in the ISOwater media from 6 to 98% and from 8 to 92% for anatase and rutile, respectively. However, the mortality rate of zebrafish larvae at 5 mM of $Na_2P_4O_7$ was almost 100% after 24 hours of exposure. Thus, $Na_2P_4O_7$ at 5mM was not selected to for further bioaccumulation studies with TiO_2NPs.

Ultrafiltration results (Table 1) revealed that humic acid (HA) was able to maintain an

important fraction of TiO_2NPs (for both allotropic structures, anatase and rutile) as NPs, with a size <100 nm. Results showed that when HA concentration increased from 2.5 to 50 mg·L^{-1}, the fraction of TiO_2NPs that was able to across the nanofilter (pore size 100 nm) increased from 3 to 64% and from 2 to 44%, for anatase and rutile, respectively. However, an increase of HA concentration to 100 mg·L^{-1} increased the aggregation of TiO_2NPs for both anatase and rutile. Same conditions were evaluated for the 75% anatase/25% rutile mixture at two concentration, 0.2 and 2 mg·L^{-1}. Around 39% and 25% of total TiO_2NPs remained in the exposure media as NPs, <100 nm, at the two NPs concentration tested (0.2 and 2 mg·L^{-1}, respectively).

On the other hand, when the bioaccumulation studies were addressed at 2 and 10 mg·L^{-1} the ultrafiltration results obtained from aqueous samples spiked with 75% anatase/25% rutile showed a slightly higher aggregation than expected. Unlike the previous results, the aqueous samples from the bioaccumulation assays in presence of eleutheroembryos revealed just around 20% of total TiO_2NPs as NPs. These results suggest that the presence of eleutheroembryos could affect the behavior of TiO_2NPs in the exposure media. This finding also indicates that the exposure of eleutheroembryos to TiO_2NPs as aggregates represents a more environmentally realistic exposure scenario and could constitute the basic of different toxic patterns of NPs against ionic TiO_2.Furthermore, TEM results were consistent with those obtained after the ultrafiltration procedure.TiO_2NPs in ISOwater media at 50 mg·L^{-1} of HA appeared as spherical individual particles that formed aggregates (Figure 1). The size of the TiO_2NP aggregates within the solutions ranged between 50 to -300 nm. TEM images also demonstrated how the storage temperature affects the aggregation rate of NPs: significant aggregation of TiO_2NP (larger than 300 nm) was seen when samples were stored at -20°C, while freshly prepared dispersions showed a lower NPs size, below 100 nm (Figure 1).

3.2. Bioconcentration experiments

3.2.1 Concentration in the exposure medium and in eleutheroembryos

Almost similar concentration of total titanium concentration in the exposure media at different sampling times were obtained for both ionic (Figure 2, A and B) and nanoparticles experiments (Figures 3, A and B). These results are in line with the OCDE Guideline 305, where a maximum variation of up to 20% from nominal concentration is allowed. Total titanium concentration in the exposure media used during the depuration phase was negligible. The total amount of titanium accumulated by eleutheroembryos in the experiment with ionic titanium increased with the exposure time until reaching a steady state of around 7673±889 ng·g^{-1} (wet weight) at 48 hours of exposure, and 2931±492 ng·g^{-1} at 92 hours of exposure with an average concentration in the exposure solution of

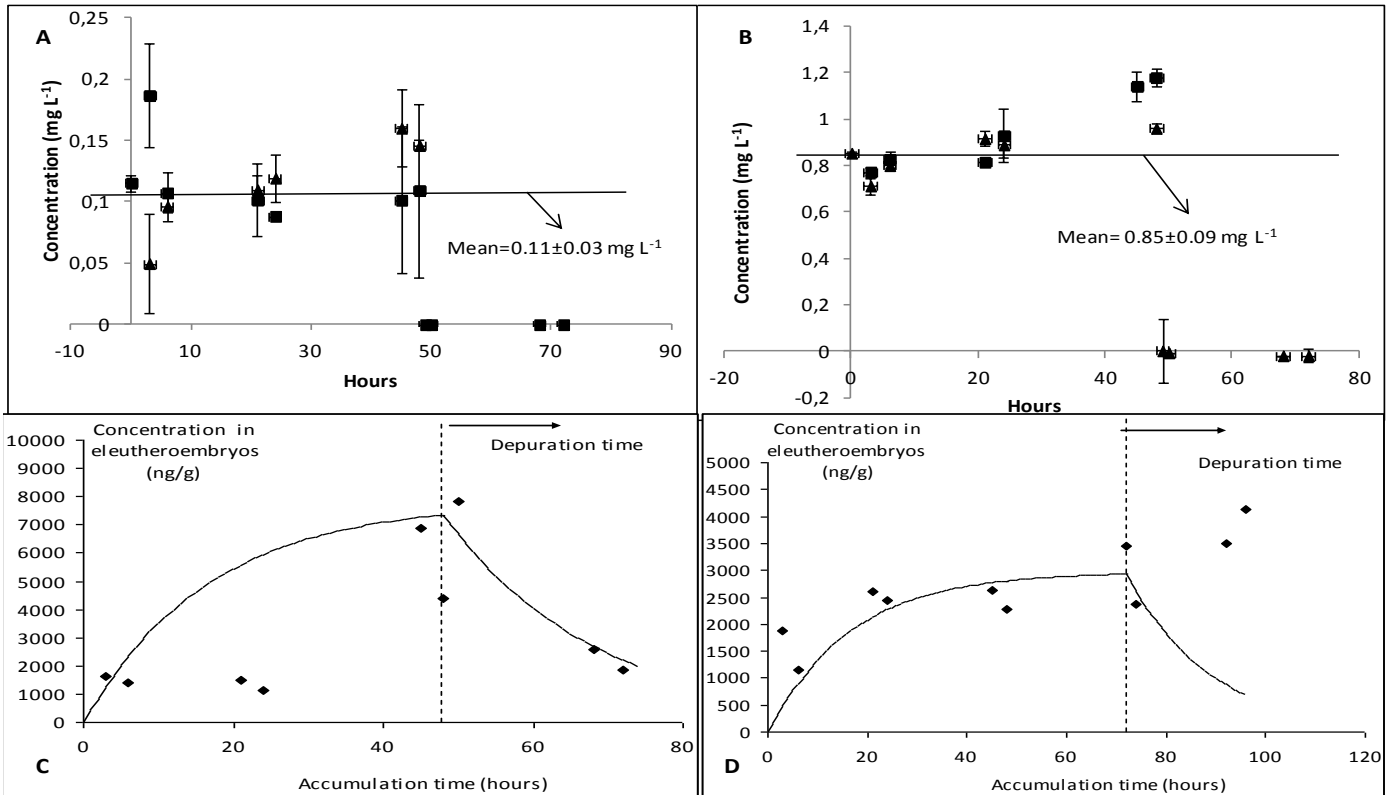

Figure 2. Total titanium content in the exposure medium and eleutheroembryos for the bulk titanium dioxide bioaccumulation experiment. (A) Ionic titanium Nominal concentration of 0.1 mg·L^{-1} in exposure media. (B) Nominal concentration of 1mg·L^{-1} in the exposure media. .(C) Profile of ionic titanium accumulation in eleutheroembryos exposed at 0.1 mg L^{1}; (D) Eleutheroembryos exposed at 1 mg L^{-1}.

0.11±0.03 and 0.85±0.09 mg·L^{-1}, respectively (Figure 2, C and D).

Titanium concentration found in eleutheroembryos during the depuration phase decreased to 2007±116 and 694±119 ng·g^{-1}, respectively in less than 24 h. The titanium accumulated by eleutheroembryos also increased over time when they were exposed to TiO_2NPs, reaching values up to 4959±341 and 26926±103 ng·g^{-1} at 72 hours when the TiO_2NPs average concentrations were 1.8±0.2 and 9.1±2.1 mg·L^{-1}, respectively (Figure 3, C and D). Titanium concentration accumulated by eleutheroembryos decreased to 2040±103 and 8744±290 ng·g^{-1}, values below the concentrations accumulated at uptake phase, evidencing that elimination of TiO_2NPs occurred during the depuration phase.

3.2.2. Bioconcentration Factors

Table 2 shows the toxicokinetic values obtained for ionic titanium and TiO_2NPs with both tested concentrations, as well as the concentrations values in the exposure medium (C_w), in eleutheroembryos (C_f), the uptake and depuration rate constants (k_1, k_2), and the bioconcentration factors (BCF_{48h} and BCF_k).

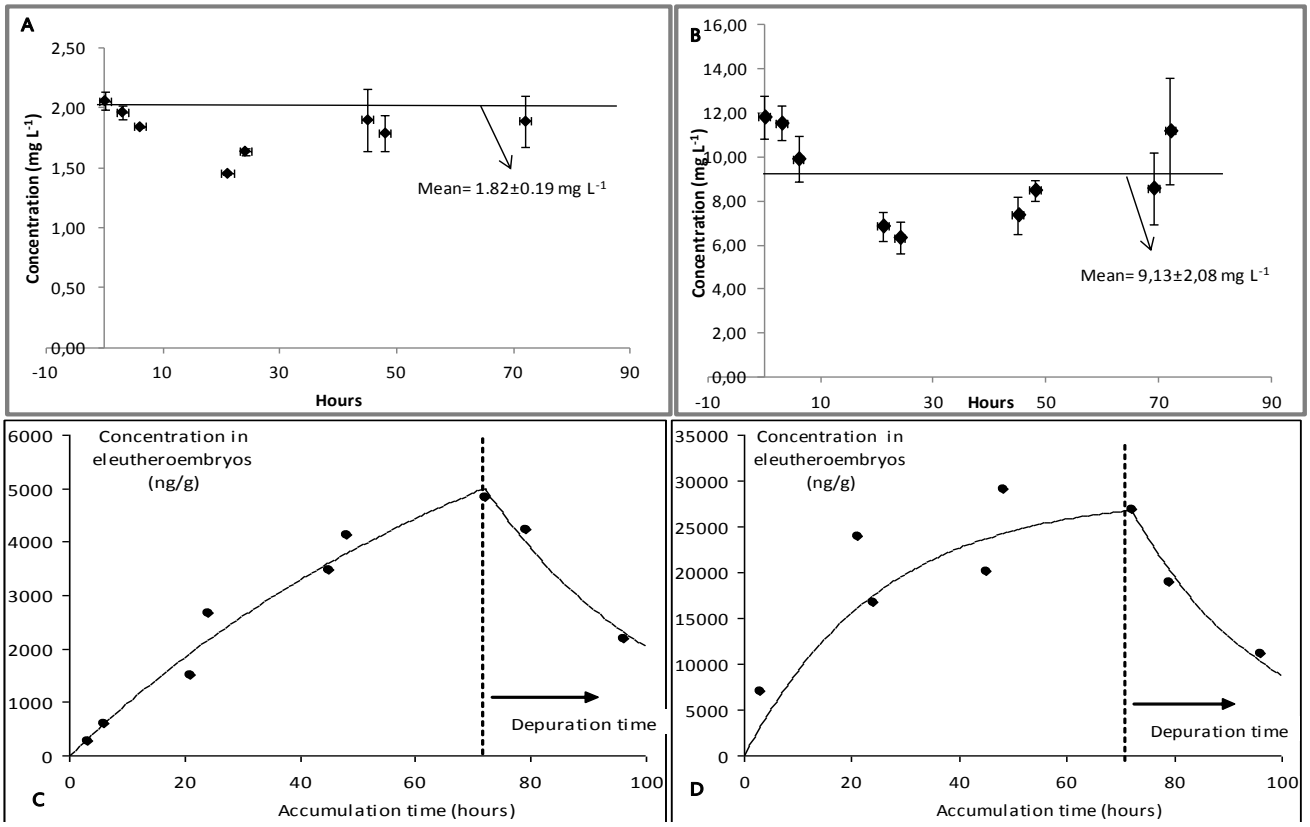

Figure3. Total titanium content in the exposure medium and eleutheroembryos for the TiO_2NP (75%anatase+25% rutile) bioaccumulation experiment. (A) TiO_2NPs concentration of 2 mg·L-^{1} in exposuremedia. (B) Nominal concentration of 10 mg·L^{-1}in the exposure media. (C) Profile of ionic titanium accumulation in eleutheroembryos exposed at 2 mg·L-1; (D) eleutheroembryos exposed at 10 mg·L^{-1}

Bioconcentration factors obtained for all the experiments highlight a non-important bioconcentration by zebrafish eleutheroembryos for either chemical species (BCF values of 77 and 3 at 0.11±0.03 and 0.85 0.09 mg L^{-1} for ionic titanium and 4.3 and 3 at 1.82±0. 19 and 9.13±2.08 for TiO_2NP mg L^{-1}).

4.Discussion

4.1. Characterization of TiO_2NPstability in the exposure media.

Ultrafiltration results revealed the high tendency of TiO_2NPs to aggregate in most of the tested aqueous media. There is an interesting controversy regarding the role of NP aggregation on toxicity (Auffan *et al.*, 2009; Maurer-Jones *et al.*, 2013).

Aggregated TiO_2NPs can be less mobile and interact with filtering and sediment-eating

organisms, or even with suspended organic matter. Here, several capping agents, commonly used in the synthesis and stability of NPs to prevent TiO_2NP aggregation, were tested to form more stable colloidal solutions and facilitate the exposure to aquatic organisms. Some groups studying TiO_2NP behavior have concluded that TiO_2NPs become larger (>100 nm) after their release into aqueous media (French *et al.*, 2009; Von de Kammer *et al.*, 2010; Xiong *et al.*, 2011; Zhu *et al.*, 2010b). Our results showed that by adding 5 mM $Na_2P_4O_7$ all TiO_2NPs were present as ultrafine NPs in standard ISOwater media. To our knowledge, this is the first time TiO_2NPs are shown to remain as NPs (NP<100nm) over 72 hours in a simulated natural media. However, the addition of this salt ($Na_2P_4O_7$) to ISOwater was not compatible with the early stages of zebrafish and other simulated environmental conditions were evaluated in order to ensure their survival. The addition of 50 $mg{\cdot}L^{-1}$ of humic acids (HA) (usually present in natural waters) maintained an important percentage of total TiO_2NPs as ultrafine NPs for anatase and rutile. HA decreased TiO_2NPs aggregation by increasing the electrostatic repulsion of anatase and rutile NPs (Phenrat *et al.*, 2010). However, the aggregation rate increased with the 75% anatase/25% rutile mixture in ISOwater at 50 $mg{\cdot}L^{-1}$ of HA. It could be hypothesized that the combination of allotropic structures resulted in a higher aggregation because there were more interactions between particles, preventing the binding of HA to the surface of the NPs.

In this study, it has been shown that TiO_2NPs under simulated realistic environmental conditions have a high tendency to form aggregates larger than 100 nm in presence of natural organic matter, which is in line with other studies (Sillanpää *et al.*, 2011; Lin *et al.*, 2012). Lin *et al.* (2012) reported that the hydrodynamic size of TiO_2NPs decreases with increased NP surface-bound HA concentration, determining that the lowest TiO_2NP hydrodynamic diameter was 510±20 nm at the highest tested HA concentration, 1500 $mg{\cdot}L^{-1}$. Li and Sun (2011) found a high degree of TiO_2NP agglomeration with a nominal size of 30 nm in aqueous suspensions. TiO_2NPs were in aqueous suspensions as micron-sized aggregates of 1514±131, 1342±23, and 1152±32 nm at pH 4, 6, and 8, respectively. TiO_2NP sizes decreased with increasing concentrations of humic substances (fulvic acids) at the same pH values (4, 6, and 8). The smallest NP sizes were detected in presence of 5 $mg{\cdot}L^{-1}$ fulvic acids, which were 424±22, 372±1, and 332±1 nm at pH 4, 6 and 8, respectively (Li and Sun, 2011).

Furthermore, the presence of larvae favors TiO_2NP aggregation in standard aqueous media. The presence of aquatic organisms modifies the bioaccessibility of chemicals from aqueous media as pointed by other authors, who found that *Daphnia magna* had an effect on the aggregation of natural colloid minerals (Filella *et al.,* 2008). This indicates the importance of monitoring NP size distribution during toxicological studies. Johnson *et al.* also observed that metallic oxide NPs formed

aggregates and precipitated out of solution in presence of fish, decreasing their bioavailability (Johnston *et al.*, 2010). Furthermore, the presence of aquatic organisms in the aqueous media could decrease the fraction of HA available to bound to the surface of the TiO_2NPs. HA may attach to the surface of the larvae, which would lead to an increase of the aggregated TiO_2NP fraction. These TiO_2NP aggregates could set

Table 2. Toxicokinetic parameters and bioconcentration factors (BCF_{48h}, BCF_k) obtained from the adjustments of the experimental data.

	Ionic Ti		TiO_2NPs	
C_w ($mg \cdot L^{-1}$)	0.11±0.03	0.90±0.10	1.8±0.2	9.1±2.1
C_f ($ng \cdot g^{-1}$)	7367	2931	5000	26802
k_1 ($mL \cdot ng \cdot h^{-1}$)	4.6	0.2	0.051	0.12
$k_{2\ (acum)}$ ($ng \cdot mL^{-1}$)	0.06	0.06	0.012	0.04
$k_{2(dep)}$ ($ng \cdot mL^{-1}$)	0.05	0.06	0.032	0.04
BCF_{48h}	73	3.3	2.5	2.9
BCF_k	77	3.3	4.2	3.0

and deposit at the bottom of the tank decreasing their bioaccessibility to aquatic organisms. This fact emphasizes the importance of understanding the behavior and fate of NPs on the bioavailability/bioaccesibility to aquatic organism.

Predicting NP chemistry in the environment is quite difficult. Many factors contribute to their stability and behavior: structure, composition, and surface chemistry of the particles, water chemistry (pH, ion concentration, ionic strength, redox chemistry, type and concentration of organic natural matter, and temperature). Here, simulated realistic conditions (standard ISOwater media, with a salt composition similar to that found in natural waters, 50 $mg \cdot L^{-1}$ of HA, and the presence of zebrafish eleutheroembryos) used led to generate TiO_2NPs larger than 100 nm. Conditions of this more realistic scenario should be considered to assess the toxicity of NPs to aquatic organisms in the environment.

4.2 Titanium bioaccumulation by zebrafish eleutheroembryos

This study has shown that titanium from ISOwater media spiked with ionic titanium and TiO_2NPs was bioaccumulated by zebrafish

eleutheroembryos over time. Furthermore, zebrafish eleutheroembryos were able to eliminate the accumulated titanium during the depuration phase. This tendency was similar with all tested concentrations for both chemical forms, except for eleutheroembryos exposed to 0.11±0.03 $mg \cdot L^{-1}$ of ionic titanium. The BCF values (BCF<75) obtained in this work

evidenced that there is not significant bioaccumulation by zebrafish eleutheroembryos of neither chemical forms.

It is worth noting that some of the obtained experimental data fitted poorly with the bioaccumulation model (Figure 2C and 2D). Data regarding the titanium accumulated by

Table 3. BCF values reported in the literature for ionic titanium and TiO_2 NP in aquatic organisms

Biological species	Compound	BCF	Reference
Adult carp *(Cyprinus carpio)*	TiO_2	<1.1-9.6 (2.0 mg·L^{-1}) <10 (0.2 mg·L^{-1})	METI-NITE, 2006
Adult Zebrafish (*Danio rerio*)	Bulk TiO_2	7 (1.0 mg·L^{-1})	Ramsden *et al.*, 2013
Eleutheroembryos zebrafish (*Danio rerio*)	Ionic Titanium	76 (0.1 µg·L^{-1}) 3.3 (0.85 µg·L^{-1})	This work
Adult carp *(Cyprinus carpio)*	TiO_2NP 80% anatase-20% rutile (21 nm)	495 (dry weight) (10.0 mg·L^{-1})	Sun *et al.*, 2006
Adult carp *(Cyprinus carpio)*	TiO_2NP 80% anatase-20% rutile (21 nm)	325 (dry weight) (10.0 mg·L^{-1})	Zhang *et al.*, 2007
Rainbow trout (*Oncorhynchus mykiss*)	TiO_2NP 80% anatase-20% rutile (21 nm)	No accumulation	Federici *et al.*, 2007
Adult carp *(Cyprinus carpio)*	TiO_2NP 80% anatase-20% rutile (21 nm)	617 (dry weight) (10.0 mg·L^{-1})	Sun *et al.*, 2009
Rainbow trout (*Oncorhynchus mykiss*)	TiO_2NP 80% anatase-20% rutile (21 nm)	No accumulation	Scown *et al.*, 2009.
Daphnia magna	TiO_2NP 80% anatase-20% rutile (21 nm)	56563 (0.1 mg·L^{-1}) 118063 (1.0 mg·L^{-1})	Zhu *et al.*, 2010a
Adult Zebrafish (*Danio rerio*)	TiO_2NP 80% anatase-20% rutile (21 nm)	25.38 (0.1 mg·L^{-1}) 181.38 (1.0 mg·L^{-1})	Zhu *et al.*, 2010b
Adult Zebrafish (*Danio rerio*)	TiO_2NP 80% anatase-20% rutile (21 nm)	4.8 (0.1 mg·L^{-1}) 7.2 (1.0 mg·L^{-1})	Ramsden *et al.*, 2013
Eleutheroembryos zebrafish (*Danio rerio*)	TiO_2NP (<25nm)	4.25 (1.82 mg·L^{-1}) 3.0 (0.9 mg·L^{-1})	This work

eleutheroembryos over the first hours for the assay with 0.1 µg·L^{-1} (Figure 2C) and the depuration time for the assay with 1.0 µg·L^{-1} (Figure 2D) distorted the model distribution, so these data were not considered in the overall analysis in order to obtain more consistent results from both experimental trials. BCF values obtained in our experiments are in agreement with the few previous studies published: the METI-NITE Japanese database listed BCF values below 1.1-9.6 and 10 when the species carp *Cyprinus carpio* were exposed to 2 and 0.2 mg·L^{-1} of dissolved titanium dioxide, respectively (METI-NITE, 2006). In another study, adult zebrafish (*Danio rerio*) were exposed to 1 mg·L^{-1} of bulk TiO_2 and a

BCF of 7.2 was obtained (Ramsden *et al.*, 2013). Contrasting BCFs values can be explained because different titanium species were used in the experiment (metallic titanium in our case and TiO_2 for the METI-NITE studies), and to the lower concentration used in our study. The existence of an inverse relationship between the BCFs and the exposure concentration has also been reported (McGeer *et al.*, 2003; Cuello *et al.*, 2012). Accumulation mechanisms are dependent of the nature of the tested compound: while passive diffusion across the lipid bilayer of biological membranes, as predicted by Fick´s Law, is the main process for neutral organic substances, metals of low lipophilicity are accumulated in biota differently than neutral organic molecules. The uptake of metals occurs by complex dynamics (specific channels in the cell membrane, active transport, or endocytosis), are stored in detoxified forms, such as inorganic granules or bound to metallothionein-like proteins, active elimination, etc. (Simkiss and Taylor, 1989). Other authors pointed out the importance of the composition of the surrounding media (salt content, pH, etc) during metal accumulation (Komjarova and Blust, 2009). Bioconcentration factors do not distinguish between essential mineral nutrient, normal background metal concentration, the capabilities of animals to vary uptake and elimination depending on exposure concentrations, nor the specific ability to sequester, detoxify, and store internalized metals from metal uptake that results in adverse effect. All these facts explain why BCFs values are dependent on the exposure concentration (McGeer *et al.*, 2003).

Our experiments with NPs were carried out using a 72-hour accumulation period (common time for an accumulation test), since previous experiments have shown a non-stationary stage using only 48 hours for accumulation. This same effect was found by other authors for *Daphnia magna* exposed to TiO_2NPs, finding that TiO_2NPs exerted minimal toxicity to *Daphnia* within the traditional 48-hour exposure time, but caused high toxicity when the exposure time was extended to 72 hours (Zhu *et al.*, 2010a). The BCF values for TiO_2NPs in the literature are quite different, from 118063 to no accumulation (Scown *et al.*, 2009) (Federici *et al.*, 2007) (Table 3). Data obtained for *Daphnia magna* (Zhu *et al.*, 2010a) were quantitatively very different from our results, since this species is a crustacean and the uptake and depuration process can substantially differ from that of fish. Reported bioaccumulation data for adult carp ranging 325-617 was obtained on dry weight basis, (78-80% water content (FAO, 2013), these BCF values should be corrected for proper comparison. Thus, two major sets of data for BCF values, one around 100 and another group around 1 or no accumulation have been reported.

The low BCF could be justified for the low bioaccessibility of TiO_2NPs to zebrafish eleutheroembryos due to the presence of humic acids. Lin *et al.* showed that HA bound to the surface of TiO_2NPs prevented the adhesion

of TiO_2NPs to the algal cells due to increased electrosteric repulsion (Lin et al., 2012). As we have stated before, composition of media can affect the size of the NPs, and consequently their bioacessibility and bioaccumulation. In summary, more effort should be put to assess the role of NP physical and chemical properties on fish muscle and/or organ bioconcentration.

5. Conclusions

Here we have shown that bioconcentration tests using zebrafish eleutheroembryos are adequate to evaluate dissolved titanium bioconcentrations. Therefore, this model is a good alternative to the OCDE Bioconcentration Test Nº 305, which requires many adult fish, implying high costs as well as complex, time-consuming experiments. The BCFs values obtained for dissolved titanium are in good agreement with those previously established using the OCDE 305 guidelines. Furthermore, the results obtained in the present study show a low and similar bioconcentration of TiO_2NPs in zebrafish eleutheroembryos, in line with the few data published to date on NPs bioaccumulation. Still, the available information on NPs accumulation is still confusing and the results of the different studies are quite difficult to compare, indicating that many important factors affecting the toxicity of NPs are not fully understood yet. The obtained data indicate the need for new standardized ecological accumulation test protocols, taking into account the special physical and chemical properties of NPs. Additionally, new methods for rapid detection of NPs in fish tissues and NPs characterization along these ecotoxicological tests should be developed. Once this is established, further studies should be carried out in order to understand and predict the real impact and hazards of NPs to health and environment. Anyhow, to begin with, new standardized ecological accumulation test protocols should be designed, taking into account the physical and chemical properties of NPs.

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Does water chemistry affect the dietary uptake and toxicity of silver nanoparticles by the freshwater snail Lymnaea stagnalis?

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Abstract

Silver nanoparticles (AgNPs) are widely used in many applications and most likely released into the aquatic environment. There is increasing evidence that Ag is efficiently delivered to aquatic organisms from AgNPs after aqueous and dietary exposures. Accumulation of AgNPs through the diet can damage digestion and adversely affect growth. It is well recognized that aspects of water quality, such as hardness, affect the bioavailability and toxicity of waterborne Ag. However, the influence of water chemistry on the bioavailability and toxicity of dietborne AgNPs to aquatic invertebrates is largely unknown. Here we characterize for the first time the effects of water hardness and humic acids on the bioaccumulation and toxicity of AgNPs coated with polyvinyl pyrrolidone (PVP) to the freshwater snail Lymnaea stagnalis after dietary exposures. We show that bioaccumulation and toxicity of Ag from PVP-AgNPs ingested with food are not affected by water hardness and by humic acids. Snails efficiently assimilated Ag from the PVP-AgNPs mixed with

diatoms (Ag assimilation efficiencies ranged from 82 to 93%). Rate constants of Ag uptake from food did not vary among the different water hardness and in the presence of humic acids, demonstrating the lack of effect of water chemistry on the dietary uptake of Ag by snails. These results suggest that correcting regulations for water quality could be irrelevant and ineffective where dietary exposure is important.

Keywords: Silver nanoparticles, Dietborne uptake, Bioavailability, freshwater invertebrate, water chemistry

Introduction

Nanotechnology is a new and fast emerging field. Nanomaterials are now used in multiple applications, including biomedicine (Brannon-Peppas and Blamchelette 2004), bioremediation (Grieger et al 2010) and electronics (Henini and Bugajski 2005). They are increasingly incorporated in numerous consumer products such as cosmetics, paints, and in food packaging (Nel et al 2006). As a result, their production is steadily growing (Maynard 2006). Yet, concerns remain about the risk they may pose to the health of the environment. Products containing silver nanoparticles (AgNPs) are numerous. For example, AgNPs are employed in a wide variety of consumer products including socks, bandages, drugs and detergents, (www. Nanotechproject.or/inventories/consumer) due to their antibacterial properties (Cho et al., 2005). Considering the possible dissolution of AgNPs in the presence of oxygen (Liu and Hurt 2010) and the toxicity of ionic silver to bacteria (Charley and Bull 1979), the increased production of AgNPs poses concerns for environment safety since discharge into the environment, of at least some of the produced AgNPs, is unavoidable.

Once in the environment, AgNPs are subject to transformations that can affect their stability, behavior and ultimately, their fate (Kumar et al 2010; Levard et al 2012; Lowry et al 2012). For example, AgNPs may form complexes with ligands, agglomerate, aggregate and sediment. Furthermore, their interaction with many inorganic (e.g., SO_4^{-2}, Cl^-, and S^{-2}) and organic (e.g., carboxylic acids and humic substances) ligands can enhance or reduce their toxicity to aquatic organisms. For example, Choi et al (2009) demonstrated that the presence of sulfides reduced nanosilver toxicity to nitrifying bacteria by up to 80%. However, increased sulfide concentrations also tended to gradually increase toxicity

because the available sulfide inhibited bacterial activity (Choi et al 2009). In addition, the organic constituents of aquatic ecosystems can increase the residence times of AgNPs (Cumberland and Lead 2009; Tejamaya et al 2012; Hitchman et al 2013). Natural organic macromolecules such as humic acids can also induce the formation of AgNPs under environmentally realistic conditions (Akaighe et al 2011; Cumberland and lead, 2013). Because they can act as a physical barrier, preventing the attachment of nanoparticles to organisms, increased concentration of humic substances can decrease the toxicity of AgNPs (Fabrega et al 2009, 2011. Gao et al 2012). Clearly, the chemical composition of an aquatic system influences the bioavailability and the toxicity of AgNPs.

Capping agents (surface coatings) influence the chemical behavior of AgNPs, thereby affecting their stability (Tejamaya et al. 2012). For example, surface coatings can control the bioavailability of AgNPs by reducing the amount of Ag^+ released, (Gao et al 2012). Polymer coated AgNPs are completely different in nature from uncoated nanoparticles, and reactivity varies among surface coatings (Tejamaya et al. 2012). Among the most common capping agents used for AgNPs are citrate and humic substances (Litvin et al 2012). Both stabilize AgNPs by charge repulsion. Polyethylene glycol (PEG) and polyvinyl pyrrolidone (PVP) are also used as capping agents and sterically stabilize AgNPs (Cumberland et al 2009; Romer et al 2011). In contrast to citrate, PVP is strongly bound to the core Ag.

Most ecotoxicological studies on AgNPs have been conducted with AgNPs (or $AgNO_3$) added to media or to water. Virtually nothing is known about the effect of water chemistry on the dietary bioavailability and the toxicity of AgNPs. Here, we describe the bioaccumulation and toxicity of Ag after dietary exposure to environmentally relevant concentrations of AgNPs coated with polyvinilpirrolidone (PVP) to the freshwater snail *Lymnaea stagnalis.* We specifically evaluate the effects of water hardness and the presence of humic acids. We use a biodynamic model to characterize the important physiological processes governing the dietary uptake of Ag from AgNPs exposure by *L. stagnalis* (Luoma and Rainbow 2005, Croteau et al. 2011a). To our knowledge, this the first study that evaluates the influence of water chemistry on the dietborne uptake of Ag after AgNPs exposures.

EXPERIMENTAL SECTIONS

Silver Nanoparticles

Silver nanoparticles were synthesized, as described in Tejamaya et al 2012. Briefly, PVP-stabilized AgNPs were prepared by reducing 60 mL of 1mM silver nitrate (1 mM) with 180 mL of 2 mM sodium borohydride (>99% purity, Sigma Aldrich) in the presence of PVP10 (Mw 10000, Sigma Aldrich). Stirring was stopped after the addition of $AgNO_3$ and the suspension was left overnight. The particle suspension was cleaned at least three times using a 1 kDa regenerated cellulose membrane and a diafiltration method to remove the excess reactants but preventing drying and subsequent aggregation and oxidation (Römer et al 2011, Cumberland et al 2009). The original concentration and volume were maintained by replacing the removed filtrate with solutions of citrate or water as appropriate.

The AgNPs were analyzed by field flow fractionation (FFF) and dynamic light scattering (DLS). The z-average hydrodynamic size for the PVP-AgNPs (4 ppm) diluted in moderately hard synthetic freshwater (Table S1) and incubated for 24 h was approximately 36 ± 1 nm and zeta potential for the particles was -9.7 ± 0.9 as measured by DLS. The hydrodynamic size measured by FFF was 32 ± 0.7 nm. Additional characterization data is included in the Supplemental Information (Table S2,S3).

Experimental Media

Synthetic freshwater of different hardness was prepared according to the US EPA (2002). The waters tested were very soft (VS), moderately hard (MOD); and very hard (VH), representing water hardness ranging from 10 -800 mg L^{-1} of $CaCO_3$, respectively (Table S1). To study the influence of humic acid (HA) on the uptake of Ag from dietborne PVP-AgNPs exposures, six different concentrations of Suwannee Humic Acid II (International Humic Substances Society, www.humicsubstances.org) were prepared in MOD water. The concentrations of HA used were chosen to be environmentally relevant, i.e., concentrations were 0, 0.25, 1, 2.5, 5 and 10 mg/L (Akaighe et al 2011).

The biodynamic model

To assess the influence of water chemistry on the dietary uptake of AgNPs by *L. stagnalis,* we used a biodynamic model (Luoma and Rainbow 2005) that deconstructs metal bioaccumulation into its mechanistic components. In general, metal accumulation from food by an organism ($[M]_{organism}$), could

be expressed as a balance between uptake and loss rates, i.e.,

$$[M]_{organism} = k_{uf} \times [M]_{food} - k_e \times [M]_{organism} \quad (1)$$

where k_{uf} is the unidirectional metal uptake rate constant from food (g g^{-1} d^{-1}), $[M]_{food}$ is the dietborne metal concentrations (nmol g^{-1}) and k_e is the rate constant of metal loss (day^{-1}). The influence of body growth dilution can be considered negligible because the duration of the experiments was much shorter than the life-span of the experimental organisms.

Specifically, the silver uptake rate constant from food (k_{uf}) can be determined from the slope of the linear regression between Ag influx into the snail's soft tissues and the dietary Ag exposure concentrations (linear portion of the curve). Silver uptake from food can also be characterized by the Ag assimilation efficiency (AE, unitless) and the food ingestion rates (IR in g g^{-1} d^{-1}). Each can be estimated from a mass-balance of Ag recovered in the snail's soft tissues (Ag_{snail} in ng) and in their feces (Ag_{feces} in ng) after 48 h depuration. Specifically, AE of Ag for each experimental organism can be calculated using equation 2, whereas food IR can be determined using equation 3,

$$\text{Ag AE} = \frac{Ag_{snail}}{Ag_{snail} + Ag_{feces}} \times 100 \quad (2)$$

$$IR = \frac{(Ag_{snail} + Ag_{feces})}{[Ag]_{diatoms} \times wt_{snail} \times T} \quad (3)$$

where $[Ag]_{diatoms}$ (ng g^{-1}) is the measured Ag concentration in the diatoms mixed with the AgNPs, wt_{snail} is the snail's dry weight (g) and T is the exposure duration (day).

Experimental organisms

The freshwater snails, *L. stagnalis*, were reared in the laboratory in MOD water. Three days prior to each experiment, snails of a similar size range (mean soft tissue dry weight of 3.7 mg ± 0.9 S.D., n=160) were transferred to a 10 L glass aquarium filled with the specific synthetic freshwater for acclimatization (Table S1, Supporting Information). Food was withheld during this period.

The background Ag concentration was estimated for each snail based on their weight using the slope for the linear relationship between the Ag burden in 70 unexposed snails and their dried weight (Croteau et al. 2011a). Using this relationship, Ag background was subtracted from measured Ag concentration for each experimental group.

Dietborne uptake experiments

To study the influence of water chemistry on the dietborne uptake of Ag from AgNPs into

L. stagnalis, we characterized Ag uptake rates after exposure to AgNPs and in the presence or absence of HA. We used the benthic diatom *Nitzschia palea* as a food source. Diatoms were grown axenically for several generations in an S-diatom medium (Irving et al. 2003). They were harvested onto 1.2-µm Isopore™ membrane filters (Millipore) and rinsed with SOFT water (Table S1) to make algal mats. We employed the protocol described by Croteau et al. (2011b) to present the algae in a form the snails would ingest. Briefly, we serially diluted suspensions of AgNPs which were poured onto algal mats and filtered using low vacuum (< 10mm Hg) to deposit particles. Small sections of the filters holding the diatoms amended with AgNPs were sampled and dried for 24 h at 40 °C prior to silver analysis.

At each exposure concentration, 8 acclimated snails were exposed to diatoms amended with AgNPs for 2-4 h. Exposure was shorter than gut residence time (Croteau et al. 2007), which minimized the confounding influences of efflux and metal recycling. The short exposures also allow determining food ingestion rates and Ag assimilation efficiency. Snails were exposed to the amended food in 150-mL acid-washed polypropylene vials that were partially submerged in a 40-L glass tank filled with 3 L of either VS, MOD, VH water (Table S1) or MOD water spiked with different concentrations of HA. The animals were allowed to ingest a bolus of food amended with AgNPs. The snails were then removed, rinsed, placed individually in acid-washed enclosures and fed "unspiked" food (lettuce) *ad libitum* for 48 h in either VS, MOD, VH water or MOD water spiked with

Table 1. Mass balance of filtration assays

Filter size (µm)	AgNP Added (ng)	RECOVERY (ng)		RECOVERY (%)		
		Diatoms	Filtrate	Diatoms	Filtrate	Total
1.2	4.5	1.5	2.5	34	56	**90**
1.2	41.3	11.5	27.6	28	67	**95**
1.2	4.6	0.6	3.6	13	78	**92**
1.2	46.6	2.2	32.1	5	69	**74**
1.2	118	18	96.1	15	81	**96**
0.1	4.2	2.7	1.6	66	38	**103**
0.1	39.9	31.1	9.4	78	24	**101**
0.1	130	95.4	28.5	74	22	**95**

HS. The silver retained after complete gut clearance defined "assimilation" (Wang and Fisher 1999). After this depuration period, snails were removed from enclosures and individually frozen. Feces produced by each snail were collected, placed in acid-washed Teflon vials and dried for 24 h at 40 °C for metal analysis. Aliquots of water were taken immediately after labeled feeding, as well as at the beginning and the end of depuration. Water samples were acidified with concentrated nitric acid.

Optimization of dietary exposure procedure

We investigated whether the pore size of the membrane filters would influence the retention of AgNPs onto the algal mats. For this, we created algal mats using two membrane filter pore sizes (1.2µm and 0.1µm). We then poured 10 ml of diluted solutions of AgNPs onto the algal mats. Total silver concentrations were measured in the original solutions, filtrates and diatom mats. Silver recovery was determined from a mass balance of the amount of Ag poured onto the diatom mats, as well as that recovered on the diatom mats and in the filtrates.

Analytical procedure

To minimize possible metal contamination, labware, vials, and Teflon sheeting were soaked for at least 24 h in acid (15 % nitric acid and 5% hydrochloric), rinsed several times in ultrapure water and dried under a laminar flow hood prior to use.

Partially thawed *L. stagnalis* were dissected to remove soft tissue, placed individually on a piece of acid-washed Teflon sheeting, and left to dry at 40 °C for 3 days. Dried snails, feces, and diatoms were digested in nitric acid following the protocol described in Croteau et al. (2011a). Similar weight samples of the certified reference material DOLT-3 (dogfish liver, National Research Council Canada) were submitted to the same digestion procedure during each analytical run. All samples, blanks and standards were analyzed for the naturally occurring stable isotopes of Ag by ICP-MS (Perkin Elmer, Elan 6000). Two analytical replicates were measured for each sample. A replicate consisted of 15 individual measurements that were averaged. External standards, serially diluted from ultra-pure, single-element stock, were used to create calibration curves for each isotope. To account for instrument drift and change in sensitivity, internal standardization was performed by addition of germanium to all samples and standards but the calibration blanks. We also reanalyzed one of our standards after every 10 samples. Deviations from standard value were less than 5%.

RESULTS AND DISCUSSION

Silver concentrations in diatoms: the influence of filter pore-size

The recovery of Ag in the diatom mats was slightly higher for the smaller pore size filter (Table 1). Specifically, total Ag recovery varied from 74 to 96 % for the 1.2 µm filters, and from 95 to 103% for the 0.1 µm filters. On average, 73% of the added Ag was retained on the diatom mats when 0.1 µm pore-size filters were used, in contrast to 19% when pore-size 1.2 µm filters were used. The fact that more Ag was captured onto 0.1 um filters than on 1.2 um filters suggests that Ag was largely in particulate form.

Influence of water chemistry on dietborne Ag uptake rates

Silver uptake rates in *L. stagnalis* increased linearly with dietary exposure concentrations (Figure 1). The uptake rate constants from food (k_{uf} ± 95% C.I. in g g^{-1} d^{-1}) were 0.30 ± 0.05 for VS, 0.21 ± 0.04 for MOD and 0.24 ± 0.04 for the VH water (Table 2). Silver delivered from the PVP-AgNPs was thus bioavailable to the snails in all three treatments. Water hardness had no detectable effect on delivery of Ag from the AgNPs via diet. Silver uptake rates in snails exposed to dietary concentrations of AgNPs, ranging from 412 to 586 nmol g^{-1}, and in the presence of varying concentrations of HA in the aqueous media, were not significantly different among treatments (Figure 2; $p<0.05$). The k_{uf} values for PVP-AgNPs in *L. stagnalis* are within the range of previous values obtained by Croteau et al. (2011a) for other AgNPs with different capping agents in MOD water: cit-AgNPs and HA-AgNPs (0.10±0.09 g g^{-1} d^{-1} and 0.27±0.12 g g^{-1} d^{-1} respectively).

The waterborne Ag concentrations were low after the dietary exposures (< 0.04 µg/L). We estimated that Ag uptake rates from solution were negligible compared to those from diet, based upon waterborne rate constants derived in previous studies (Croteau et al 2011a).

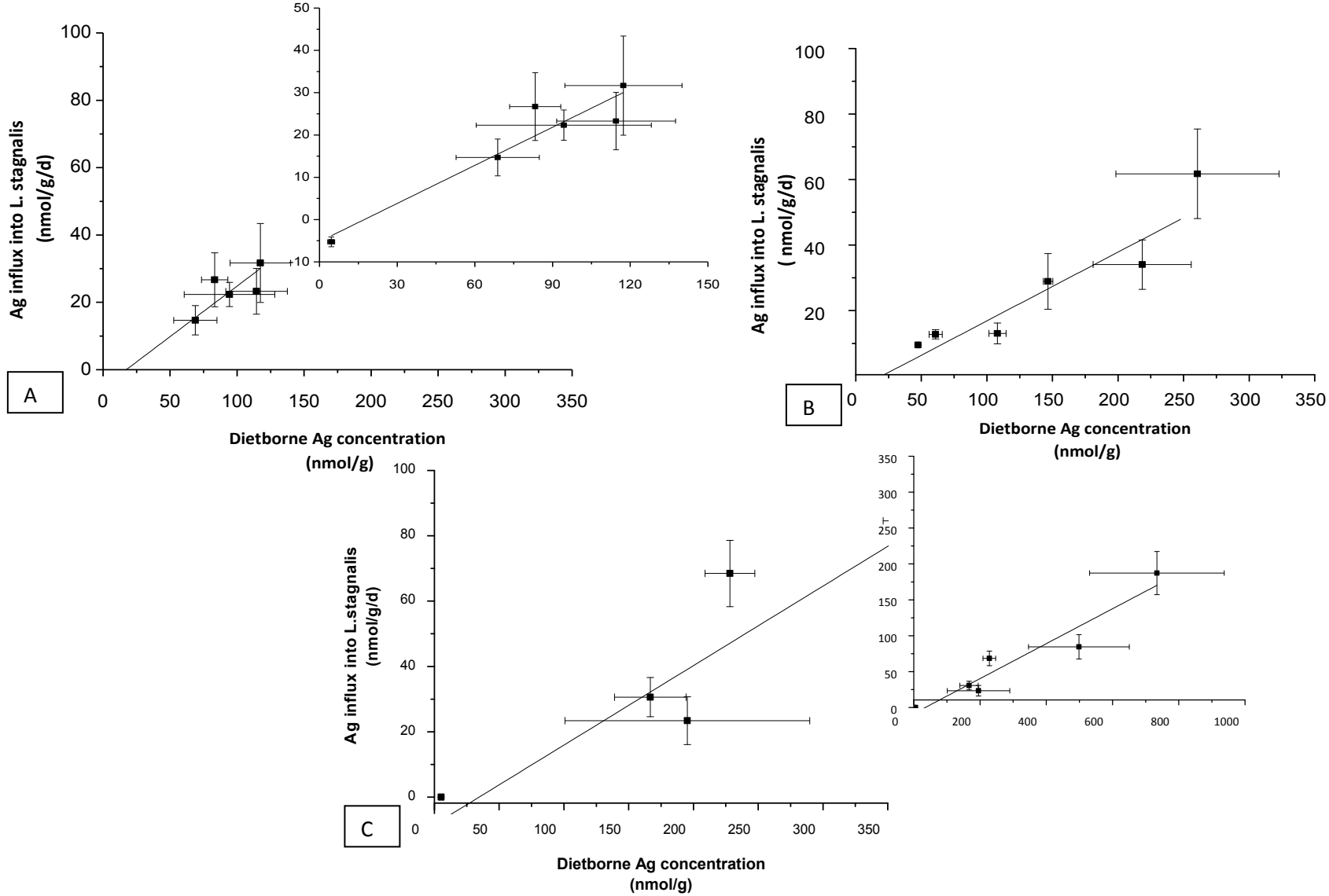

Figure 1. Silver uptake rates (nmol g-1d-1 ±95% C.I.) in L. stagnalis soft tissue after dietborne exposures in very soft water (A), moderately hard water (B) and very hard water (C). Each symbol represents Ag concentrations (from which background Ag was removed) for 8 individuals. Gray lines represent linear regression relationships. Inset in panels A and C shows data a smaller and bigger scale, respectively.

Biodynamic parameters for dietary Ag uptake

Silver was efficiently assimilated from the PVP-AgNPs mixed with the diatoms. The AE values ranged from 84 to 100 % in VS water, from 67 to 92 % in MOD water, and from 90 to 94% in VH water (Table S4, Figure S1). Silver AEs were similar when HA was present in the overlying media (71-85%). Silver AE from the PVP-AgNPs was higher than that reported by Croteau et al. (2011a) for citrate-capped (49±7%) and humic acids-capped AgNPs (58±8%) in the same species. These differences in AEs could be partly attributed to the particle coatings that differ in terms of their stability (Oberdorster et al 2004). For example, Tejamaya et al. (2013) showed that citrate-capped AgNPs are more unstable than PVP-AgNPs. As a result, citrate-capped AgNPs are more prone to aggregation, which reduces their bioavailability.

Food IR ranged from 0.06 to 0.30 g g^{-1} d^{-1} in VS water, from 0.01 to 0.24 g g^{-1} d^{-1} in MOD water and from 0.13 to 0.32 g g^{-1} d^{-1} in VH water (Table S5, Figure S2). Feeding rates were slightly faster in the presence of HA (0.27-0.44 g g^{-1} d^{-1}) (Figure S2). There was evidence of feeding inhibition when snails were fed with PVP-AgNPs in diet (as compared to control snails that feed at a rate of about 0.9 g g^{-1} d^{-1}, Croteau et al. 2011a). However there was not a dose-dependent decline in IR with increasing exposure concentrations, as previously observed for citrate-capped AgNPs (Croteau et al. 2011a). The food IR values for the PVP-coated AgNPs in *L. stagnalis* are similar to that reported for snails exposed to citrate-capped and humic-acid capped AgNPs (Croteau et al. 2011a). All IRs determined for food amended with AgNPs are lower than reported for snails exposed to diatoms pre-exposed to Ag from $AgNO_3$ (Croteau et al 2011a), suggesting a nanomaterial-specific effect. It appears that addition of AgNPs to diet, including PVP-coated AgNPs, either reduced how palatable the food was and/or affects digestive processing.

To summarize, our results showed that there was very little or no impact of water chemistry

Table 2. Biodynamic parameters(±95% C.I.) for PVP-AgNPs by *L.stagnalis* after dietborne in different water hardness and in presence of humic acids[1]

	VSW		MOD		VHW		MOD with HA	
Kuf ($g\ g^{-1}d^{-1}$)	0.30±0.05	(6)	0.21±0.04	(6)	0.24±0.04	(6)	ND	
AE (%)	93±7	(5)	82±11	(5)	90 ±4	(5)	79±5	(6)
IR ($g\ g^{-1}d^{-1}$)	0.19±0.09	(5)	0.15±0.10	(4)	0.23±0.08	(5)	0.36±0.07	(6)

[1]The number of individual replicates samples containing 8 individual snails is in parentheses. ND, not determinated

on uptake of Ag from PVP coated AgNPs from food, although further studies comparing other NPs types are needed. It is often recommended that water quality regulations account for the reduced bioavailability and toxicity as influenced by high major ion concentrations, high concentrations of dissolved organic matter, or the presence of dissolved sulfide. The present study suggests this may apply only to bioavailability and toxicity of waterborne AgNPs. Further, in many cases diet is a more important source of exposure than is waterborne Ag (Croteau et al 2011b; Garcia-Alonso et al 2011). In those cases concentrations of AgNPs in food are the primary driver of bioavailability. It should also be noted that studies have shown that even small additions of AgNPs to food appear to inhibit ingestion rates in aquatic organisms, with implications for growth and energy budgets (Zhao and Wang, 2011; Croteau et al 2011b) . Lower ingestion rates will also affect delivery rates of Ag to snails, which is an important consideration for toxicity.

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Supporting Information for A. López-Serrano [1] et al

Does water chemistry affect the dietary uptake and toxicity of silver nanoparticles by the freshwater snail *Lymnaea stagnalis*?

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8 pages (8 figures, 5 tables)

Table S1.Ionic composition, hardness, pH and alkalinity of water tested from US EPA 2002 U.S. Environmental Protection Agency:

Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms; U.S.

EPA, Washington DC, 2002; EPA-821-R-02-012..

	Reagents added ($mg\ L^{-1}$)				**Approximate Final Water Quality**		
	$NaHCO_3$	**$CaSO_4 \cdot 2H_2O$**	**$MgSO_4$**	**KCl**	**pH**	**Hardness ($mg\ CaCO_3\ L^{-1}$)**	**Alkalinity ($mg\ CaCO_3 L^{-1}$)**
Very Soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	57-64
Very Hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

Characterization Data of PVP-AgNPs

Table S2. Hydrodynamic diameter of PVP AgNPs (by DLS and FI-FFF) and zeta potential. Stock diluted to 4 mg L^{-1} with DI and MOD water were analyzed.

Sample	By DLS		By FI-FFF	Zeta potential
	z-average (nm)	PdI*	Peak maximum (nm)	(mV)
PVP in DI	33 ± 1	0.409 ± 0.01	30.45 ± 2.19	-13.9 ± 0.9
PVP in MOD	34 ± 2	0.487 ± 0.08	30.43 ± 0.74	-9.3 ± 0.6
PVP in MOD (24h)	36 ± 1	0.403 ± 0.01	32.08 ± 0.74	-9.7 ± 0.9

*PdI= polydispersity index

Table S3. Core diameter of PVP particles in MOD measured by TEM.

PVP	Average Core diameter± SD (nm)	Number of particles measured
0.43µM (4 mg L^{-1})	11.3± 2.6	113
75 nM	11.1 ± 2.4	100
50 nM	11.4 ± 2.6	103
25 nM	11.9± 3.1	101

Table S4. Assimilation efficiency (%) for each treatment at different water tested.

Metal AE can be calculated as:

$$\mathbf{AE(\%)}=\frac{Morg}{Morg+Mfeces}\,x\,100$$

where M_{org} (ng) is the accumulated amount of metal in the organism after depuration, $M_{feces\ (}$ng) the amount of metal egested in the feces during depuration, $[M]_{food\ (}$ ng g^{-1}) the metal concentration in the food, wt_{org} (ng)and the organism's dry weight and T (day) the exposure duration

	VERY SOFT WATER			MOD WATER			VERY HARD WATER			MOD WATER IN PRESENCE OF HUMIC ACID		
Treatment												
	AE (%)	**SD**	**n**	**AE (%)**	**SD**	**n**	**AE (%)**	**SD**	**n**	**AE (%)**	**SD**	**n**
T1	100	5	8	67	24	1				71	21	4
T2	84	30	4	79	9	4	90	3	8	79	4	5
T3	95	1	7	75	19	2	84	10	8	82	7	4
T4	91	3	8	89	6	7	93	2	8	80	7	6
T5	100	9	6	88	6	8	91	4	8	76	15	6
T6	100	31	3	92	10	7	94	2	6	85	3	6

T1-T6 corresponding to different assays.

Table S5. Ingestion rate from food (g^{-1} d^{-1}) for each treatment at different water tested.

Food IR (g of ingested food g^{-1} (body tissue) day^{-1}) can be determined during pulse-chase feeding experiment by mass-balance calculations and is calculated as:

$$IR=\frac{(Morg+Mfeces)}{[M]food\times wtorg\times T}$$

	VERY SOFT WATER			MOD WATER			VERY HARD WATER			MOD WATER IN PRESENCE OF HUMIC ACID		
Treatment												
	IR	**SD**	**n**	**IR**	**SD**	**n**	**IR**	**SD**	**n**	**IR**	**SD**	**n**
T1	0.13	0.15	4	0.10	0.11	4	0.20	0.04	8	0.44	0.13	4
T2	0.22	0.10	7	0.01	0.05	2	0.13	0.04	8	0.30	0.12	5
T3	0.30	0.11	8	0.18	0.12	7	0.32	0.05	8	0.36	0.12	4
T4	0.24	0.19	6	0.24	0.30	8	0.19	0.03	8	0.27	0.08	6
T5	0.06	0.13	3	0.24	0.10	7	0.31	0.10	6	0.33	0.12	6
T6										0.44	0.14	6

*IR negative values have been removed from table. T1-T6 corresponding to different assays

FIGURE S1. Silver AE (% ±95% C.I) in snails exposed to diatoms mixed with PVP-AgNPs in Very Soft (a), Moderately Hard (b), Very Hard (c) water and at different HA concentrations (d). Each symbol represents AE for 8-3 individuals (Table S2.) at different PVP-Ag NPs concentrations.

a) **b)** **c)** **d)**

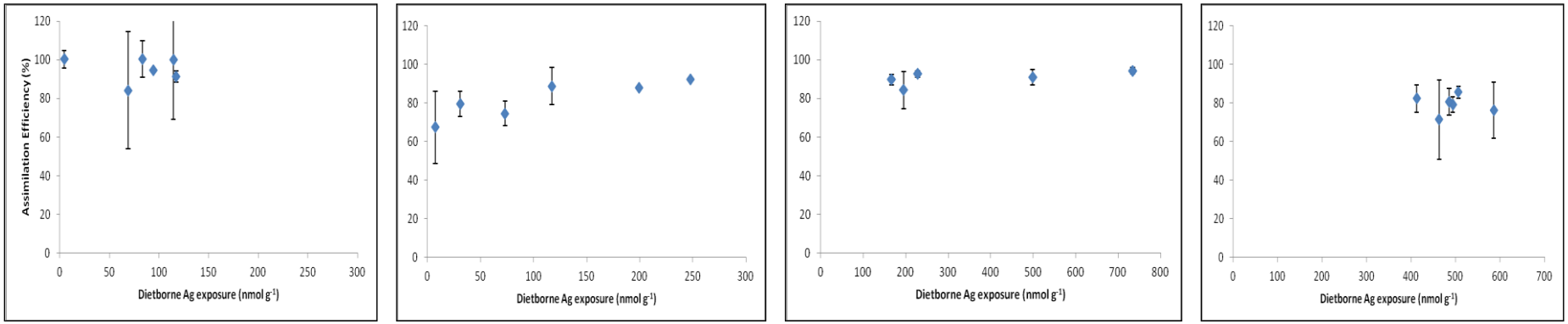
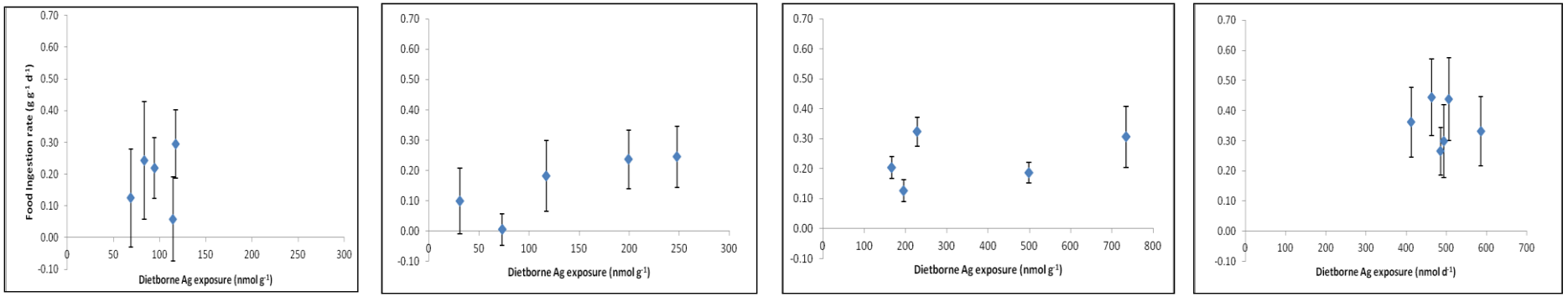


Figure S2. Food Ingestion Rate (IR) (g g $^{-1}$ d^{-1} ±95% C.I) in snails exposed to diatoms mixed with PVP-AgNPs in Very Soft (a), MOD (b), Very Hard (c) and Moderately Hard water at different Humic Acid concentrations (d). Each symbol represents IR (g g $^{-1}$ d^{-1}) for 8-3 individuals (Table S3.) at different PVP-Ag NPs concentrations

a) **b)** **c)** **d)**



5. DISCUSIÓN INTEGRADORA

Modelo alternativo al Test OECD 305

Como se ha comentado en la introducción, los ensayos de toxicidad tradicionales se realizan generalmente mediante la exposición de un sistema biológico (líneas celulares, modelos animales etc.) a la sustancia que se quiere evaluar y una posterior identificación de los efectos provocados por su exposición. Hasta la fecha, la mayoría de ensayos validados requieren experimentos complejos y laboriosos de larga duración, implicando un gran número de animales de experimentación lo que lleva asociado un elevado coste económico. El test de bioconcentración empleado en este trabajo se presenta como ensayo simplificado y alternativo a la Guía de ensayo Test OECD 305 aportando importantes ventajas sobre el mismo. Entre ellas, se incluyen una significativa reducción en el número de sistemas vivos modelo, necesarios en el desarrollo de los experimentos de bioconcentración, así como una importante reducción de la duración del ensayo que, en definitiva, irá asociada a una importante reducción del coste económico.

En un principio, el Grupo de Investigación en el que he llevado a cabo este trabajo inició la investigación sobre la evaluación de los Factores de Bioconcentración (BCF) de sustancias consideradas peligrosas empleando embriones del pez cebra como alternativa al empleo de peces adultos. Sin embargo, la problemática en su manejo y tratamiento analítico de este tipo de muestras biológicas, y principalmente la inconsistencia de los resultados obtenidos con los BCF ya publicados extraídos de Guías de Ensayo normalizadas y validadas en peces adultos, llevaron a descartar esta opción. Por ello se pasó al empleo de larvas (eleuteroembriones) como sistema modelo, para las cuales estudios previos realizados por otros miembros del grupo de investigación demostraron a través de los datos de BCFs encontrados de algunos contaminantes clásicos que eran concordantes con los publicados en la bibliografía utilizando peces adultos y/o otros animales.

Por todo ello, el trabajo desarrollado en la parte experimental se ha centrado en el empleo de larvas como modelo alternativo al uso de animales en investigación para el estudio de los efectos tóxicos causados por la exposición a diversas sustancias químicas que puedan ser consideradas, o se ha demostrado que son peligrosas. Es

importante resaltar que los analitos se han testado individualmente puesto que así lo requiere la validación del test, ya que el empleo de mezclas de contaminantes podría dar lugar a efectos sinérgicos proporcionando resultados y conclusiones erróneas. Los analitos seleccionados fueron arsenito, tributilestaño, plata y titanio (estos dos últimos en su forma iónica y también en forma de nanopartículas de plata y de dióxido de titanio). La discusión integradora se va a realizar en tres bloques diferenciados que forman parte de la presente Tesis Doctoral:

- Desarrollo de metodologías analíticas y su validación frente a las normalizadas para la detección de los analitos seleccionados en muestras biológicas y medioambientales: larvas del pez cebra y medios de exposición.

- Optimización de condiciones apropiadas que mantengan estable la concentración del analito objeto de estudio en el medio de exposición durante la fase de absorción del Test de Bioconcentración propuesto en esta memoria.

- Aplicación de los métodos analíticos desarrollados para la obtención de los factores de bioconcentración de los analitos estudiados y su validación con los datos publicados en la bibliografía.

Desarrollo y optimización de las metodologías analíticas empleadas en la determinación de los analitos de estudio

El cálculo de los factores de bioconcentración requiere determinar la concentración del analito objeto de estudio tanto en el medio de exposición de nuestro sistema modelo como en el tejido de las larvas expuestas. La consecución de dicho objetivo requiere afrontar y resolver previamente problemas analíticos asociados a:

- Complejidad de la matriz (tanto del medio de exposición como de las larvas). La composición del medio de exposición en el que se incubaron las larvas presentaba un alto porcentaje de sales que en la mayoría de los casos se traducía en la presencia de un efecto matriz importante o en la formación de complejos y que era necesario

eliminar o minimizar para la cuantificación del analito en estudio. Por otro lado, el elevado contenido lipídico de las larvas constituía un problema importante en cuanto a la eficiencia del proceso de extracción del analito o del tratamiento de muestra seguido. Por todo ello, se llevó a cabo una optimización y desarrollo del procedimiento analítico en función del tipo de muestra, del analito a determinar así como de la técnica analítica seleccionada para el estudio.

- La baja concentración del analito en los medios de exposición constituyó un segundo aspecto importante a considerar en el desarrollo de la metodología analítica. Como se ha descrito en la introducción, de acuerdo a la Guía de Ensayo OECD 305, la concentración máxima de estudio no debe ser superior al 1% del LC_{50}. Considerando que los ensayos se realizan a dos concentraciones diferentes que deben diferenciarse en un factor de 10 nos encontramos con una concentración del 0.1% del LC_{50}, que puede estar próximo al límite de detección de las técnicas analíticas empleadas en la detección.

- El reducido tamaño de las larvas constituyó otro problema adicional ya que la cuantificación de analitos en aquellos casos en los que los BCFs no sean elevados, requiere de técnicas sumamente sensibles con unos límites de cuantificación adecuados para la determinación de cantidades muy pequeñas de analito. Esto mismo hace que no sea tarea fácil desarrollar métodos de tratamiento de muestra.

A continuación, se detallan algunas de las medidas abordadas más relevantes tras la optimización del tratamiento de muestra y las condiciones experimentales en la determinación de cada uno de los analitos estudiados mediante las técnicas analíticas ZGF-AAS y ICP-MS.

La composición salina de los medios de exposición lleva asociado un alto efecto matriz, traduciéndose en la mayor parte de los casos en una importante disminución o incluso la supresión total en la intensidad de la señal instrumental en el momento de su detección. Este efecto matriz se ha eliminado eficientemente añadiendo a la muestra un porcentaje óptimo de ácido nítrico. Esta solución resultó exitosa para la determinación de arsénico, plata y titanio en los medios de exposición. Sin embargo, en el caso del tributilestaño (TBT), debido a su naturaleza lipofílica, el contenido total del TBT o sus productos de degradación no se mantenían homogéneamente distribuidos

en el medio de exposición lo que se traducía en una alta irreproducibilidad en la señal analítica. Por ello, se realizó una extracción de este analito en fase orgánica, tolueno, dando como resultado una preconcentración de un factor de 20 y un aumento de la intensidad de la señal analítica obtenida por cámara de grafito. De esta manera, todo el TBT y sus productos de degradación quedaban concentrados en 200 μL de extracto final. La señal de intensidad obtenida correspondiente al estaño total se traducía en un pico bien definido y libre de interferencias.

La cuantificación, por cámara de grafito (como cantidad total de metal), de los dos tipos de nanopartículas estudiadas en los medios de exposición, nanopartículas de plata y de dióxido de titanio, resultó ser adecuada en las condiciones simuladas optimizadas sin necesidad de ningún tratamiento de muestra adicional. Estas condiciones simuladas se optimizaron tras un exhaustivo estudio de caracterización y evaluación del comportamiento químico de las nanopartículas en distintos medios compatibles con los organismos acuáticos para conseguir su estabilización en el medio de exposición. (Se discute en el apartado siguiente).

La cuantificación de cada uno de los analitos en las larvas resultó ser mucho más compleja y difícil que en los medios debido a la baja cantidad de muestra de la que disponíamos (alrededor de 20 mg) y al alto contenido lipídico de las mismas.

En la mayoría de los analitos ensayados, salvo en el caso de las nanopartículas de titanio, como resultado del tratamiento de las larvas mediante sonda de ultrasonidos focalizada se obtuvo una suspensión del analito en el extracto correspondiente. Sin embargo, dado que las propiedades químicas eran diferentes, se optimizó la adición de diversas sustancias químicas que ayudasen en la preparación de una suspensión homogénea del analito en el extracto. En dicho estudio hubo que considerar las características instrumentales de la técnica analítica empleada en su cuantificación. A continuación, se detallan algunas de las medidas abordadas más relevantes en cada caso particular.

Para el arsénico, se adicionó un 0,04 % de agente tensioactivo, Triton X-100, que ayudó a formar una suspensión homogénea y de menor viscosidad que la materia orgánica procedente de las larvas digeridas con sonda de ultrasonidos. La adición de dicho tensioactivo proporcionó no solo un incremento de la intensidad de la señal de

absorbancia en su detección por ZGF-AAS, es decir, en un incremento de la sensibilidad sino también contribuyó a aumentar la reproducibilidad de los resultados.

La determinación de TBT en las larvas resultó uno de los retos analíticos más complejos y laboriosos; básicamente porque su naturaleza lipofílica hacía difícil su extracción a partir de la materia orgánica de las larvas. Por ello, se llevó a cabo una eliminación previa de la grasa en metanol/acético y posteriormente se centrifugó la muestra para su deposición en el fondo del recipiente contenedor. De esta manera el contenido total del compuesto de estaño que presentaba mayor afinidad por el metanol permaneció disuelto en el sobrenadante permitiendo su detección por ZGF-AAS con la consiguiente mejora en la reproducibilidad y sensibilidad.

Con el fin de evaluar el metabolismo del TBT en las larvas (y transformación en sus derivados di y mono-butilados) se llevó a cabo un tratamiento de muestra que permitiese la posterior especiación de los compuestos de estaño en los extractos por GC-FPD. Tras la optimización de las condiciones instrumentales de la técnica se procedió a la etilación con tetraetilborato de sodio para la formación de especies volátiles capaces de ser detectadas por GC-FPD. A partir del tratamiento de muestra desarrollado para el estudio de bioconcentración, se extrajeron las diferentes especies de estaño, tras la digestión de las larvas con sonda de sonidos focalizada, en una mezcla de acético/metanol. El desarrollo de la metodología fue optimizado y validado con un candidato de material de referencia, la ostra T-37. El estudio de una extracción secuencial de las especies de estaño en diferentes proporciones de ácido acético/metanol, puso de manifiesto que el tratamiento de la muestra no daba lugar a alteración de especies y que con dos extracciones secuenciales se aseguraba la cuantitatividad del proceso. Asimismo, se demostró que la suma de la concentración de cada una de las especies individuales determinada por GC-FPD era igual a la concentración total de estaño en las larvas determinado por ZGF-AAS y por ICP-MS.

El tratamiento analítico de muestra para determinación de plata y nanopartículas de plata, por ZGF-AAS, fue muy sencillo. La extracción desde las larvas se llevó a cabo una vez más con la ayuda de la sonda de ultrasonidos focalizada y la adición de 5% de ácido nítrico que ayudaba a la destrucción de la matriz y a la disminución de las interferencias en su determinación posterior. Dado que para ambos experimentos la

plata, tanto iónica como nanopartículas, se determinó como contenido total en plata, la adición de ácido nítrico a la muestra provocaba la oxidación a plata iónica en el caso de las larvas tratadas con nanopartículas. Por todo ello, en ambos casos el tratamiento resultó ser adecuado y muy simplificado, ya que la digestión y extracción de la muestra se desarrollaba en tan solo un minuto y medio a diferencia de los métodos tradicionales de digestión con ácidos que implican en la mayoría de los casos la utilización del horno microondas.

La determinación del titanio total absorbido por las larvas se llevó a cabo por ICP-MS. El tratamiento de muestras se llevó a cabo en presencia de ácido nítrico mediante el empleo de la sonda de sonidos focalizada. La separación del contenido lipídico por centrifugación permitió eliminar interferencias y facilitar la nebulización del analito por ICP-MS. Sin embargo, el tratamiento para la determinación de las nanopartículas de dióxido de titanio requirió un proceso más fuerte de digestión de la muestra a temperatura ambiente mediante el empleo de la mezcla de ácidos fluorhídrico y bórico más peróxido de hidrógeno. La centrifugación de la muestra, como en el caso del titanio iónico, para la separación de la materia orgánica procedente de las larvas daba lugar a la deposición de las nanopartículas en el fondo del recipiente contenedor sin poder, por lo tanto, detectarse en el sobrenadante por la técnica ICP-MS. Este hecho, además, puso de manifiesto que el contenido total de titanio observado en las larvas se encontraba como nanopartículas o agregados de nanopartículas de titanio, puesto que al centrifugar la muestra la señal correspondiente al titanio en el sobrenadante era nula. De esta manera, tras la digestión de las larvas con la mezcla de ácidos fuertes las NPs se transformaban en Ti disuelto, eliminando así las interferencias posibles para su posterior cuantificación por ICP-MS.

En resumen, se han establecido y validado unas metodologías analíticas que permiten la determinación de la concentración de diversas especies metálicas y dos tipos de nanoparticulas en matrices complejas. La metodología analítica desarrollada empleando la sonda de sonidos focalizada se aplicó con éxito (exceptuando las TiO_2NPs) para la posterior determinación de todos los analitos ensayados, proporcionando una recuperación y reproducibilidad muy adecuadas a la vez que una considerable reducción del tiempo requerido para el tratamiento de muestra por otros procedimientos convencionales.

Estabilización de los analitos en los medios de exposición

Hasta la fecha, la mayoría de ensayos validados y normalizados no suelen llevar a cabo un seguimiento riguroso del comportamiento químico de la sustancia a testar en el transcurso del experimento, a pesar de que el Test OECD 305 establece que la concentración nominal no debe variar en el transcurso del ensayo en más de un 20% de su valor inicial. En todos los experimentos realizados en la presente memoria se ha hecho un estudio exhaustivo sobre la estabilidad de las sustancias en el medio de exposición y en los casos en que éstas no hayan sido estables se ha recurrido a distintos procedimientos para su estabilización. Es importante recalcar que si la sustancia no es estable en el medio de exposición, determinar la bioconcentración del compuesto es un proceso sumamente complejo. La sustancia química estudiada puede sufrir transformaciones en el medio de exposición que se traducen en una menor o mayor bioaccesibilidad hacia el organismo considerado y en consecuencia a una estimación errónea de su bioconcentración, así como de su riesgo para la salud medioambiental y humana.

El estudio de caracterización y búsqueda de unas condiciones óptimas que ayuden a la estabilización de la concentración nominal del analito en el medio de exposición ha sido más complejo y difícil en el caso de las nanopartículas, para las cuales, poco se conoce acerca de su comportamiento una vez son liberadas a medios acuáticos, y por tanto, del impacto y daños que este tipo de materiales podrán suponer para la salud y el medioambiente. Numerosas líneas de investigación se están encaminando al estudio y elucidación de sus posibles mecanismos de toxicidad en sistemas modelo una vez las NPs han sido liberadas al medio ambiente. No obstante, las conclusiones publicadas hasta la fecha tanto desde el punto de vista de bioconcentración como de toxicidad resultan confusas. Es difícil establecer modelos de predicción de las mismas ya que no se comportan de manera similar a sus análogos de composición, principalmente porque poseen diferente reactividad derivada de su alta relación superficie/volumen. Por todo ello, antes de llevar a cabo estos estudios,

resultará primordial la caracterización de su química y conocer su comportamiento, movilidad, persistencia y destino en el medioambiente al que son liberadas para así poder predecir su impacto global.

A continuación, se discuten algunos de los problemas y soluciones encontrados en el estudio de estabilidad de la concentración nominal inicial en los medios de exposición para cada uno de los analitos estudiados:

En la evaluación de la estabilidad química del As (III) en el medio empleado y en las condiciones bajo las que se realiza el ensayo se demostró una elevada estabilidad, para las dos concentraciones estudiadas: 5 y 50 μg L^{-1}. Se mantuvo casi el 100 % de su concentración nominal inicial durante al menos las 48 horas de duración de las que constaba la fase de absorción. Estos resultados coinciden con estudios ya publicados que tratan la estabilidad de arsénico en este tipo de muestras acuosas medioambientales. Este hecho, a su vez, pone en evidencia la necesidad de tomar medidas en la eliminación del arsénico de las aguas naturales, puesto que debido a su alta persistencia y su baja degradación, se pueden originar problemas asociados a la contaminación por arsénico en las aguas.

Por el contrario, en los primeros ensayos con TBT en los medios de exposición, se observó una inestabilidad superior al 60%, forzando la repetición de los experimentos. La preparación de los medios se realizó esta vez con especial cuidado y al final se logró mantener hasta un 70% de la concentración nominal de TBT durante los experimentos. Esta disminución de la concentración de TBT en el medio de exposición de las larvas puede atribuirse a su elevada volatilidad, su alta degradación, potencial capacidad de adsorción en las paredes del recipiente del experimento y a las bajas concentraciones empleadas en los ensayos de bioconcentración: 2 y 0.2 μg L^{-1}. Este problema ha sido muy común en la mayoría de los trabajos que tratan el estudio de la toxicidad del TBT, incluso en algunos desarrollados incluso a concentraciones superiores.

Los estudios llevados a cabo con plata iónica, pusieron de manifiesto una pérdida importante de la concentración nominal de la misma durante los experimentos. Este hecho puede ser explicado debido a la gran adsorción que presenta la plata en las paredes del contenedor. Por ello, se evaluaron varios tipos de contenedores para

garantizar su estabilidad en el proceso de absorción. Los resultados obtenidos revelaron una mayor estabilidad de la concentración de plata en los distintos niveles de concentración ensayados con el empleo de contenedores de polietileno. Aun así, existían pérdidas de la concentración nominal que parecía inevitable por tratarse de concentraciones tan bajas que podían ser consecuencia de la complejación de la plata iónica con algunas de las sales del medio de exposición, de composición similar a las aguas de los ríos, disminuyendo su biodisponibilidad para las larvas del pez cebra.

El titanio en disolución resultó ser estable en el medio de exposición durante las 72 horas en que se estudió su bioconcentración, lo que pone de manifiesto su baja reactividad en el medio ambiente. A pesar de que su persistencia podría ser objeto de preocupación, los escasos efectos adversos asociados a contaminación por este analito, hace que su efecto, desde el punto de vista medioambiental, no sea preocupante hasta la fecha.

En lo que se refiere al comportamiento químico de las nanopartículas en el medio de exposición, es importante mencionar que actualmente es uno de los retos analíticos de las líneas de investigación focalizadas en conocer sus mecanismos de acción. Los dos tipos de nanopartículas metálicas estudiadas en este trabajo presentan gran tendencia a la agregación una vez han alcanzado el medio acuático, transformándose en partículas de mayor tamaño y por tanto alejándonos del marco nano y perdiendo así sus propiedades como tales. Puesto que esto es lo que realmente ocurre en el medioambiente, no parece necesaria la búsqueda de unas condiciones simuladas y forzadas para estabilizarlas y proteger las nanopartículas de su agregación. Sin embargo, dado el amplio intervalo de condiciones medioambientales existentes, pueden existir otras condiciones en las cuales las nanopartículas se encuentren desagregadas en un tamaño más cercano al tamaño original en el momento de su liberación al medio. En tal caso resultaría importante establecer su toxicidad dentro del rango correspondiente a la escala nano. En este trabajo, se ha demostrado que el empleo de unas condiciones simuladas compatibles con la vida de los organismos acuáticos han sido capaces de mantener a ambos tipos de nanopartículas desagregadas, en la medida de lo posible, para poder llevar a cabo los ensayos y evaluar si la reactividad y eficiencia resultantes de su alta relación superficie/volumen implica una alta bioconcentración. La evaluación y optimización del

empleo de diferentes agentes estabilizantes que modifiquen su superficie a través de fuerzas electrostáticas y a su vez impidan su transformación química manteniendo la integridad de las mismas, es una de las soluciones más recurridas y que en nuestro caso permitió estabilizarlas en gran medida en el medio y en el tiempo de exposición correspondiente a nuestro Test de Bioconcentración.

Hoy día, en base a la bibliografía publicada sobre la estabilidad de las nanopartículas de plata su alta tendencia de agregación una vez son liberadas al medio es bien conocida. Su elevada cinética de oxidación a plata iónica constituye un problema adicional. Por tanto, para este tipo de nanopartículas nuestra investigación en la caracterización de nanopartículas de plata perseguía dos objetivos en paralelo: evitar su agregación/aglomeración y evitar su oxidación a plata iónica. El medio aquí propuesto consistió en la adición de 0,1 % citrato + 0,1% almidón (m/V). Este medio resultó no solo compatible con la vida de las larvas de pez cebra sino que a la vez también fue capaz de mantener a las nanoparticulas de plata en una distribución estrecha de tamaños comprendidos entre 100-50 nm con una mínima tasa de oxidación de las mismas. Posteriormente, se corroboró la estabilidad de la concentración nominal durante las 48 horas de la fase de absorción en el medio de exposición en presencia de las larvas. Aunque la presencia de las larvas podía alterar el comportamiento predicho inicialmente, para el caso de nanopartículas de plata a las dos concentraciones estudiadas (0,1 y 1 mg L^{-1}) se mantuvo su concentración nominal en un intervalo de tamaño inferior a 100 nm.

Respecto a la estabilidad de las nanopartículas de dióxido de titanio, los estudios realizados han puesto de manifiesto una elevada tendencia a formar agregados/aglomerados una vez entran en el medio ambiente. En este trabajo, como en el caso de las nanopartículas de plata, se llevó a cabo un estudio de caracterización encaminado a lograr mantener ambas estructuras alotrópicas, anatasa y rutilo, como nanopartículas de dióxido de titanio desagregadas con un tamaño inferior a 100 nm en el medio de exposición. Tras varios estudios con agentes estabilizantes, utilizados frecuentemente, se logró evitar su agregación para ambas estructuras alotrópicas durante más de 72 horas tras la adición al medio del agente $Na_2P_2O_7$ a una concentración 5mM. Se pensó en un principio que este compuesto sería compatible con la vida de las larvas por su posible disociación a fosfatos, condiciones similares a

las medioambientales, ya que en los ríos se pueden encontrar altas concentraciones de los mismos. Sin embargo, estas condiciones resultaron no ser compatibles con la vida de las larvas, causando la muerte de la población en menos de 24 horas. De todos los estudios realizados para lograr estabilizar las nanoparticulas de TiO_2, anatasa y rutilo, la adición de ácidos húmicos, permitió mantener un alto porcentaje de nanopartículas desagregadas. La interacción entre partículas disminuía significativamente debido a las fuerzas electrostáticas de repulsión que le confieren los ácidos húmicos una vez se han unido a la superficie de ambos tipos de nanopartículas. La tasa de agregación entre anatasa y rutilo, sin embargo, era ligeramente diferente, debido probablemente a la diferente naturaleza de cada una de ellas. La anatasa es más hidrofílica, por lo que los ácidos húmicos evitaban su agregación en más de un 60 %; mientras que para rutilo solo un 40% de nanopartículas presentaban tamaños inferiores a 100 nm. Estas condiciones de estabilidad se evaluaron en la composición más comercializada y comúnmente estudiada (75% anatasa+ 25% rutilo) observando tan sólo un 40% de partículas con un tamaño inferior a 100 nm. A pesar de ello y teniendo en cuenta la compatibilidad de estas sustancias naturales presentes en el medioambiente, se eligieron estas condiciones, como adecuadas para el estudio de bioconcentración.

En el transcurso del experimento la concentración nominal inicial se mantuvo estable en el contenido total de titanio, al igual que ocurría con su forma iónica, sin embargo la tasa de agregación fue mayor a la prevista inicialmente, poniendo de manifiesto que la presencia de las larvas contribuía a la aglomeración de las mismas en el medio acuoso. Este resultado, descrito por otros autores, podría estar indicando que una menor fracción de ácidos húmicos estuviese disponible para interaccionar con la superficie de nanopartículas de TiO_2 ya que estas sustancias de naturaleza orgánica presentan a su vez una alta afinidad para adherirse al tejido orgánico del pez. Como consecuencia, la eficiencia en la función de los ácidos húmicos encargados de establecer fuerzas electrostáticas de repulsión entre las nanopartículas de TiO_2 y evitar así su aglomeración se vería reducida, dando lugar a un mayor porcentaje de agregados de nanopartículas de TiO_2 en el medio de exposición.

Factores de Bioconcentración

Tras el desarrollo y optimización de la metodología analítica apropiada para la detección de cada uno de los analitos y la caracterización y búsqueda de las condiciones experimentales compatibles con la vida acuática capaces de mantener invariable la concentración nominal de ensayo, se pasó al estudio e interpretación de los factores de bioconcentración. Además, dado que se trata de un parámetro identificador del peligro inherente a una sustancia química, dependerá de las propiedades intrínsecas de dicha sustancia ya que las condiciones experimentales fueron inicialmente definidas. Por tanto, diferencias entre los BCFs de cada sustancia estarán directamente relacionadas a las variaciones en bioconcentraciónn como resultado neto de la absorción, transformación y eliminación de la sustancia de estudio por un organismo. La interpretación de la bioconcentración de cada uno de los analitos estudiados en este trabajo se ha realizado mediante el empleo de un modelo matemático, que difiere ligeramente entre cada una de las especies estudiadas en función de la respuesta de las larvas al contaminante expuesto. El ajuste de los datos a una cinética de primer orden, permite obtener el valor de parámetros biodinámicos influyentes, como son sus constantes de absorción y depuración. Estas constantes son función principalmente del organismo acuático empleado como sistema modelo y de factores ambientales tales como el pH y la temperatura. A su vez, el cociente entre las constantes de absorción (k_1) y depuración (k_2) permite obtener los valores de BCF. Esta cinética de primer orden es a menudo utilizada para el cálculo de los BCF, aunque en algunos casos los datos experimentales no ajustan a tal modelo y en tal caso, se emplean modelos más complejos para la valoración de la bioconcentración de una sustancia.

En este trabajo no resultó necesario el desarrollo de modelos matemáticos más complejos para ninguno de los analitos estudiados puesto que los resultados de las concentraciones encontradas tanto en los medios de exposición como en las larvas ajustaban perfectamente a una cinética de primer orden excepto en el caso del arsénico. En la tabla 4 se ilustran los BCF obtenidos para cada analito estudiado, así como algunos BCF ya publicados obtenidos con peces adultos.

Como ya se ha mencionado, el arsénico fue el único analito de entre los estudiados para el cual las concentraciones del mismo encontradas en las larvas fueron tan bajas que no fue posible ajustar estos resultados obtenidos a ningún modelo experimental. A pesar de que las concentraciones de exposición utilizadas eran consistentes con los contenidos de arsénico en las aguas subterráneas y superficiales consideradas como zonas no contaminadas, 5 μg L^{-1}, y con las concentraciones encontradas en zonas altamente contaminadas por arsénico, 50 μg L^{-1}, no se observó un aumento significativo de la concentración de este analito durante la exposición al mismo. Los resultados de BCF se calcularon a partir de la máxima concentración de arsénico encontrada en las larvas, a las 48 horas de exposición, obteniendo valores comprendidos entre 2.2- 9.5, encontrándose en los mismos intervalos encontrados para peces adultos (Tabla 4). Por tanto, se concluyó de este trabajo que no existía bioconcentración para el As (III) por parte de las larvas del pez cebra.

Por el contrario, el tributilestaño resultó ser altamente tóxico para nuestro sistema modelo empleado, siendo el de mayor bioconcentración entre los analitos estudiados. En un principio, en base a la amplia literatura publicada para esta sustancia, inicialmente se expusieron las larvas del pez cebra a concentraciones de 10 μg L^{-1} lo que originó la mortandad de toda la población tras 24 horas de exposición. Teniendo en cuenta este incidente se seleccionaron concentraciones de exposición de 2 y 0.2 μg L^{-1}. Tanto la determinación total de TBT como su especiación en el medio de exposición de esta sustancia a las larvas demostraron la rápida cinética de degradación a su metabolito MBT a cualquiera de las concentraciones testadas. Sin embargo, su capacidad de depuración no anuló su efecto tóxico para las larvas que llegaron a acumular concentraciones del orden de 138 ± 3 y 1137 ± 30 ng g^{-1} tras su exposición a 0.2 y 2 μg L^{-1}, respectivamente. El ajuste a una cinética de primer orden proporcionó valores de BCF comprendidos entre 840-1280. Este resultado podría explicarse por la naturaleza lipofilica de esta sustancia manufacturada, que favorece su difusión a través de la membrana celular, resultando en una hiperacumulación en órganos y tejidos. Los valores de BCF obtenidos en este trabajo para larvas del pez cebra, eran además consistentes con muchos de los trabajos publicados en peces adultos, como puede verse en la Tabla 4. Teniendo en cuenta los resultados obtenidos en este trabajo, el TBT pertenece al rango de clasificación definido como sustancia peligrosa

por su alta acumulación a través de la cadena trófica. Aunque como ya se ha mencionado, la legislación actual prohíbe el uso de esta sustancia en cualquiera de sus aplicaciones, todavía se detectan concentraciones de alto riesgo para esta sustancia en el medioambiente. Dado que sus mecanismos de toxicidad siguen sin estar bien establecidos, se requiere del desarrollo de posteriores estudios así como de la toma de medidas para su eliminación total del medioambiente.

La plata iónica resultó ser el segundo analito de mayor bioconcentración de entre los estudiados, encontrándose valores de BCF comprendidos entre 233-666 para niveles de exposición a la misma tan bajos como 0,01 y 0,05 μg L^{-1}. Estos valores eran consistentes con los publicados en la literatura, lo que permite clasificar a la plata como una sustancia peligrosa para larvas del pez cebra en un período corto de exposición a la misma. Estos resultados fueron además comparados con los encontrados con la plata en forma de nanopartículas de plata, ya que hasta la fecha son pocos los estudios de toxicidad realizados que hayan discernido sobre si los efectos tóxicos encontrados provenían de las nanopartículas de plata en sí, de sus agregados o de su posible oxidación a plata iónica. En este trabajo, aunque las condiciones propuestas sean simuladas, sí se ha estudiado la toxicidad proveniente de la exposición a nanoparticulas de plata de un tamaño inferior a 100 nm. En el estudio de la bioconcentración se vio cómo la cantidad total de plata acumulada por larvas del pez cebra incrementaba con el tiempo de exposición. Sin embargo, no fue significativa encontrándose BCF< 1 para las dos concentraciones ensayadas en este caso: 100 y 1000 μg L^{-1}. Estos valores son coherentes con la escasa literatura existente que presenta a las nanopartículas de plata como sustancias no peligrosas para el medio acuático a concentraciones incluso superiores a las evaluadas aquí, que son de un orden de magnitud similar a la concentración de las nanopartículas en sus aplicaciones, pero son de al menos dos órdenes de magnitud superiores a la concentración esperada en el medioambiente.

Comparando la bioconcentraciónde las nanoparticulas de plata con la del ión plata, las nanoparticulas de plata resultaron menos tóxicas para las larvas del pez cebra. Existe una importante controversia en este punto, dado que la mayoría de los trabajos publicados no evalúan el comportamiento, evolución y destino de las nanoparticulas una vez son liberadas al medio, estableciéndose en algunos casos

conclusiones confusas sobre si la toxicidad observada puede ser consecuencia del ión plata disuelto liberado por las nanopartículas. Por ello, y para este tipo de nanopartículas, para las que la cinética de oxidación se encuentra tan favorecida, la evaluación de su impacto y toxicidad para el medio ambiente debería ir acompañada de un estudio previo de caracterización en el medio al que son liberadas. Será necesario ampliar este tipo de estudios para validar y certificar el patrón de toxicidad observado para las nanopartículas de plata, evaluando para ello otros muchos factores y condiciones medioambientales influyentes en su toxicidad.

De acuerdo con lo concluido en estos estudios, y en concordancia con gran parte de los trabajos publicados hasta la fecha, son muchos los fenómenos que pueden influir en la toxicidad/bioconcentración de las nanopartículas de plata. Uno de los factores más importantes contribuyentes a su posible toxicidad será su biodisponibilidad para organismos acuáticos. El estudio con AgNPs realizado con otro tipo de organismos vivos evaluó la influencia de distintas condiciones medioambientales en la bioconcentración de nanopartículas de plata cubiertas con polivinilpirrolidona (PVP-AgNP), uno de los agentes comúnmente empleados para garantizar sus propiedades derivadas de su elevada relación superficie/volumen. En este caso el caracol *Lymnaea stagnalis* se expuso a las PVP-AgNPs a través de la dieta, adicionadas sobre la especie alga diatomea *Nitzschia palea,* proporcionada como fuente de alimentación al caracol. El posible efecto de tales condiciones medioambientales se valoró a partir de un modelo biodinámico, mediante el cálculo de una serie de parámetros fisiológicos que pueden ser cuantitativamente comparados entre metales, especies y condiciones medioambientales. Estos parámetros han sido: K_{uf}, constante unidireccional de absorción desde la dieta; eficiencia de asimilación, AE, (*assimilation efficiency*) definida como la fracción total de sustancia contaminante retenida en el animal una vez completadas su digestión y asimilación; y la tasa de ingestión desde la comida, IR (*ingestion rate*). Las condiciones medioambientales testadas fueron la dureza y la presencia de ácidos húmicos, demostrando no tener influencia alguna en la plata acumulada por este organismo, especie altamente sensible a la contaminación por metales así como a las condiciones medioambientales en los que se desarrollan. La concentración de plata acumulada por el caracol aumentaba paralelamente a la concentración de PVP-AgNPs adicionada a la comida y

resultó ser independiente a la dureza del agua de composición similar a aguas de río o la variación en la concentración de los ácidos húmicos. Se concluyó por tanto que las condiciones testadas no tenían influencia alguna para la biodisponibilidad y o bioaccesibilidad de este tipo de nanoparticulas de plata por el caracol *Lymnaea stagnalis*. Los resultados proporcionados por este estudio suponen una gran contribución a la comunidad científica ya que hasta la fecha no existe ningún trabajo realizado que evalúe el papel de las condiciones medioambientales sobre la bioaccesibilidad, biodisponibilidad y bioconcentración de las nanoparticulas de plata para los organismos acuáticos en aguas de río en la que las condiciones medioambientales no están siempre bien establecidas. Como ya se ha mencionado, existe además un amplio intervalo de condiciones medioambientales que pueden afectar al impacto y toxicidad que tienen para la salud y para el medioambiente. Las conclusiones de este trabajo aportan una pequeña contribución al enorme intervalo de factores concernientes a la comprensión de la toxicidad de nanopartículas. Dado el carácter especifico de las propiedades y características de las nanoparticulas, descubiertas en las últimas décadas, será necesario evaluar un sinfín de parámetros ambientales para establecer las teorías referentes a su modo de acción, comportamiento, toxicidad, etc.

Por último, los resultados de bioacumulación encontrados para el titanio iónico y las nanopartículas de dióxido de titanio evidencian ambas sustancias como sustancias no peligrosas y de bajo impacto para las larvas del pez cebra. Para ambas formas químicas además se prolongó el Test en base a previos trabajos en organismos acuáticos que demostraron un significativo incremento de la concentración en los organismos tras 72 horas de exposición. A pesar de que la concentración de titanio acumulada por larvas de pez cebra incrementaba progresivamente en el transcurso de la etapa de absorción alcanzándose valores elevados tras su exposición a ambas formas químicas, el ajuste de la concentración total de titanio encontrada tanto en las larvas como en el medio de exposición a una cinética de primer orden proporcionó resultados de BCF entre 3-77 y 2,5- 4.2 para el titanio iónico y nanopartículas de dióxido de titanio, respectivamente. Los valores de BCF aquí obtenidos además coincidían con algunos de los publicados por la base de datos japonesa METI y algunos otros estudios (Tabla 4). Todos ellos ponen de manifiesto una

bioconcentración insignificante para las larvas del pez cebra, a pesar de exponer a las larvas a concentraciones altas. Estos resultados, revelan que no hay un peligro directamente asociado a estas nanopartículas. En principio, es una buena evidencia debido al elevado número de aplicaciones actuales. También puede contribuir a que se investiguen nuevos campos de aplicación tanto para el titanio iónico como para las TiO_2NPs que reemplacen a otras sustancias químicas más tóxicas y peligrosas dada su baja toxicidad e impacto global para la salud y el medioambiente. Sin embargo, en el caso de las nanopartículas, especial cuidado debe ponerse en su formulación para sus múltiples aplicaciones dado que hasta la fecha sus mecanismos de acción son todavía desconocidos. Se recomienda la realización de estudios en tiempos superiores ya que los existentes hasta ahora no son suficientes.

Tabla 4. Ajuste de primer orden para el cálculo de parámetros representativos en el Test de Bioconcentración.

	Ti^{+4}		TiO_2 NP		Ag^{+}		AgNPs		As (II)		TBT	
C_w ($\mu g \cdot L^{-1}$)	110,41	902,43	184,06	910,12	0,011	0,059	99,9	1171,6	5	50	1,18	0,195
C_f ($ng \cdot g^{-1}$)	7367	2931	5000	26802	7,01	11,84	59,9	1162,0				
k_1 ($mL \cdot ng \cdot h^{-1}$)	4,6	0,2	0,051	0,12	100	14	0,09	0,1			37,85	35,12
$k_{2\ (acum)}$ ($ng \cdot mL^{-1}$)	0,06	0,06	0,012	0,04	0,15	0,06	0,15	0,1			0,029	0,042
$k_{2(dep)}$ ($ng \cdot mL^{-1}$)	0,05	0,06	0,032	0,04	0,02	0,06	-	-			0,025	0,028
$BCF_{48h\ larvae}$	73	3,3	2,5	2,9	665,3	220,2	0,599	0,991	8,6	2,2	723	970
$BCF_{k\ larvae}$	77	3,3	4,2	3,0	666	233,3	0,6	1			840	1280
Valores de BCFs en peces adultos publicados en trabajos anteriores												
	<1,1-10 (METI-NITE)		No accumulation-617 (Sun et al 2009)		250-460 (Laban et al 2009)		0,0096-42 (Bar-Ilan et al 2009, Asharani et al 2008)		<4(METI-NITE)		500-5000 (METI-NITE)	

6. CONCLUSIONES

En el trabajo presente se ha conseguido desarrollar una nueva metodología para la determinación de compuestos contaminantes clásicos, As (III), TBT, Ag^+ y TiO_2, así como de contaminantes emergentes: nanopartículas de plata y de dióxido de titanio, empleando muestras biológicas: larvas del pez cebra. Además, el método propuesto se muestra como posible alternativa al Test de la OECD 305, que requiere, gran cantidad de peces adultos, un elevado coste económico y el desarrollo de experimentos complejos. Para ello, se ha desarrollado un procedimiento de trabajo simplificado que presenta importantes ventajas:

Una de las ventajas más importantes de la metodología analítica desarrollada es el procedimiento simplificado del tratamiento de muestra. Es importante destacar que se resuelve la problemática inherente a la matriz de la muestra de las larvas y al elevado contenido en sales de los medios de exposición.

Se ha desarrollado un método de lixiviación mediante el empleo de la sonda de ultrasonidos focalizada con un porcentaje de ácido relativamente bajo, con la consecuente disminución en la generación de residuos. Es un método amistoso con el medio ambiente que resulta altamente eficaz y sumamente rápido.

La metodología analítica desarrollada se ha validado en los casos en que ha sido posible con materiales certificados de referencia y los resultados han evidenciado la exactitud del método.

Se propone el ensayo desarrollado como una alternativa al Test de la OECD 305, para el estudio de la bioconcentración de contaminantes clásicos y emergentes por organismos acuáticos, ya que los resultados obtenidos en los cálculos de BCFs son concordantes con los datos publicados en la bibliografía.

7. TRABAJOS FUTUROS

Los estudios realizados en esta tesis y los resultados obtenidos abren nuevas vías de investigación como son:

- Aplicación de las metodologías desarrolladas para estudios en otro tipo de muestras biológicas y medioambientales.

- Aplicación del Test de Bioconcentración aquí desarrollado para el estudio de otros contaminantes empleando como modelo larvas del pez cebra.

- Estudio de la bioconcentración de las nanopartículas en tiempos más largos de exposición, empleando para ello peces cebra en lugar de larvas, puesto que algunos estudios ya han demostrado la toxicidad de las mismas en tiempos prolongados. Además, así se demostraría experimentalmente la validez del test propuesto aquí con larvas.

- Estudio del perfil genómico y proteómico en las larvas expuestas a ambos tipos de nanopartículas, para entender sus mecanismos de metabolización de este organismo acuático y poder así extrapolar los resultados a la especie humana.

8. ANEXO

Como Anexo se incluye en esta tesis doctoral un trabajo de investigación con carácter multidisciplinar realizado en colaboración con el grupo de investigación del Departamento de Bioquímica de la Universidad Autónoma de Madrid. Este trabajo tenía como objetivo la comprensión de los mecanismos intracelulares y de la posible actividad reductasa de *Schizosaccharomyces pombe* para transformar la especie As (V), altamente tóxica, en As (III) como mecanismo de detoxificación.

Nuestra contribución analítica implicó la optimización y aplicación de herramientas y estrategias analíticas de especiación de As en las muestras de células y levaduras. Se llevó a cabo el análisis de especiación identificando distintas formas de dicho elemento involucradas en la reducción de As (V) a As (III) por la levadura: As (III), As (V), monometil arsénico (MMA) y dimetil arsénico (DMA).

Los resultados obtenidos de este trabajo demostraron por primera vez la actividad reductasa de *Schizosaccharomyces pombe* contribuyendo a la comprensión de los posibles cambios metabólicos sufridos por el As y su biotransformación en las distintas especies dentro de las células, así como la relación de cada una de estas especies con los mecanismos de toxicidad y adaptación a este elemento.

Response to Arsenate Treatment in Schizosaccharomyces pombe and the Role of Its Arsenate Reductase Activity (PlosONE, 2012)

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Response to Arsenate Treatment in *Schizosaccharomyces pombe* and the Role of Its Arsenate Reductase Activity

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Abstract

Arsenic toxicity has been studied for a long time due to its effects in humans. Although epidemiological studies have demonstrated multiple effects in human physiology, there are many open questions about the cellular targets and the mechanisms of response to arsenic. Using the fission yeast *Schizosaccharomyces pombe* as model system, we have been able to demonstrate a strong activation of the MAPK Spc1/Sty1 in response to arsenate. This activation is dependent on Wis1 activation and Pyp2 phosphatase inactivation. Using arsenic speciation analysis we have also demonstrated the previously unknown capacity of *S. pombe* cells to reduce As (V) to As (III). Genetic analysis of several fission yeast mutants point towards the cell cycle phosphatase Cdc25 as a possible candidate to carry out this arsenate reductase activity. We propose that arsenate reduction and intracellular accumulation of arsenite are the key mechanisms of arsenate tolerance in fission yeast.

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Introduction

Arsenic is a metalloid which is present in the environment both naturally and anthropogenically. As in the case of other metals and metalloids, such as cadmium and chromium, arsenic has been shown to be a health risk at low concentrations. In nature, arsenic is presented in many different oxidation states, being the inorganic ones, arsenite, As (III), and arsenate, As (V), the two main forms. While arsenite is presented mainly in anaerobic and alkaline environments, arsenate is more typical of aerobic and acid environments [1].

According to the World Health Organization (WHO), arsenic poisoning is one of the major health problems in several undeveloped countries, although cases have occurred in countries with a higher level of development [2]. In some areas of countries such as India and Bangladesh, arsenic poisoning is especially worrying and is usually caused by groundwater contamination, reaching levels above 10 μg/L which is the limit established as safe by the United States Environmental Protection Agency (EPA). There is a clear evidence of an association between the intake of arsenic and an increased risk of several types of cancer, miscarriages [3], as well as problems in cognitive development in growth stages [4,5,6].

At intracellular level, both arsenate and arsenite work differently. For instance, arsenate can enter the cell via a phosphate transporter due to its structural similarity to phosphate. For the same reason, arsenate can also alter several biochemical reactions such as cellular respiration. On the other hand, several reports have described the capacity of arsenite to damage DNA since arsenite inhibits base- and nucleotide-excision repair mechanisms [7,8,9,10].

Throughout the evolution, several mechanisms of response have been developed by organisms against different stressors. For instance, in the fission yeast *Schizosaccharomyces pombe*, the stress response is mainly directed by MAPKs and more specifically by the Spc1/Sty1 pathway. Spc1 is analogous to mammalian p38, and is activated when different types of stress such as UV radiation, heat shock and hyperosmolarity are present. In addition, it has been described that p38-like pathways are activated in response to arsenic stress in both *S. pombe* and *Saccharomyces cerevisiae* [11,12].

Regarding arsenate, several reports have shown that MAPK pathway is not the only mechanism of response used by eukaryotic organisms against arsenate. Some organisms can reduce arsenate to arsenite through the activity of arsenate reductases. Arsenite resulting from this reduction is removed from the cell through specific transporters (*Escherichia coli* Arsb, *S. cerevisiae* Acr3p, etc). This reducing capacity has been described in unicellular organisms, such as *Leishmania major* and *S. cerevisiae*, and pluricellular organisms, such as the fern *Pteris vittata* and human [13,14,15,16]. In the latter, arsenic reduction is carried out by the cell cycle phosphatase Cdc25, which also regulates G2/M transition by activating dephosphorylation of CDKs (cyclin dependent kinases) [17].

Studies about the mechanisms of stress response, signal transduction and cell cycle regulation using model organisms such as the yeasts *S. pombe* and *S. cerevisiae* have provided an important framework for investigating analogous mechanisms in higher eukaryotes.

In this report, we will attempt to unravel the intracellular mechanisms established in *S. pombe* in response to arsenic, more specifically to its pentavalent form. The conclusions drawn from this paper, taken in conjunction with previous works, could be useful to achieve a deeper understanding of the mechanisms of arsenic toxicity and detoxification in higher eukaryotes.

Materials and Methods

Strains and Media

All strains of *Schizosaccharomyces pombe* used in this study are listed in table 1. All different strains were cultivated in yeast extract medium (YES), at a temperature of 30°C with shaking. Deionized water was used to prepare the media. Media was sterilized in autoclave at 1 atm/121°C for 15 minutes. A spectrophotometer Spectronic 20D (Milton and Roy Company, France) was used to determine the number of cells of each culture (1 OD is about 10^7 cells/mL).

Viability Assays

For plate survival assays, different concentrations of both arsenate and arsenite (25 μM to 100 μM) were added depending on the experiment. Once the culture reached 0.3 OD, serial dilutions of yeast cultures were spotted in plates. Plates were incubated at 30°C for 48–72 hours.

Stress Treatment of Cells

Cells were cultivated up to 0.3–0.5 OD as explained before and arsenate was added at a final concentration of 100 μM. For immunoblotting analysis and mRNA extraction and quantification, cells were harvested by either filtration or centrifugation, respectively, and immediately stored at −80°C.

Immunoblotting

To purify the Spc1:HA6His protein we followed previously described protocol [18]. Purified Spc1:HA6His protein was loaded in SDS-PAGE and phosphorylation detected by immunoblotting. Phosphorylation was detected using anti-phospho p38 MAPK antibody (Cell Signaling Technology, USA) and the amount of Spc1:HA6His loaded was measured with an anti-HA antibody (Amersham, USA). Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibodies (Amersham, USA). Cdc25:myc was detected using anti-myc epitope antibodies (Cell Signaling Technollogy, USA) and actin with anti-actin antibodies (MP Biomedicals, USA).

mRNA Extraction and Quantification

Cells were harvested by centrifugation at OD = 0.5. Both mRNA extraction and purification were performed as previously described [19]. To quantify the amount of Cdc25 mRNA, total RNA was used as template for reverse transcription and preparation of total cDNA (Reverse Transcription System, Promega Corporation, USA). Finally, the *S. pombe cdc25* gene transcription level was determined by a quantitative PCR (qPCR) using that cDNA as template.

Arsenic Speciation Studies

Arsenic speciation studies were performed as described in [20]. An ultrasonic homogenizer, model SONOPLUS HD 2200 (Bandelin, Germany), equipped with a converter UW 2200, SH 213 G horn as amplifier and sonotrode MS 73 (3 mm titanium microtip) was used for cell extracts treatment. A centrifuge model 5804 Eppendorf (Hamburg, Germany) was used for phase separation after the extraction step.

Table 1. Genotypes of *Schizosaccharomyces pombe* strains used in this work.

Strain Name	Genotype	Source
PR109	*h- leu1-32 ura4-D18*	Paul Russell's laboratory
KS1366	*h+ leu1-32 ura4-D18 spc1::ura4*	Paul Russell's laboratory
JM544	*h- leu1-32 ura4-D18 wis1::ura4*	Paul Russell's laboratory
KS2136	*h- leu1-32 ura4-D18 wis4::ura4*	Paul Russell's laboratory
KS2185	*h- leu1-32 ura4-D18 his7-366 win1-1wik1::his7*	Paul Russell's laboratory
PR1337	*h- mcs4-13*	Paul Russell's laboratory
KS1376	*h- spc1:HA6His*	Paul Russell's laboratory
PS2759	*h- leu1-32 ura4-D18 spc1:HA6His (ura4) wis1::ura4*	Paul Russell's laboratory
KS1891	*h- leu1-32 ura4-D18 spc1:HA6His (ura4) wis1::myc*	Paul Russell's laboratory
KS2086	*h- leu1-32 ura4-D18 spc1:HA6His (ura4) wis1-AA::myc*	Paul Russell's laboratory
KS2149	*h+ leu1-32 ura4-D18 his7-366 spc1:HA6His (ura4) win1-1*	Paul Russell's laboratory
KS2138	*h- leu1-32 ura4-D18 spc1:HA6His (ura4) wis4::ura4*	Paul Russell's laboratory
KS2189	*h- leu1-32 ura4-D18 his7-366 spc1:HA6His (ura4) win1-1 wik1::ura4*	Paul Russell's laboratory
2209	*h- leu1-32 ura4-D18 his7-366 spc1:HA6His (ura4) pyp1::leu2 win1-1 wik1::his7*	Paul Russell's laboratory
MR218	*h- leu1-32 ura4-D18 his7-366 spc1:HA6His (ura4) pyp2::ura4 win1-1 wik1::his7*	Laboratory collection
MR15	*h- ura4-D18 cdc25:12 myc*	Laboratory collection
GL125	*h- leu1-32 ura4-D18 cdc2-3w*	Paul Russell's laboratory
MR661	*h-leu1-32 ura4-D18 cdc2-3w cdc25::ura4*	Laboratory collection

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A Perkin-Elmer 4100 ZL atomic absorption spectrometer with a longitudinal Zeeman background correction, equipped with a transversely heated graphite tube atomizer (THGA) with L'vov platforms was used for arsenic quantification. A Perkin Elmer arsenic electrodeless discharge lamp (EDL) with wavelength 197.3 nm and instrument slit width 0.7 nm was used. A Perkin Elmer EDL System was used to stabilize the lamp current between 349–351 mA. As alternative analytical technique for the determination of As, an ICP-MS HP-7700 Plus (Agilent Technologies, Analytical System, Tokyo, Japan) was used. It was equipped with a Babington nebulizer, Fassel torch and double pass Scott-type spray chamber cooled by a Peltier system. Single ion monitoring at m/z 75 was used for data collection.

The chromatographic system employed for As speciation consisted of a model PU-2080 Plus Pump, (JASCO Corporation, Tokyo, Japan) and PRP-X100 analytical and guard anion-exchange column (Hamilton, Reno, NV, USA). The column effluent was directly introduced into the nebulizer of the ICP/MS previously described via a PTFE capillary tube (0.5 mm i.d.). The samples were injected through a six port-valve (Rheodyne 9125, USA).

Reagents and Standards Employed for As Analysis

High-purity deionized water (Milli-Q Element system, Millipore, USA) was used for sample and standard solutions preparation. Ten milligrams per liter stock solutions, expressed as metal, of MMA and DMA, were prepared in 4% HNO_3 by dissolving adequate amounts of $CH_3AsO_3Na_2$ (MMA) and $(CH_3)_2AsO_2Na{\cdot}3H2O$ (DMA), both 98% purity from Merck (Darmstadt, Germany). Ten milligrams per liter stock solutions of As (V) and As (III) were prepared from$As_2O_5{\cdot}2H_2O$ (98.5%) from Merck (Darmstadt, Germany) and As_2O_3 (99.5%) from J.T. Baker (Deventer, Holland), respectively. All these solutions were kept at 4°C and stored in high density polyethylene (HDPE) bottles until use. Working solutions were prepared daily. The $Pd(NO_3)_2$ matrix modifier solution employed for GFAAS analysis was made from a dilution of 10 g/L Pd solution (Merck, Germany) with water to 2 g/L. HPLC-grade methanol from SDS (Barcelona, Spain) and $(NH_4)H_2PO_4$ from Merck (Darmstadt,Germany) were the reagents employed as mobile phase of the chromatographic system.

Samples

The samples prepared were kept frozen (−80°C) until analysis. Total arsenic and arsenic species were determined in two types of samples: yeast *Schizosaccharomyces pombe* extract and yeast extract (YES).

Analytical Procedures

Total arsenic was determined in *YES* by ZGF AAS by diluting one hundred times and adding five percent of nitric acid to eliminate matrix effects as well as Pd $(NO_3)_2$. It was necessary to modify the thermal furnace program respect to recommended conditions by the manufacturer. The furnace program finally employed is showed in Table 2. A YES volume of 20 µL was injected together with 3 µL of 2 g/L $Pd(NO_3)_2$.

Arsenic speciation was carried out in yeast *Schizosaccharomyces pombe* extracts by LC-ICP/MS. The yeast extracts were diluted two hundred times with deionizer water and introduced into a vial Teflon. The ultrasonic probe was then introduced into the solution and sonication was applied during 30 seconds at 30% amplitude. The extracts were centrifuged at 5000 rpm for 10 minutes and the supernatant was passed through a 0.22 µm nylon syringe filter before analysis. The chromatographic conditions were previously optimized (Sanz et al., 2005). Briefly, a polymeric anion-exchange column, PRP-X100 and mobile phase of 10 mM HPO_4^{-2}/H_2PO_4 at pH 8.5 plus 2% of methanol was added to the 10 mM phosphate mobile. The flow rate was 1 mL/min. Under these conditions appropriate separation of the four targeted species (As (III), MMA, DMA and As (V)) in 9 min can be obtained as shown in a typical chromatogram like figure 1. The instrumental parameters for total As determination and speciation analysis have been summarized in table 3.

Table 2. Graphite furnace programme.

Step	T (°C)	Ramp (s)	Hold (s)	Flow (mL/min)
1	90	5	10	250
2	110	3	20	250
3	300	20	10	250
4	1100	30	20	250
5	1200	1	2	0
6	2100	0	4	0
7	2300	1	4	250

Results

Spc1 MAPK Pathway Components are Required for the Response to Arsenate

We have previously described that trivalent arsenic is able to activate the MAPK Spc1 in *Schizosaccharomyces pombe* and cells deficient in this MAPK are sensitive to As (III) [11]. Arsenate, As (V) is the most abundant form of arsenic in many sources of drinking water and is thought to be responsible for many of the chronic effect of arsenic. We decided to study arsenate behavior and compare it with arsenite effects on cellular physiology using a simple eukaryote as *Schizosaccharomyces pombe* as model organism.

First, we monitored the sensitivity to arsenate of different fission yeast strains deficient in one or more genes participating in the activation of the MAPK Spc1 (Figure 2A). We compared the viability under chronic exposure to arsenate of those strains using serial dilutions in plates containing rich media.

Using this experimental approach we observed that cells deficient in the MAPKK Wis1 and a double mutant lacking

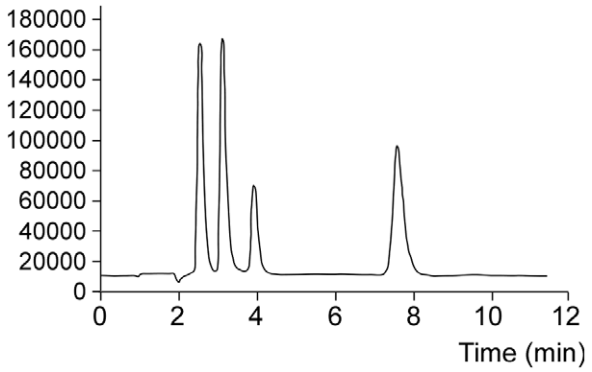

Figure 1. Typical Chromatogram obtained for a standard solution of As species at 2.5 µg L^{-1} using the experimental parameters summarized in Table 3. Peak 1: As (III); Peak 2: DMA; Peak 3: MMA; Peak 4: As (V).
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Table 3. Instrumental parameters for As determination by LC/ICP/MS.

ICP MS	
RF power	1550 W
Ar flow rate	Plasma gas: 15 L min-1
	Nebulizer: 1 L min-1
Isotope monitored	75 As
Integration time	0.1 s (spectrum) per point
Points per peak	3
HPLC	
Column	PRP-X100 anion Exchange
	Dimensions: 250 mm×4.1 mm, particle size 10 μm
Guard column	PRP-X100 anion exchange
	Dimensions: 4.6 mm
Mobile phase	10 mM $HPO_4^{-2}/H_2PO_4^-$; 2% (v/v) MeOH; pH 8.5
	100 μL
Flow rate	1.5 mL min-1
Mode	Isocratic

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MAPKKKs Wis4 and Win1, were very sensitive to arsenate. However, mutants deficient in Mcs4 or in each one of the MAPKKKs did not show any increased sensitivity to arsenate.

These results indicate that the activation of the MAPK Spc1 is essential for the efficient response to arsenate and the activations requires full function of the MAPKK.

Activation of Spc1 is Wis1 Dependent but can be Mediated through a MAPKKK Independent Mechanism

We had previously described that arsenite activation of Spc1 was mediated through a mechanism that depends on Wis1 activation, but also on a Wis1-activation independent mechanism [11]. In order to monitor the presence of a similar mechanism after arsenate treatment, we took advantage of different fission yeast mutant strains available. Under arsenate treatment, Spc1 is strongly activated trough a mechanism that requires Wis1 (Figure 2B). However, this activation was still present when the treatment was performed in a mutant strain where both activating phosphorylation sites from Wis1 where changed to the non-phosphorylable aminoacid alanine (Figure 2B). Similar situation was observed in mutants lacking either MAPKKK or both. This result indicates that Spc1 activation under arsenate treatment depends (like in the case of arsenite), on the presence and activation of Wis1, but also on a Wis1-activation independent mechanism.

To further advance in our knowledge of the mechanism of Spc1 activation under arsenate treatment, we also monitored the activation of Spc1 in mutants lacking Wis4 and Win1 activities and each of the phosphatases Pyp1 and Pyp2 (Figure 2C).

We reasoned that if the activation of Spc1 was independent of Wis1 phosphorylation, it may be dependent on Pyp1 or Pyp2 inhibition. As seen in Figure 2C, in mutants lacking Wis4, Win1 and Pyp2 activities, Spc1 activation still occurs in the presence of arsenate, indicating that its activation depends on other mechanism. However, cells lacking Pyp1 activity in a *wis4*Δ *win1-1* genetic background showed a decrease capacity to phosphorylate Spc1 upon arsenate treatment.

This result is consistent with a mechanism where Spc1 regulation is achieved both, through activation of Wis1 and inhibition of Pyp1.

Fission Yeast Displays Arsenate Reductase Activity

The results described above using arsenate as a stress source, resemble those previously obtained with arsenite. One possible explanation for these similar responses could be that arsenate is transformed into arsenite through a biochemical transformation performed by the fission yeast *Schizosaccharomyces pombe*. However, such arsenate reductase activity has not been described in fission yeast yet.

We expected that if such arsenate reductase activity existed in fission yeast, intracellular arsenite should appear in the course of an experimental treatment. We obtained whole cell extracts of fission yeast cells treated with As (V) and determined the intracellular concentrations of As (V) and As (III) at different time points. As seen in Figure 3A, intracellular As (III) concentrations increases with time, indicating that the arsenate added to the media has been transformed into arsenite by a cellular activity.

Cdc25 is Required for Arsenate Response in Fission Yeast

We have determined that fission yeast presents arsenate reductase activity *in vivo*. Our next question was what protein or proteins were carrying out such activity.

One of our approaches was to look for *S. pombe* genes similar to known arsenate reductases in other organisms. We found that arsenate reductases and Cdc25 proteins share similarities in their catalytic domain. We compared *S. pombe* Cdc25 sequence with rice Cdc25 and arsenate reductase Acr2 from *Saccharomyces cerevisiae* and human arsenate reductase Cdc25.

As observed in Figure 3B, the similarity between the 4 proteins in their catalytic domains is very high with a strong conservation in several key aminoacids.

If Cdc25 is an arsenate reductase we would expect that cells deficient in Cdc25 would be sensitive to arsenate. However, Cdc25 is an essential gene that cannot be eliminated in a haploid wild type genetic background because is indispensable for the advance of cell cycle through the dephosphorylation and activation of the CDK, Cdc2. However, it has been described that cells carrying a hyperactive allele of Cdc2, the *cdc2-3w* allele, were able to survive in the absence of Cdc25. We therefore monitored the sensitivity of *cdc2-3w cdc25Δ* strain to arsenate treatment (Figure 3C).

As observed in Figure 3C, cells deficient in Cdc25 were more sensitive to arsenate than wild type or *cdc2-3w* strains. Interestingly the abundance and mobility of Cdc25 protein was altered after arsenate treatment (Figure 3D), and the mRNA encoding Cdc25 also suffers fluctuations after arsenate treatment (Figure 3E), indicating that Cdc25 expression may be regulated by arsenate.

Arsenate Reductase Activity Requires Wild Type Activity of Spc1, Cdc2 and Cdc25

The results described before indicated that Cdc25 has a role in the response to arsenate, perhaps through its arsenate reductase activity. In order to test this hypothesis, we determined the arsenic species As (III) and As (V) in cellular extracts and growth media obtained from wild type, *spc1*Δ, *cdc2-3w* and *cdc2-3w cdc25*Δ strains after arsenate treatment.

In Figure 4A, As (V) appears to accumulate in cell extracts from wild type and mutant strains treated, like *spc1*Δ, *cdc2-3w* and *cdc2-*

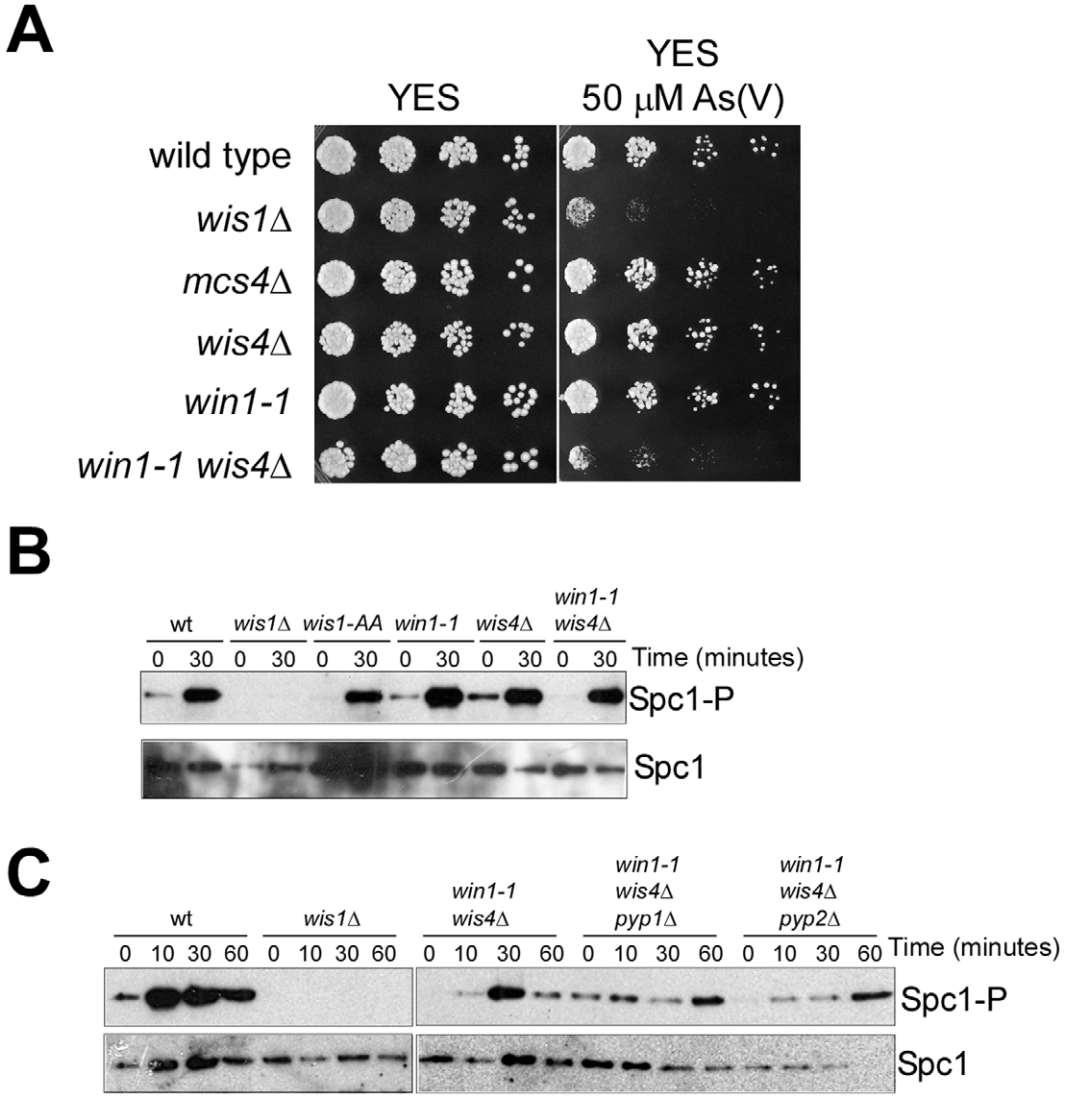

Figure 2. Spc1 MAPK pathway and the response to arsenate. A. Serial dilutions of wild type, *wis1*Δ, *mcs4*Δ, *wis4*Δ, *win1-1* and *wis4*Δ *win1-1* strains were plated in rich media (YES) or rich media containing 50 μM sodium arsenate. Pictures were taken after incubation at 30°C for 48 hours. B. Western blotting of purified Spc1 extracts from wild type, *wis1*Δ, *wis1-AA*, *win1-1*, *wis4*Δ, and *win1-1 wis4*Δ treated with 100 μM sodium arsenate for 0 to 30 minutes. Antibodies against phosphorylated p38 were used. As a control, antibodies against HA epitope were used. C. Western blotting of purified Spc1 extracts from wild type, *wis1*Δ, *win1-1 wis4*Δ, *win1-1 wis4*Δ *pyp1*Δ and *win1-1 wis4*Δ *pyp2*Δ treated with 100 μM sodium arsenate for 0 to 30 minutes. Antibodies against phosphorylated p38 were used. As a control, antibodies against HA epitope were used.
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*3w cdc25*Δ strains, after 3 or 9 hours. This accumulation is specially high in mutants lacking Cdc25, that is consistent with a role of this phosphatase in As (V) removal from fission yeast cytoplasm. After 9 hours, all strains have similar As (V) levels in their cytoplasms. During this period (3–9 hours after treatment), the growth media did show a slight decrease in the total concentration of As (V).

In Figure 4B we show the result of quantifying the amount of As (III) in the same experiment. After 3 hours treatment with As (V), a noticeable amount of As (III) appeared in the interior of fission yeast cells. This As (III) resulted from the cellular reduction of As (V) into As (III). The accumulation of As (III) was significantly higher in wild type cells than in the other mutants assayed (Figure 4B, 9 hours). Interstingly, the amount of As (III) present in the cellular exterior (growth media) was detectable, but very low.

Together with the accumulation of As (III), these results indicate that Cdc25 might be required for arsenate reductase activity, and that this activity could be affected by the presence of Cdc2 in the cell.

Discussion

In this report, several mechanisms by which *S. pombe* is able to respond to arsenate have been analyzed. The overall conclusion from these studies is that the response of fission yeast to arsenate and arsenite is different to the response to other types of stress like, for example, high osmolarity. Besides this, we have described for the first time that *S. pombe* has an arsenate reductase activity. We discuss the possible role of Cdc25 phosphatase as the leading candidate to perform this activity in *S. pombe* and its possible functional interaction with Cdc2 kinase.

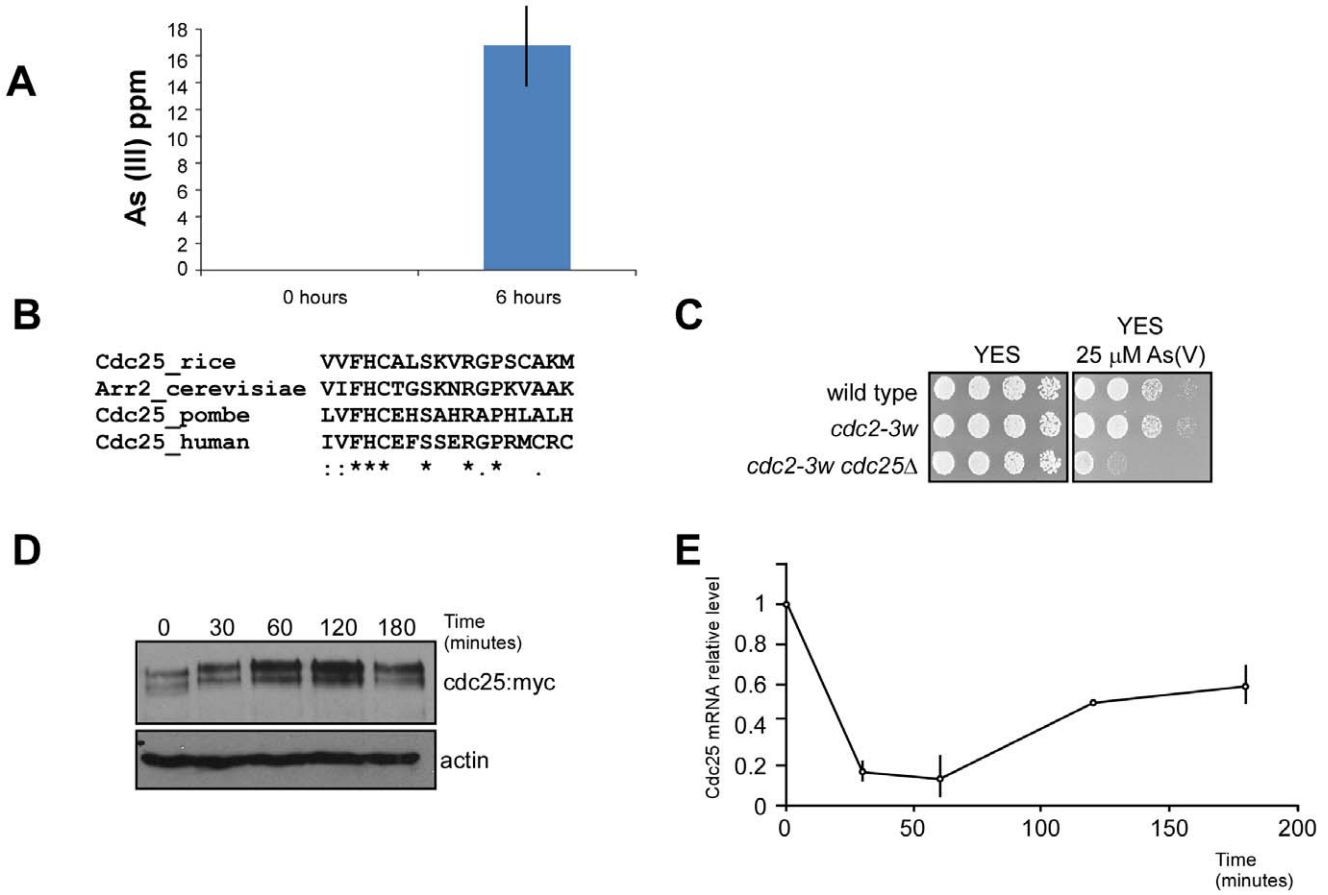

Figure 3. Cdc25 is essential for the response to arsenate. A. Arsenate to arsenite conversion in fission yeast. Cell extracts from cells treated with 100 μM sodium arsenate were analyzed for the presence of As (III) at different time points. Graph represents parts per million (ppm) As (III). B. Protein alignment of a fragment of *S. pombe* Cdc25, rice Cdc25 and *S. cerevisiae* Acr2 and human Cdc25. Asterisks indicate full conservation. C. Serial dilutions of wild type,*cdc2-3w* and *cdc2-3w cdc25*Δ strains were plated in rich media (YES) or rich media containing 25 μM sodium arsenate. Pictures were taken after incubation at 30°C for 48 hours. D. Western blotting of whole cell extracts from Cdc25:myc strains treated with 100 μM sodium arsenate for 0 to 180 minutes. Anti-myc antibodies were used to detect Cdc25:myc and anti-actin as a control. E. Total RNA from the experiment presented in (D) was purified and the total amount of Cdc25 mRNA quantified by qPCR. Actin mRNA was used as an internal control.
doi:10.1371/journal.pone.0043208.g003

Activation of the Spc1/Sty1 Stress Response Pathway by Arsenate

Viability under arsenate treatment assays have shown that a correct Spc1 MAPKs pathway is essential for cell survival against this type of stress. In contrast to arsenite, *S. pombe* has a much higher sensitivity to arsenate, reaching growth inhibition at micromolar concentrations (This work and [11]).

As observed in Western blotting experiments, it is quite possible that Spc1 could be activated by alternative mechanisms to the MAPK pathway, mainly at level of the MAPKK Wis1. Interestingly, Pyp1 phosphatase appears to be directly involved in this process. The lack of Pyp1 along with a defective MAPK Spc1 pathway does prevent further activation of Spc1 when arsenate is present. In strains with a functional Pyp1, but deficient in Pyp2, activation of Spc1 seems to occur. Therefore, Pyp1 is a good candidate to be inhibited by arsenate *in vivo*.

Comparisons with previous reports studying the role of MAPK pathways in response to arsenic in *S. cerevisiae*, showed similar results to those obtained in our experiments, where MAPK Hog1 is activated in response to arsenite and Slt2 does so in response to arsenate [12,21]. These results show that this type of arsenic stress response not only appears in *S. pombe*, but has been conserved throughout the evolution, although the mechanisms may be slightly different, at least at the level of MAPK specificity.

Arsenate Reductase Activity of the Cell Cycle Phosphatase Cdc25

Arsenite found in the arsenic speciation experiments, raises the possible existence of alternative response mechanism to the Spc1 pathway, by which *S. pombe* is able to respond to the stress by arsenate. This mechanism could be the reduction of arsenate into arsenite, ability that has already been described in other organisms [13,14,15]. More recently, experiments focused on the human cell cycle phosphatase Cdc25 have also described this reducing capacity for this protein [16].

As observed in our results, arsenate reduction occurs in *S. pombe*. This arsenate reduction activity is affected by the presence of Spc1, Cdc2 and Cdc25.

Like *spc1*Δ strain, *cdc2-3w*, which presents hyperactivated Cdc2, shows a diminished capacity to reduce arsenate into arsenite in the cell. Because of this, it could be assumed an inhibitory role to Cdc2 on arsenate recution activity. Interestingly, Cdc25 activates Cdc2 by removing an inhibitory phosphate previously placed by the kinase Wee1. Given this result, it could be assumed that in the

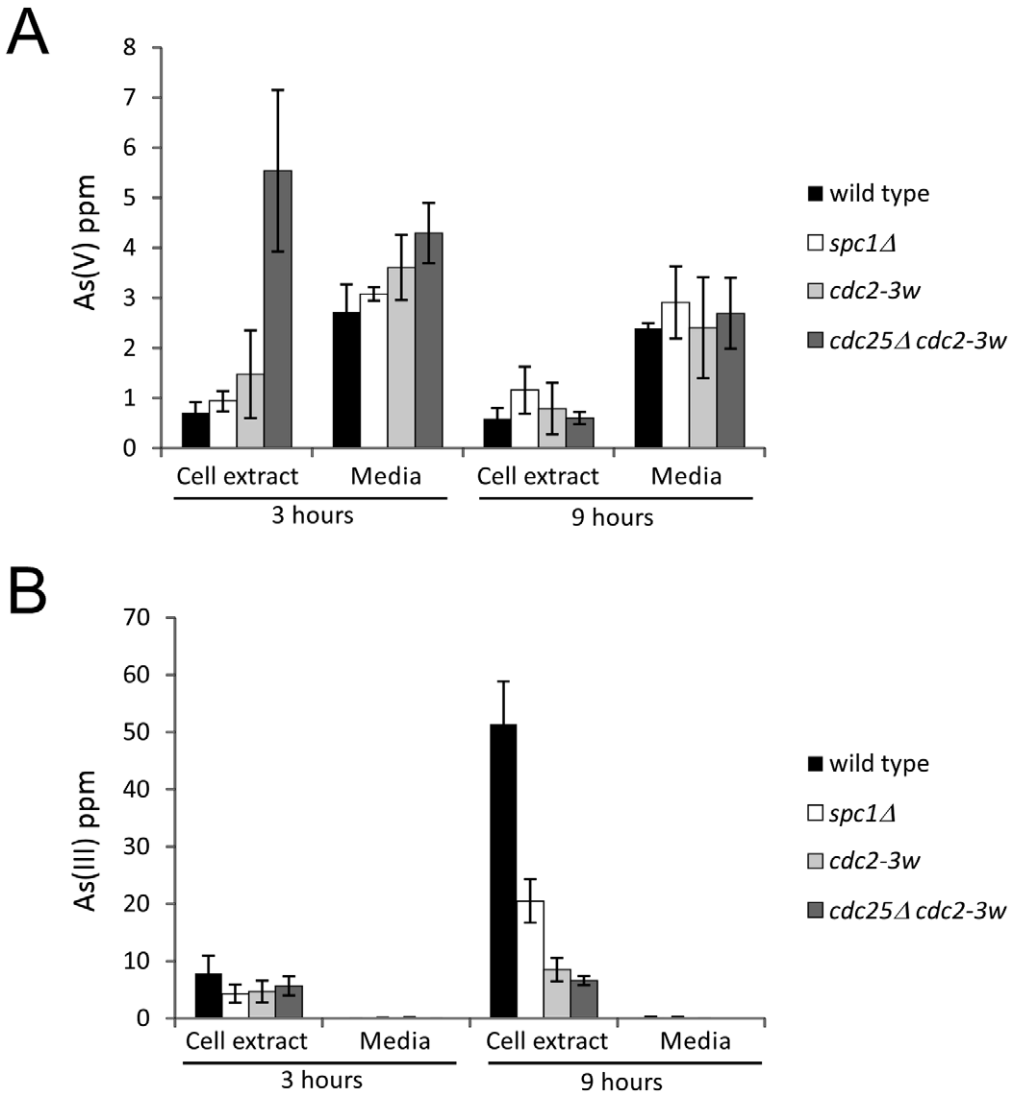

Figure 4. Arsenic speciation in different fission yeast mutants. Total cell extracts from 5×10^7 cells and growth media from wild type, *spc1*Δ, *cdc2-3w* and *cdc25*Δ *cdc2-3w* strains were obtained after treatment for 3 or 9 hours with 100 μM sodium arsenate. Graph shows the amount of As (V) (A) or As (III) (B) present in the extracts or growth media.
doi:10.1371/journal.pone.0043208.g004

double mutant *cdc2-3w cdc25*Δ the kinetics of arsenate reduction would increase, as Cdc2 would be activated at a lower level. As observed in the results, the increase does not occur, therefore, Cdc25 could exert an activating role in this reduction independently of Cdc2 activation. On the other hand and despite these results, still remain to be cleared whether Cdc2 activity is regulated by Cdc25 in the reduction of arsenate, as has been described in the cell cycle [22].

These data show the complex mechanism by which *S. pombe* is able to reduce arsenate into arsenite. In the model we propose, Cdc25 and Cdc2 proteins play an activator and inhibitor role in the regulatory mechanism of the arsenate reduction, respectively. More studies are required in order to the molecular mechanism regulating arsenate into arsenite reduction, a key step for cell survival against this type of stress.

It is also interesting to notice that, although As (III) accumulates inside the cells, very little As (III) appears to accumulate in the growth media. This lack of arsenite accumulation could be explained by two different models:

a) The export of As (III) to the cell exterior is not very efficient.
b) There is an spontaneous oxidation of As (III) to As (V) in the growth media.

We consider that the second possibility is very unlikely because we have experienced very different responses in sensitivity from fission yeast to arsenate to arsenite. If arsenite would spontaneously oxidized to arsenate, the response to both forms of arsenic would be identical.

Therefore, we favour a model where the mechanisms of As (III) removal from the cytoplasm in fission yeast are not based in extracellular elimination, but on vacuolar accumulation, like the mechanism described previously [23,24].

We have described the mechanisms that lead to activation of the MAPK Spc1 by arsenate and the presence of an arsenate reductase activity in *S. pombe*. Future research will determine the regulation of this arsenate reductase activity and the possible interplay with other cellular stress response mechanisms.

Author Contributions

Conceived and designed the experiments: RMO CC MARG. Performed the experiments: AS ALSO AMMG JS SZF. Analyzed the data: AS ALSO AMMG JS SZF RMO CC MARG. Contributed reagents/materials/analysis tools: AS ALSO AMMG JS SZF RMO CC MARG. Wrote the paper: RMO CC MARG.

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