Doctoral Thesis

Ismael Rodea Palomares
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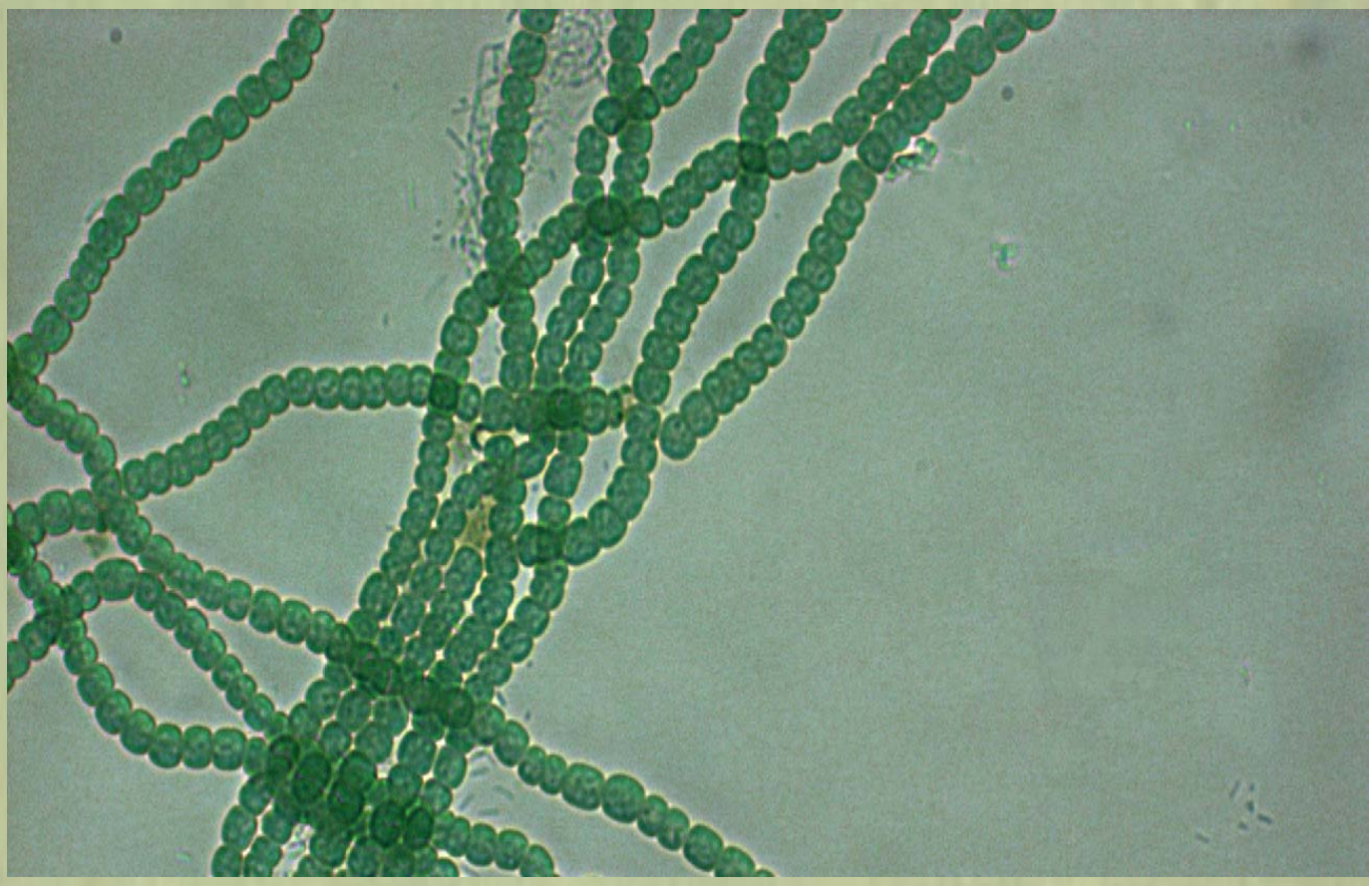
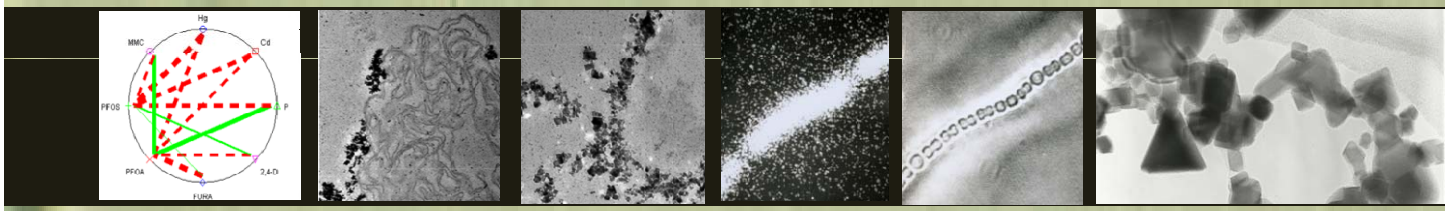


# Application of a novel *lux*-based cyanobacterial bioreporter in environmental toxicity: Assessment of individual and combined toxicity of priority and emerging pollutants




UNIVERSIDAD AUTÓNOMA DE MADRID FACULTAD DE CIENCIAS DEPARTAMENTO DE BIOLOGÍA


Ph.D. Thesis

# APPLICATION OF A NOVEL *LUX*-BASED CYANOBACTERIAL BIOREPORTER IN ENVIRONMENTAL TOXICITY: ASSESSMENT OF INDIVIDUAL AND COMBINED TOXICITY OF PRIORITY AND EMERGING POLLUTANTS

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# APPLICATION OF A NOVEL *LUX*-BASED CYANOBACTERIAL BIOREPORTER IN ENVIRONMENTAL TOXICITY: ASSESSMENT OF INDIVIDUAL AND COMBINED TOXICITY OF PRIORITY AND EMERGING POLLUTANTS

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**Programa de Doctorado en Bioquímica, Biología Molecular, Biomedicina y Biotecnología (Biociencias Moleculares)**.

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## Abbreviations

(AA) Allen and Arnon culture medium
(AA-EQs) Annual Average values of EQs
(Add) Additivity
(AGM) Algal Growth Medium
(AhR) Arylhydrocarbon receptor
(ANOVA) Analyses of variance
(Ant) Antagonism
(AR) Androgen
(ATP) Adenosine 5´-triphosphate
(BC) Bacteriological Code
(BET) Brunauer-Emmet-Teller
(BOD) Biochemical oxygen demand
(Bz) Bezafibrate
(CA) Concentration Addition
(CAS) Chemical Abstracts Service
(CANARY) (Cellular Analysis and Notification of Antigen Risks and Yields)
(CI) Combination Index
($CI_L$) Lower Confidence Limit, $\alpha = 0.05$
($CI_u$) Upper Confidence Limit, $\alpha = 0.05$
(CMP) Chemomechanical planarization
(COP) Conference of the Parties
(CLRTAP) Convention on Long-Range Transboundary Air Pollution
(CV) coefficient of variation
(D) Doses
(*Dm*) Median doses
($D_{ow}$) Apparent octanol-water partition coefficient
($ddH_2O$) Double distilled water
(DDT) dichlorodiphenyltrichloroethane
(DLS) Dinamic Light Scattering
(DNA) Deoxyribonucleic acid
(DMSO) Dimethyl sulfoxide
(EC) European Community
($EC_x$) Effective Concentration
(ECHA) European Chemicals Agency
(EDTA) Ethylenediaminetetraacetic acid
(ELS) Electrophoretic Light Scattering
(EPS) Exopolymeric substance
(EQs) Environmental Quality Standards
(EMEA) European Medicines Agency
(ENP) engineered nanoparticle
(EPA) Environmental Policy Agency
(ER) Estrogene
(ERA) Environmental risk assessment
(E[Y | x]) Average response at dose X
(*fa*) Fraction affected
(*fu*) Fraction unafected
(Fn) Fenofibric acid
(FURA) furazolidone
(GFP) Green fluorescent protein
(Gm) Gemfibrozil
(HEPES) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(HPLC) High-performance liquid chromatography
(IA) Independent Action
(IC) Índice de Combinación
(ICBN) International Code of Botanical Nomenclature
(ICP-MS) Inductively coupled plasma mass spectrometry
(IPTG) Isopropyl-β-D-thio-galactoside
(ISO) International Organization for Standardization
($K_{ow}$) Octanol water partition coefficient
(LC) Liquid chromatography
($LC_x$) Lethal Concentrations
(MACs) Maximum Allowed Concentrations
(MES) [2-(N-morpholino) ethanesulfonic acid]

(MMC) Mitomycin C
(MOA) Mechanism of Action
(MOPS) 3-(N-morpholino)propanesulfonic acid
(MS) Mass spectrometry
(NDH-1) respiratory complex I
(Nm) Neomycin sulfate
(NOECs) No Observed Effect Concentrations
(NTU) Nephelometric Turbidity Units
(pKa) Acid dissociation constant
($p$) $p$ – value
(PAHs) Polycyclic Aromatic Hydrocarbons
(PBDEs) Polybrominateddiphenilethers
(PEC) Predicted Environmental Concentrations
(PCBs) Polychlorinated biphenyls
(PFCs) Perfluorinated surfactants
(PFBS) Perfluorobutanesulfonate
(PFOA) Perfluorooctanoic acid
(PFOS) Perfluorooctanesulfonate
(POPs) Persistent organic pollutants
(PPAR α) Peroxisome proliferator-activated receptor α
(PPB) Propylparaben
(PPCPs) Pharmaceuticals and personal care products
(PS) Photosystem
(PSA) Polar Surface Area
(OD) Optical density
(OECD) Organisation for Economic Co-operation and Development
(PBT) Persistence, bioaccumulation and toxicity
(PW) Pure water
(Q)SAR Quantitative structure-activity relationship
(REACH) Registration, Evaluation and Authorization of Chemicals
(RLU) Relative Light Units
(ROS) Reactive Oxygen Species
(SAR) Structure activity relationship
(SDA) Sequential deletion analysis
(SD) Standard deviation
(SDS) Sodium dodecyl sulfate
(STPs) Sewage treatment plant
(Syn) Synergism
(TCP) 2,4,6-triclorophenol
(TEM) Transmission electron microscopy
(TOC) Total Organic Carbon
(TXRF) Total Reflection X-ray Fluorescence
(UNECE) United Nations Economic Commission for Europe
(UNEP) United Nations Environmental Programme
(UMT) Urogen III methyltransferase
(USEPA) United States Environmental Policy Agency
(vPvB) Very persistent and very bioaccumulative
(WFD) Water Framework Directive
(WHO) World Health Organization
(WW) Wastewatrer
(X-Gal) bromo-chloro-indolyl-galactopyranoside

## CONTENTS

# RESUMEN/SUMMARY

**RESUMEN/ SUMMARY**

## RESUMEN

La degradación del medio natural es uno de los problemas y retos más importantes de la humanidad actualmente. Dicha degradación es una consecuencia directa de la actividad humana, especialmente desde la revolución industrial del Siglo XIX que sentó las bases del sistema económico moderno y que tuvo como consecuencia un crecimiento exponencial de la población, su masificación en grandes ciudades, y un intenso desarrollo tecnológico basado en la industria química. Todos estos factores han traído como consecuencia la producción y desecho de un sinfín de productos (naturales o artificiales) que finalmente acaban llegando a los ecosistemas acuáticos a través de las aguas de vertido de industrias y ciudades o la escorrentía de los campos de cultivo.

Como consecuencia de los efectos de la contaminación detectados sobre la salud humana y el medio ambiente, los distintos estados y organizaciones internacionales han ido implementando durante los últimos 20 años distintos estándares de calidad que poco a poco han ido desarrollándose dando lugar a un enfoque multidisciplinar basado en tres pilares que se retroalimentan: primero, la investigación de los efectos sobre la salud y el medio ambiente de las distintas sustancias empleadas en la industria; segundo, el establecimiento de límites y criterios legales de máximos permitidos de las distintas sustancias para el consumo humano y la protección del medio ambiente en base a la evaluación de su peligrosidad; y tercero, la vigilancia y revisión de dichos criterios para conseguir un adecuado nivel de protección de la salud y del medio ambiente. En el caso de los ecosistemas acuáticos, este enfoque ha implicado un continuo desarrollo de las técnicas analíticas requeridas para poder detectar el creciente número de contaminantes que pueden acabar en las aguas, y un desarrollo de métodos biológicos para poder medir los posibles efectos adversos de esas sustancias sobre los distintos niveles del ecosistema.

El objetivo fundamental de la presente tesis fue evaluar la aplicabilidad de un nuevo bioensayo de toxicidad general en ecotoxicología; para ello el bioensayo se aplicó para determinar la toxicidad individual y en mezclas de distintos contaminantes prioritarios y emergentes y de aguas residuales. Este nuevo bioensayo de toxicidad se basa en una cepa derivada de la cianobacteria filamentosa (de agua dulce) del Orden *Nostocales*: *Anabaena* sp. PCC 7120, llamado *Anabaena* CPB4337. Dicha cepa porta una integración cromosómica del operón *luxCDABE* de la bacteria terrestre naturalmente bioluminiscente *Photorhabdus luminescens*

(antes *Xenorhabdus luminescens*). En el caso de la cepa de cianobacteria en estudio, el operón *lux* se expresa de forma constitutiva y por tanto se trata de un organismo autoluminiscente, su luminiscencia es alta y estable en un rango de temperaturas entre 20 º C y 30 ° C (Pinas Fernández y Wolk, 2000; Szittner y Meighen, 1990), y la viabilidad celular y el crecimiento no se han visto afectados por la integración cromosómica ni por la generación endógena de aldehído (Pinas Fernández y Wolk, 1994). El bioensayo, al igual que los bioensayos clásicos de toxicidad, ha sido diseñado para obtener información sobre la toxicidad general de una muestra, pero su principal diferencia con los anteriores radica en que el parámetro toxicológico medido no está relacionado con la inhibición del crecimiento o el contenido en pigmentos, sino en la medida de la inhibición de la bioluminiscencia. En este aspecto, este bioensayo es similar al bien establecido bioensayo de inhibición de la luminiscencia de la bacteria marina naturalmente luminiscente *Vibrio fischeri* (Microtox ®), pero con dos ventajas principales sobre el mismo: es más relevante ecológicamente en el caso de muestras de aguas continentales, ya que se basa en un organismo que vive en aguas dulces; y además, el ensayo desarrollado necesita una menor manipulación de la muestra que éste último, generando pocos problemas de alteración de la muestra para su análisis.

**CAPÍTULO I: Introducción general**

Este capítulo está dedicado a presentar un estado del arte en los siguientes temas: *En Acuatic contamination and regulation,* se hace una revisión de la legislación Europea actual en los ámbitos de la producción de productos químicos y de la protección ambiental de ecosistemas acuáticos continentales; de esta forma se hace una presentación a los lectores de los principales problemas ambientales de contaminación acuática, y las estrategias y herramientas desarrolladas para combatirlos y prevenirlos por los gobiernos e instituciones internacionales. En el epígrafe *Priority and emerging pollutants*, se describen los principales grupos de contaminantes que pueden presentarse en los ecosistemas acuáticos, así como las principales diferencias entre los contaminantes clásicos, cuyas propiedades son más o menos bien conocidas, y los contaminantes emergentes. El epígrafe titulado *Assessment of mixture toxicity* está dedicado a presentar los diferentes métodos disponibles para analizar la toxicidad de las mezclas de contaminantes, así como los conceptos más importantes en toxicología de mezclas (incluyendo los conceptos de aditividad, sinergia y antagonismo). En el epígrafe *Toxicity and ecotoxicity tests* se describen los distintos tipos de bioensayo que se han desarrollado para el análisis de la toxicidad en muestras acuáticas, y los conceptos más importantes que relacionan y diferencian la ecotoxicología de la toxicología clásica. En el epígrafe dedicado a *Microbial biorreporters,* se introduce al lector en el campo de las distintas aplicaciones que han tenido los microorganismos

como elementos sensores (biosensores) para la detección de distintas sustancias, principalmente se describen los distintos elementos sensores del sistema promotor de genes que se inducen por algún estrés o analito, y los genes *reporter* que producen la señal medible. Finalmente, en el epígrafe titulado *Cyanobacteria and their applications in environmental monitoring*, se describen las principales características de las cianobacterias que las convierten en organismos especialmente bien situados para su aplicación para la detección y el seguimiento de contaminantes en ambientes acuáticos y especialmente para la construcción de organismos *bioreporter*.

**CAPÍTULO II: Metales pesados: Toxicidad, especiación y primera aproximación para definir la interacción de tóxicos en una mezcla: el papel de los niveles de efecto.**

Este capítulo está integrado por dos manuscritos:

- *Use of lux-marked cyanobacterial bioreporters for assessment of individual and combined toxicities of metals in aqueous samples.* pp: 283-304. **Handbook on Cyanobacteria: Biochemistry, Biotechnology and Applications. Editors: Percy M Gault & Harris J Marler. Nova Science Publishers, Inc. New York. USA. ISBN: 978-1-60741-092-8 (2009).**
- *Effect of pH, EDTA, and anions on heavy metal toxicity towards a bioluminescent cyanobacterial bioreporter.* **Archives of Environmental Contamination and Toxicology. 57: 477-478 (2009).**

En este capítulo se realizó una optimización y calibración de las condiciones para aplicar el bioensayo de toxicidad basado en *Anabaena sp.* PCC 7120 CPB4337 (en adelante *Anabaena* CPB4337) al estudio de la toxicidad de muestras acuosas, y fue aplicado para evaluar la toxicidad individual y en mezclas de varios metales pesados y metaloides. Actualmente la contaminación por metales pesados continúa siendo una de las más peligrosas y preocupantes a nivel global, debido a la gran toxicidad de los metales pesados para los organismos acuáticos y terrestres, sus complejas dinámicas de biodisponibilidad marcada por su especiación y debido a que ordinariamente se presentan en escenarios de contaminación multimetálica.

En el primer manuscrito se optimizaron las condiciones para aplicar el bioensayo de toxicidad basado en *Anabaena* CPB4337 y se realizó una calibración del bioensayo frente a un tóxico de referencia (Cu) a distintos tiempos de exposición (1h y 24h). Se evaluó la toxicidad aguda y crónica de varios metales pesados y metaloides y se realizó, por primera vez en ecotoxicología,

una aplicación del método del Índice de Combinación (IC) para el estudio de la toxicidad conjunta de 3 metales pesados: Cu, Zn y Cd; dicho Índice de Combinación (IC) es un método ampliamente utilizado en farmacología para determinar la naturaleza (sinergia, aditividad o antagonismo) y la intensidad de la interacción entre fármacos. Los resultados de este trabajo demostraron que el nuevo bioensayo de toxicidad basado en *Anabaena* CPB4337 puede ser aplicado exitosamente en ensayos de toxicidad agudos (de 1h de tiempo de exposición) y crónicos (24h de tiempo de exposición); la calibración del ensayo utilizando cobre como toxico de referencia demostró que el bioensayo tiene unos niveles de reproducibilidad similares a los publicados por la EPA (USEPA, 2002) para ensayos estándar de ecotoxicidad acuática y por tanto puede ser aplicado con seguridad en análisis ambiental. El Índice de Combinación (IC) demostró ser un método adecuado para su aplicación en el estudio de la toxicidad ambiental de mezclas de contaminantes. El estudio de la toxicidad de mezclas de metales pesados empleando el método del Índice de Combinación nos permitió concluir que el tipo de interacción de los metales pesados presentaba una dependencia de los niveles de efecto ($EC_x$) producidos sobre la cianobacteria: En general, el tipo de interacción mostrada por los metales pesados en las mezclas binarias y la mezcla ternaria fue antagónica a niveles bajos y medios de efecto, pero devino sinérgica a altos niveles de efecto.

En el segundo manuscrito se realizó un estudio sobre el efecto de varios factores modificantes de la biodisponibilidad de los metales pesados sobre la toxicidad para *Anabaena* CPB4337 del Hg, Zn, Cd. Los factores modificantes elegidos fueron pH, EDTA (como modelo de ligando orgánico), y algunos aniones presentes en las aguas naturales ($PO_4^{3-}$, $CO_3^{2-}$y $Cl^-$). Se empleó un programa de especiación química para estudiar la distribución y abundancia de las posibles formas iónicas de los metales en presencia de los distintos factores modificantes, y se realizaron análisis de correlaciones para intentar ligar toxicidad y especiación. Los resultados de este trabajo mostraron que en general, la toxicidad de los metales pesados está relacionada con la cantidad de ión libre en la solución. Sin embargo, ciertos complejos de Zn-EDTA y ciertas sales ácidas cloradas de mercurio parecieron presentar también cierta toxicidad para *Anabaena* CPB4337. Un hecho interesante detectado en este trabajo, es que pequeñas cantidades de $PO_4^{3-}$ y $CO_3^{2-}$, producían una mayor toxicidad de los metales sin estar relacionado aparentemente con cambios en la especiación. Dichas variaciones en la toxicidad de los metales pesados se atribuyeron tentativamente a un posible efecto de modulación de dichas sales sobre la toma o el metabolismo de esos metales pesados. Este estudio pone de manifiesto que la combinación de los estudios de toxicidad y un conocimiento más profundo sobre la especiación y dinámica de los metales pesados y de los factores que pueden modular su toxicidad puede ser muy útil a la hora de interpretar datos de toxicidad complejos y aparentemente contradictorios.

**CAPÍTULO III: Eco-toxicidad de contaminantes emergentes I: Toxicidad individual y combinada de reguladores lipídicos.**

Este capítulo está integrado por dos manuscritos:

- *Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms*. **Environmental Science and Pollution Research. 17: 135-144 (2010).**
- *Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms.* **Water Research. 44: 427-438 (2010).**

Este trabajo fue desarrollado en colaboración con el grupo de investigación del Dr. Roberto Rosal, del departamento de Ingeniería Química de la Universidad de Alcalá. Madrid (España).

En el presente capítulo se evaluó la toxicidad mostrada sobre varios organismos acuáticos de un grupo de compuestos derivados del ácido fíbrico llamados fibratos. Los fibratos son medicamentos que se engloban dentro del grupo de los reguladores lipídicos y son, junto con las estatinas, los medicamentos más utilizados en los países desarrollados para el control de la hiperlipidemia. Los fibratos, junto con otros muchos productos farmacéuticos y cosméticos han sido detectados repetidamente en los últimos 20 años en los efluentes de las depuradoras, lagos, ríos, embalses, e incluso en aguas de consumo humano. Estas sustancias son actualmente consideradas como una amenaza potencial para la salud y el medio ambiente y son denominadas conjuntamente como *contaminantes emergentes*, ya que son diferentes de los contaminantes clásicos (herbicidas, pesticidas, etc.), no existe ninguna regulación ambiental sobre ellos, y cuyos mecanismos farmacológicos de acción son bien conocidos, pero sus posibles efectos sobre organismos no diana son actualmente desconocidos.

En el primer manuscrito se llevó a cabo una evaluación ecotoxicológica de los efectos agudos y crónicos de cuatro fibratos: el ácido fenofíbrico, el gemfibrozil, el bezafibrato y el ácido clofíbrico, sobre tres organismos acuáticos: la bacteria naturalmente luminiscente *Vibrio fischeri*, el macroinvertebrado de agua dulce *Daphnia magna* (ambos son ensayos clásicos de toxicidad), y el nuevo test de toxicidad basado en la cianobacteria autoluminiscente *Anabaena* CPB4337. La toxicidad individual de cada fibrato fue evaluada en su medio de ensayo y en muestras fortificadas, en las que los fibratos eran disueltos en un agua residual procedente del clarificador secundario de una depuradora de Alcalá de Henares (Madrid) que recibe aportes

urbanos y de algunas industrias de la zona. De entre los fibratos, la toxicidad del ácido fenofíbrico fue especialmente elevada para los tres organismos, y especialmente para *Vibrio fischeri* con una $EC_{50}$ de 1,72 mg/L. El nuevo test basado en *Anabaena* CPB 4337 resultó ser particularmente sensible a los fibratos bezafibrato, gemfibrozil y al ácido clofíbrico y su introducción en la batería de organismos ensayados permitió reclasificar el bezafibrato y el ácido clofíbrico de *no tóxico* ($EC_{50}$ > 100 mg/L) a *dañino para los organismos acuáticos* (10 mg/L < $EC_{50}$ < 100 mg/L). La fortificación de las muestras resultó en una disminución de la toxicidad para los cuatro compuestos analizados en los tres organismos. En el caso del agua residual *per se,* esta resultó ser inocua para los test de toxicidad clásicos empleados (*Vibrio fischeri* y *Daphnia magna*), pero muy tóxica para *Anabaena* CPB4337 con una inhibición de la luminiscencia del 87%. Los resultados obtenidos enfatizan la necesidad de desarrollar nuevos tests de ecotoxicidad de forma que los posibles efectos adversos para grupos ecológicos importantes de sustancias que puedan llegar al medio natural no sean pasados por alto.

En el segundo manuscrito se realizó un estudio de la naturaleza de la interacción de los tres fibratos que presentaron una mayor toxicidad en el estudio anterior, en los dos organismos acuáticos bioluminiscentes: *Vibrio fischeri,* y el nuevo test de ecotoxicidad *Anabaena* CPB4337. Para ello, se aplicó el método del índice de Combinación (IC) al estudio de la interacción de los tres fibratos entre sí en mezclas binarias y su mezcla ternaria, y de los fibratos con un agua residual en el caso de *Anabaena* CPB4337. Las conclusiones de este estudio son que compuestos con un mecanismo farmacológico de acción similar pueden mostrar un alto grado de interacción (sinergias y antagonismos) en sus efectos toxicológicos sobre organismos no diana; también se descubrieron en el presente estudio, que el tipo de interacción que muestran dos sustancias puede variar de forma extrema en un mismo organismo dependiendo del nivel de efecto. También se observó que, dependiendo del tipo de organismo que se use, el tipo de interacción de las sustancias puede variar en gran medida: para *Vibrio fischeri*, la interacción de los fibratos resultó ser básicamente antagónica a niveles de efecto bajos y medios, pero devino aditiva o incluso sinérgica a altos niveles de efecto; en el caso de *Anabaena*, los fibratos fueron extremadamente sinérgicos a bajos niveles de efecto, y extremadamente antagónicos a altos niveles de efecto. En el caso de las mezclas con agua residual, todos los fibratos resultaron ser sinérgicos en todo el rango de niveles de efecto para *Anabaena* CPB4337.

**CAPÍTULO IV: Ecotoxicidad de contaminantes emergentes II: Interacción de surfactantes perfluorados con contaminantes prioritarios y emergentes.**

Este capítulo está integrado por dos manuscritos:

- *Ecotoxicological assessment of surfactants in the aquatic environment: Combined toxicity of docusate sodium with chlorinated pollutants*. **Chemosphere. 81: 288-293. (2010).**
- *Ecotoxicological interaction of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants.* **ENVIADO PARA SU PUBLICACIÓN.**

Este trabajo fue desarrollado en colaboración con el grupo de investigación del Dr. Roberto Rosal, del departamento de Ingeniería Química de la Universidad de Alcalá. Madrid (España).

Los surfactantes son productos sintéticos empleados en una gran variedad de procesos industriales y como constituyentes de materiales de uso y consumo. Los surfactantes perfluorados (PFC) están constituidos por un esqueleto alifático, típicamente de 4 a 14 átomos de carbono, en el que todos los hidrógenos han sido sustituidos por átomos de fluor, y una cabeza polar que les dota de sus propiedades anfipáticas. Los PFC han llamado la atención de la comunidad científica y los organismos internacionales de regulación ambiental debido a su persistencia, toxicidad, y a su capacidad de bioacumulación en la vida silvestre y los seres humanos. En el año 2000, la US-EPA retiró del uso el ácido perfluorooctanoico (PFOA) y el ácido perfluorosulfónico (PFOS) para evitar la contaminación ambiental y los riesgos potenciales para la salud humana, la OCDE, en 2002, declaró estas sustancias biopersistentes, bioacumulativas y tóxicas para los mamíferos. El PFOS fue finalmente prohibido en Europa por la Directiva 2006/122 / CE y recientemente ha sido añadido al anexo B del Convenio de Estocolmo sobre Contaminantes Orgánicos Persistentes.

En el primer manuscrito, se evaluó la toxicidad individual del PFOS y el PFOA, junto con la de algunos de sus principales potenciales sustitutos comerciales, empleando para ello tres organismos acuáticos: la bacteria marina naturalmente luminiscente *Vibrio fischeri*, el alga verde de agua dulce *P. subcapitata*, y la cianobacteria recombinante bioluminiscente *Anabaena* CPB4337. Los posibles sustitutos de PFOA y PFOS ensayados fueron: el sulfonato de perfluorobutano (PFBS), el PolyFox PF-656 y el sodio sulfosuccinato (docusato de sodio). El Docusato sódico presentó la toxicidad más elevada para los tres organismos analizados y fue

seleccionado para investigar sus posibles interacciones toxicológicas con dos compuestos clorados: el triclosán y el 2,4,6-triclorofenol (TCP) utilizando para su análisis el método del Índice de Combinación (IC). En general, la mezcla binaria de los compuestos clorados triclosán y TCP exhibió antagonismo, mostrando el grado más alto de antagonismo en el caso del alga verde *P. subcapitata*. Las mezclas binarias de docusato sódico con TCP o triclosán fueron sinérgicas en niveles medios y altos de efecto para los dos organismos procarióticos bioluminiscentes, pero fue antagonista en el caso del alga verde. Curiosamente, la mezcla ternaria de los tres compuestos (docusato, TCP y triclosán) resultó ser sinérgica para los tres organismos analizados. Los resultados de este trabajo ponen de manifiesto el riesgo potencial asociado con la presencia de este surfactante con otros contaminantes.

En el segundo manuscrito se evaluó la interacción toxicológica de PFOS y PFOA con una serie de contaminantes prioritarios y emergentes: Hg, Cd, ácido 2-(2,4-dichlorophenoxiacético), propilparabén, mitomicina C y furazolidona, en mezclas binarias, ternarias y complejas empleando el índice de combinación (IC), utilizando la cianobacteria recombinante bioluminiscente *Anabaena* CPB4337. De acuerdo a sus toxicidades individuales, Hg, Cd, mitomicina C y furazolidona pudieron ser clasificados como *muy tóxicos para los organismos acuáticos*, según el Reglamento (CE) n º 1272/2008. La mezcla binaria de PFOA y PFOS resultó ser antagónica en todo el rango de niveles de efecto, esto puede explicar en parte el hecho de que PFOA y PFOS tuvieron una interacción inversa con los contaminantes orgánicos ensayados, pudiendo ser relacionado con su grado de hidrofobicidad: PFOA, más hidrofóbico que PFOS, interaccionó sinérgicamente con el compuesto más hidrofóbico (propilparabén), y antagónicamente con el más hidrofílico (2,4-D), mientras que el PFOS, más hidrofílico que PFOA, interaccionó con ellos de forma inversa, es decir sinérgicamente con el 2,4-D, y antagónicamente con el propilparabén. La interacción de PFOA y PFOS con los metales pesados fue antagónica en todos los casos, lo que puede explicarse por un efecto quelante de ambos surfactantes aniónicos. Con el incremento de la complejidad de las mezclas, el IC predijo sinergia a bajos niveles de efecto, dicha predicción fue comprobada experimentalmente realizando mezclas de los contaminantes al nivel de sus NOEC (*No Observed effect Concentration*).

**CHAPTER V: *Physicochemical Characterization and Ecotoxicological Assessment of $CeO_2$ Nanoparticles Using Two Aquatic Microorganisms*. Toxicological Sciences 119: 135-145. (2011).**

Este trabajo fue desarrollado en colaboración con el grupo de investigación del Dr. Roberto

Rosal, del departamento de Ingeniería Química de la Universidad de Alcalá de Henares. Madrid (España).

Las nanopartículas o partículas ultrafinas siempre han existido en la naturaleza, pero la gran expansión en tipos y cantidades de nanopartículas de síntesis que se está produciendo actualmente ha producido un clima general de preocupación en la comunidad científica y los organismos de regulación internacional debido a sus posibles propiedades toxicológicas desconocidas y su posible impacto potencial en el medio ambiente. Un aspecto clave para la evaluación de la ecotoxicidad de la nanopartículas de síntesis es la necesidad de abordar su comportamiento físico-químico en medios biológicamente relevantes. De entre las nanopartículas de óxidos metálicos, las nanopartículas de oxido de cerio tiene una amplia gama de aplicaciones, incluyendo la formulación de pastas para la planificación quimio-mecánica (CMP) de láminas de silicona en la producción de circuitos impresos. La posible toxicidad de las nanopartículas y sus mecanismos de acción siguen siendo actualmente aspectos esencialmente desconocidos. En el caso de las nanopartículas metálicas aún no está claro si la internalización de las partículas es importante para la manifestación de efectos tóxicos, o si estos se deben sólo a la adsorción de las nanopartículas a las membranas o paredes celulares. Otros factores clave para dilucidad las propiedades tóxicas de las nanopartículas son la importancia del tamaño de partícula en su toxicidad, su carga neta y la químico-física de superficies, así como las causas para la toxicidad detectada en partículas de óxidos metálicos de tamaño no nano. En el presente trabajo, se realizó una caracterización ecotoxicológica de cuatro tipos distintos de nanopartículas de oxido de cerio empleando dos organismos acuáticos fotosintéticos: el alga verde *pseudokirchneriella subcapitata* y la cianobacteria *Anabaena* CPB4337. Los óxidos de cerio empleados incluían materiales comerciales y se distinguían básicamente por el tamaño nominal de partícula, que iba de 10 nm para N10 hasta los 50 nm para N50. Su toxicidad fue comparada con la de un oxido de cerio particulado de tamaño micrométrico y con cloruro de cerio como controles. Conjuntamente a la caracterización toxicológica, se realizó una caracterización físico-química de los materiales empleados (formación y distribución de tamaño de agregados, potencial Z, composición química y área superficial), dicha caracterización se llevó a cabo en los distintos medios de ensayo para poder relacionar la toxicidad producida en los organismos con las condiciones físico-químicas de las nanopartículas. La toxicidad de las nanopartículas de óxidos de cerio expresada como $EC_{50}$ para *Anabaena* a 24 h de exposición en agua destilada varió entre de 0,27 y 6,3 mg/L, siendo menor conforme aumentaba el pH y la concentración de sales en los medios ensayados; en el caso de *Pseudokirchneriella subcapitata* los valores de $EC_{50}$ variaron entre 2,4 a 29,6 mg/L. Las imágenes de microscopía óptica y electrónica mostraron disrupción de membranas y células

altamente dañadas en ambos organismos. No se encontraron evidencias de internalización de las nanopartículas, pero en el caso de *Anabaena*, se encontraron células completamente cubiertas por nanopartículas con las cubiertas celulares en distintos niveles de degradación. Los resultados del presente trabajo sugieren que es necesario un contacto directo de las nanopartículas con las células para producir efectos tóxicos.

**SUMMARY**

Ecological degradation of environmental ecosystems is a consequence of human activity, especially since the industrial revolution in XIX Century which implied an exponential growth of population, conglomeration in big cities and industrial and technological development based on chemical industry. All these factors have caused the massive entry of natural and xenobiotic byproducts in aquatic ecosystems as residues of agriculture, industrial activities and residual runoffs from cities.

As a consequence of environmental degradation and human health effects, states and international regulatory entities have established in the last 20 years different quality standards which implied a multidirectional approach based on research on health and ecological effects of substances, the establishment of legal quality criteria for human and ecosystem protection and the watchfulness for the performance of these quality criteria and their goodness to get the desired level of health and environmental protection. In the case of aquatic ecosystems, this implies the continuous development of analytical techniques in order to be able to measure the increasing number of substance used in human activity, and the development of biological methods to determine the possible adverse effects of these substance on aquatic ecosystems.

The aim of the present study was to evaluate the applicability of a new general ecotoxicity test to determine the individual and combined toxicity of different classes of environmental pollutants. This new ecotoxicity test is based on a freshwater filamentous cyanobacterium of the Order Nostocales: an *Anabaena* PCC 7120 derivative strain named *Anabaena* CPB4337 which carries the whole operon *luxCDABE* of the terrestrial luminescent bacteria *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) which are expressed constitutively, being self-luminescent. The luminescence is high and stable in a range of temperatures between 20 ºC and 30 °C (Fernandez Pinas and Wolk, 2000; Szittner and Meighen, 1990), and cell viability and growth were not affected by the chromosomal integration or by the generation of endogenous aldehyde (Fernandez Pinas and Wolk, 1994). The test has been designed to get general toxicity information of a sample as classical toxicity tests but the main difference with these tests is that the measured end-point is not related with growth inhibition or pigment content but with direct measure of bioluminescence inhibition. In this regards, this test is similar to the well established *Vibrio fischeri* based toxicity test (as Microtox®) but is more ecologically relevant since it is based on a freshwater organism and the assay developed needs a minimal sample manipulation.

**CAPTER I: General introduction**

In this chapter a state of the art is presented on the following topics: In *Aquatic contamination and regulations,* the actual European legislations on chemical production and aquatic environment protection are reviewed; readers are introduced to the main problems related to the aquatic contamination and the main strategies of governments and international agencies to deal with these problems. In *Priority and emerging pollutants*, the main aquatic contaminants are presented, and the main differences between the classical well known contaminants and the emerging pollutants are exposed. *Assessment of mixture toxicity* and *toxicity*, is dedicated to introduce the different available methods to analyze the toxicity of mixtures and the main relevant concepts on mixture toxicology (including the concepts of additivity, synergism and antagonism); and *Toxicity and ecotoxicity test* to describe the different biotests developed for the assessment of the toxicity of aqueous samples and the most fundamental concepts of ecotoxicology related to classical toxicology. In *Microbial bioreporters*, readers are introduced to the field of the different applications that microorganisms have as sensing elements (bioreporter) for the detection of different substances, mainly based on genetic fusions of stress/analyte-responsive promoters and reporter genes. And finally, in *Cyanobacteria and their applications in environmental monitoring* the main characteristics of cyanobacteria are reviewed focusing on their particular skills to be chosen as model organisms for environmental application, especially as bioreporters of environmental pollution in aquatic ecosystems.

**CHAPTER II: Heavy metals: speciation, toxicity and first approach to define toxicant interactions in a mixture: the role of effect levels.**

This chapter is integrated by two manuscripts:

- Use of l*ux*-marked cyanobacterial bioreporters for assessment of individual and combined toxicities of metals in aqueous samples. pp: 283-304. **Handbook on Cyanobacteria: Biochemistry, Biotechnology and Applications. Editors: Percy M Gault & Harris J Marler. Nova Science Publishers, Inc. New York. USA. ISBN: 978-1-60741-092-8 (2009).**
- Effect of pH, EDTA, and anions on heavy metal toxicity towards a bioluminescent cyanobacterial bioreporter. **Archives of Environmental Contamination and Toxicology. 57: 477-478 (2009).**

In this chapter we evaluate the toxicity of heavy metals to *Anabaena sp*. PCC 7120 CPB4337. Heavy metal contamination is actually a major worry due to their high toxicity to aquatic and soil organisms, their complex speciation which mark their bioavailability and the common multi-metal contamination episodes.

In the first manuscript, a toxicity assay based on the bioluminescent *Anabaena sp.* PCC7120 CPB4337 to determine single and combined toxicity of pollutants is developed and applied to investigate the toxicity of heavy metals. Calibration of the bioassay is established for different times of exposure (1h and 24h) with Cu as reference toxicant. Individual toxicities of heavy metals and some metalloids are assessed in acute and chronic toxicity assays. Combination Index (CI) method, a widely used method to determine the nature (synergism, additive effect and antagonism) and the degree of drug interactions in pharmacology is applied by the first time in ecotoxicology to assess the nature of the interaction of three heavy metals (Cu, Cd and Zn) in binary and ternary mixtures. Results of this work revealed that the bioassay allowed for acute and chronic toxicity testing. The calibration of the assay using copper as reference toxicant has demonstrated that the levels of test reproducibility are within those reported by USEPA (USEPA, 2002) for other standardized toxicity test. The combination index-isobologram equation has proven to be a suitable method for combined toxicity assessment. The study of the combined effect of heavy metals revealed a dependence on the level of the effect (that is effective concentrations, $EC_x$) of the nature of the interaction of heavy metals. In general, the toxicity of binary and ternary combinations of Cd, Cu and Zn was antagonistic at low effect levels but synergistic at high effect levels.

In the second manuscript, the effect of several modifying factors of heavy metal bioavailability was evaluated. on the toxicity of Hg, Cu, Zn and Cd towards Anabaena PCC 7120 CPB4337 The selected modifying factors were pH, EDTA (as organic ligand), and some anions present in different concentrations in fresh waters ($PO_4^{3-}$, $CO_3^{2-}$ and $Cl^-$). Chemical modeling and correlation analyses were used to predict metal speciation and link it with toxicity. In general, metal toxicity significantly correlated to the predicted metal free-ion concentration, although Zn–EDTA complexes and certain Hg chloro-complexes could also exhibit some toxicity to *Anabaena*. An interesting feature of metal toxicity to strain Anabaena CPB4337 was that low amounts of $PO_4^{3-}$ and $CO_3^{2-}$ increased metal toxicity; this effect could not be related to significant changes in metal speciation and could be attributed to a modulating effect of these anions on metal/uptake toxicity. A conclusion of this study is that the combination of toxicity studies that take into account a range of factors that might modulate metal toxicity with

chemical modeling to predict changes in metal speciation might be useful for interpreting complex toxicity data.

**CHAPTER III: Eco-toxicity of emerging pollutants I. Individual and mixture toxicity of lipid regulators.**

This chapter is integrated by two manuscripts:

- Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms. **Environmental Science and Pollution Research. 17: 135-144 (2010)**
- Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms. **Water Research. 44: 427-438 (2010).**

This work was developed in collaboration with the research group of Dr. Roberto Rosal from the department of Chemical Engineering of the Universidad de Alcalá. Madrid (Spain).

In this chapter we evaluated the ecotoxicity of lipid regulators named fibrates to several aquatic organisms. Fibrates are a class of drugs derived from fibric acid widely used to reduce plasma triglycerides and raise the level of high density lipoprotein (HDL) in order to reduce free cholesterol. Together with statins, they are they are the most widely used antilipidemic drugs in developed countries. Fibrates, together with other pharmaceutical and cosmetical care products have been repeatedly reported in last 20 years in waste water treatment plants effluents, freshwaters and even in drinking waters. They have been considered as emerging contaminants (ECs) because they are different from the classical priority pollutants; they are not regulated in the existing water legislation and their pharmacological mode of actions is usually well documented but their possible effect on non-target organisms and their toxicological mode of action remains essentially unknown.

In the first report, individual toxicity of four fibrates: Chlorfibric acid, fenofibric acid, gemfibrozil and bezafibrate was evaluated in acute and chronic toxicity assays using the following aquatic organisms: the naturally bioluminescence bacteria *Vibrio fischeri,* the fresh water macroinvertebrate *Daphnia magna* (both classical toxicity tests), and the self-luminescent *Anabaena* CPB4337 toxicity test. The individual toxicity of each compound was evaluate in the assay media and in fortified samples in which the fibrates were dissolved in real wastewater

samples taken from the effluent of a wastewater treatment plant. The toxicity of fenofibric acid was particularly high with $EC_{50}$ values of 1.72 mg/L for *V. fischeri*. The new test *Anabaena* CPB4337 showed a greater sensitivity for bezafibrate, clofibric acid and gemfibrozil. The introduction of *Anabaena* bioassay in ecotoxicity testing would allow to reclassify bezafibrate and clofibric acid from "non-toxic" ($EC_{50}$ > 100 mg/L) to "harmful to aquatic organisms" (10 mg/L < $EC_{50}$ < 100 mg/L). For the three toxicity tests fortification resulted in lower measured toxicity for the four compounds. The wastewater itself was found to be very toxic to *Anabaena* CPB4337 (84 % of bioluminescence inhibition) whereas it did not have any negative effect on *Daphnia magna* or *Vibrio fischeri*. The introduction of a new toxicity test (*Anabaena* CPB4337) revealed a certain risk associated with the three less toxic compounds tested. These results emphasize the need to develop new and more sensitive toxicity tests for the detection of unwanted toxic effects that might become overlooked using conventional bioassays.

In the second report, we assess the nature of the toxicological interactions of three fibrates, gemfibrozil, bezafibrate and fenofibric acid, by the method of combination index (CI)-isobologram equation. The three pharmaceuticals were used singly and in two- and three-drug combinations. As toxicity test we used the naturally luminescent bacterium *Vibrio fischeri* and the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC 7120 CPB4337. For the last one, we also evaluated the nature of the interactions of the three fibrates with the wastewater sample used in the previous study. Findings of this work were that drugs with the same pharmacological mode of action can have important synergistic/antagonistic toxicological effects on non-target organisms. These effects can greatly vary depending on the effect level in the same organism, and also differs with the organism used. In the *Vibrio* test, the fibrate combinations showed antagonism at low effect levels that turned into an additive effect or synergism at higher effect levels; by contrast, in the *Anabaena* test, the fibrate combinations showed a strong synergism at the lowest effect levels (with combined effects ten-fold higher than those expected from an additive effect) and a very strong antagonism at high effect levels. The interaction of all fibrates with the wastewater sample in *Anabaena* sp. PCC 7120 CPB4337 was synergistic in the whole range of effect levels.

**CHAPTER IV: Eco-toxicity of emerging pollutants II. Interaction of perfluorinated surfactants with priority and emerging pollutants.**

This chapter is integrated by two manuscripts:

- Ecotoxicological assessment of surfactants in the aquatic environment: Combined toxicity of docusate sodium with chlorinated pollutants. **Chemosphere. 81: 288-293. (2010).**
- Ecotoxicological interaction of perfluorooctane sulfonate (PFOS) and perfluorooctanaic acid (PFOA) with selected pollutants. **SUBMITTED.**

This work was developed in collaboration with the research group of Dr. Roberto Rosal from the department of Chemical Engineering of the Universidad de Alcalá. Madrid (Spain).

Surfactants are synthetic chemicals used in large amounts in a variety of industrial cleansing processes as well as in consumer products. Among surfactants, Perfluorinated chemicals (PFCs) are synthetic fluorinated compounds composed of a carbon backbone (typically 4-14 atoms in length) and a charged functional group. PFCs have caught the attention of regulatory agencies because of their persistence, toxicity, and widespread occurrence in the blood of general populations and wildlife. In 2000, the US-EPA declared PFOS and PFOA withdrawal to avoid environmental pollution and potential health risks; the OECD in 2002 declared these substances as biopersistent, bioaccumulative and toxic to mammalians; PFOS was finally banned in Europe by the Directive 2006/122/EC and recently added to the Annex B of the Stockholm Convention on Persistent Organic Pollutants.

In the first manuscript, we assessed the individual toxicity of PFOS and PFOA, together with some of their main commercially available potential substitutes such as perfluorobutane sulfonate (PFBS), the PolyFox PF-656 and the sulfosuccinate derivative docusate sodium by using three aquatic organisms: the marine bacterium *Vibrio fischeri*, the fresh water alga *P. subcapitata* and the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC 7120 CPB4337. Docusate sodium exhibited the highest toxicity for the three tested organisms and was selected to investigated its toxicological interactions with two chlorinated compounds, triclosan and 2,4,6-trichlorophenol (TCP), in their binary and ternary mixtures using the method of the combination index (CI). In general, the binary mixture of the chlorinated compounds triclosan and TCP exhibited antagonism, which was stronger for *P. subcapitata*. The binary mixtures of docusate sodium with TCP or triclosan showed synergism at medium to high effect levels for the prokaryotic organisms, but antagonism for the green alga. Interestingly, the synergistic behavior predominated in the ternary mixture for the three tested organisms. This result highlights the potential toxicological risk associated with the co-occurrence of this surfactant with other pollutants.

In the second manuscript, we evaluated the toxicological interaction of PFOS and PFOA with selected priority and emerging pollutants [$Hg^{2+}$, $Cd^{2+}$, 2-(2,4-dichlorophenoxi) acetic acid, propyl paraben, mitomycin C and furazolidone] in binary, ternary and multicomponent mixtures to the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC 7120 CPB4337 using the Combination Index (CI) method. According to individual toxicities, $Hg^{2+}$, $Cd^{2+}$, mitomycin C and furazolidone could be classified as "very toxic to aquatic life" according to Regulation (EC) No 1272/2008. Potential toxicological interactions of PFOA, PFOS with these pollutants in binary, ternary and multicomponent mixtures were studied using the Combination Index (CI) method. PFOA and PFOS showed an antagonistic interaction at the whole range of effect levels, this may explain in part the fact that PFOA and PFOS interacted in an inverse way with the organic pollutants; the relative hydrophobicity of the tested compounds would also explain this interaction pattern as PFOA, more hydrophobic than PFOS, interacted synergistically with the most hydrophobic compound propylparaben and antagonistically with the most polar one, 2,4-D, while PFOS interacted just in the opposite way with both chemicals. The interaction of both PFOS and PFOA with heavy metals was mostly antagonistic, decreasing metal toxicity. The most plausible explanation could be the stabilization of the cations in solution by the negatively charged surfactants. With increasing complexity of the mixtures, the CI method predicted synergism at low to very low levels of effect; pollutant combinations at their mixture NOECs were tested and confirmed the predicted synergism.

**CHAPTER V: Physicochemical Characterization and Ecotoxicological Assessment of $CeO_2$ Nanoparticles Using Two Aquatic Microorganisms. Toxicological Sciences 119: 135-145. (2011).**

This work was developed in collaboration with the research group of Dr. Roberto Rosal from the department of Chemical Engineering of the Universidad de Alcalá de Henares. Madrid (Spain).

Nanosized particles have always been present in nature, but the accelerating penetration of engineered nanoparticles (ENP) in the market is raising serious concerns over their potential impact on the environment. A key aspect for the ecotoxicity assessment of ENP is the need to address their physicochemical behavior in biologically-relevant media. Cerium oxide nanoparticles have a wide range of applications including the formulation of slurries for the chemomechanical planarization (CMP) of silicon wafers in the production of integrated circuits. Fundamental questions regarding metal oxide nanoparticle toxicity remain unsolved. It is not clear whether the internalization of particles is relevant to the induction of intracellular effects

or toxicity may be due to the adsorption of nanoparticles onto the cellular membrane. The questions about the importance of particle size, surface chemistry and the reason for toxic effects observed in non-nano particles are still unsolved. In this work, four different cerium oxide nanoparticles, including commercial materials, were characterized and compared with a micron-size ceria. The formation of aggregates as well as ζ-potential, surface area, and chemical composition were determined and related with the toxicity of cerium oxide for the self-luminescent cyanobacterial recombinant strain *Anabaena* CPB4337 and the green alga *Pseudokirchneriella subcapitata*. The toxicity for *Anabaena* exposed to nanoparticles in pure water for 24 h ranged from 0.27 to 6.3 mg/L; P*seudokirchneriella subcapitata* yielded $EC_{50}$ values in the 2.4-29.6 mg/L range. Bright field and TEM images of both organisms showed membrane disruption and highly damaged cells. We found no evidence of nanoparticle uptake by cells, but our observations suggested that their toxic mode of action required direct contact between nanoparticles and cells; in the case of the cyanobacterium, cells completely coated by layers of ceria nanoparticles were observed. Cell damage most probably took place by cell wall and membrane disruption.

# CHAPTER I
# General Introduction

## CHAPTER I: General Introduction

### 1. *Aquatic contamination and regulation*

Ecological degradation of environmental ecosystems is a consequence of human activity, especially since the industrial revolution in the XIX Century. This degradation is a consequence of the exponential growth of population, the accumulation of population in big cities, the industrial development and the increasing consumption of natural resources and the consequent production of residues. All these factors have caused an increasing anthropic pressure on natural ecosystems. In the case of aquatic ecosystems it is especially intense since the massive entry of natural and xenobiotic byproducts from agricultural, industrial and domestic activities.

Due to the increasing environmental degradation and human health effects, states and international regulatory entities have established different regulatory strategies with the aim of preventing human health and the environment from adverse effects. In the beginning, these strategies were very limited and fragmented translated in short-range laws which regulated a few number of compounds, specific uses or special activities due to the low scientific knowledge about the possible adverse effect, and driven by the benefits of their use. With the increasing human development and the gradual degradation of natural environment, more efforts were made by the international community in order to measure, evaluate and prevent the risk associated with chemical substances. In the last 20 years, more protective legislations have been developed (2000/60/EC; 2006/1907/EC). They implied a multidirectional approach based on research on health and ecological effects of substances, the establishment of legal quality criteria for human and ecosystem protection and the surveillance for the performance of these quality criteria and their goodness to get the desired level of health and environmental protection. At international level, the most successful international legally binding instruments are coordinated by the United Nations Environmental Programme (UNEP): the Protocol to the regional UNECE Convention on Long-Range Transboundary Air Pollution (CLRTAP) on persistent organic pollutants (POPs), opened for signatures in June 1998 and which entered into force on 23 October 2003, and the global Stockholm Convention on POPs, opened for signatures in May 2001 and which entered into force on 17 May 2004. These instruments establish strict international regimes for an initial list of POPs (16 in the UNECE Protocol and 12 in the Stockholm Convention) including prohibition, restriction of use and programmes for the elimination of their waste. Recently they have been revised in the 4th Conference of the Parties (COP) to the Stockholm Convention in 2009, incorporating 9 new POPs to the existing list and proposing new candidates for further investigation.

In the case of aquatic ecosystems, different net regulations have been developed all around the world in the last years in order to protect not only physicochemical water quality but ecological status of water bodies, from marine to freshwater environments. In Europe, Water Framework Directive (Directive 2000/60/EC) was born with the objective of *establishing a framework for the protection of inland surface waters, transitional waters, coastal waters and ground waters* based on the following three lines of action: a) prevents further deterioration and to enhance the status of aquatic ecosystems, b) to promote sustainable water use based on long-term protection of available water resources and c) to enhance protection and improvement of the aquatic environment through specific measures for the progressive reduction of discharges of priority substances and the cessation of emissions of priority hazardous substances. A key factor in the improvement of water quality is the identification of priority and hazardous substances responsible of the toxic chemical pressure on aquatic ecosystems and the establishment of EQs (Environmental Quality Standards) and MACs (Maximum Allowance Concentrations) which will lead to get the goals of desired water quality.

The chemical compounds which will finally reach the natural environment and to which population will be exposed is directly dependent on the products introduced in the markets and used in industrial and human activity and which will finally reach the aquatic ecosystems via sewage treatment plant (STPs); so that the safety assessment of these substances needs to be performed not only to their possible direct use but taking into account their whole life cycle (Finnveden et al, 2009), including their disposal. Based on this line of thought, the European Commission adopted two framework directives to regulate the authorization and put in the market of chemical compound: the **Directive 2001/83/EC** of the European Parliament and of the Council of November 6th 2001 on the Community code relating to medicinal products for human use which states in their Guideline on environmental risk assessment (EMEA, 2005), that is very important that an environmental risk assessment (ERA) includes not only an environmental exposure assessment to estimate predicted environmental concentrations (PEC), but also appraisal factors that indicate the toxicological or pharmacological effects on biological systems of chemicals in the environment (Huscheck, 2004; EMEA, 2005). The referred guideline is actually in draft version and under revision. With the same philosophy, but one step further, REACH regulation (Registration, Evaluation and Authorization of Chemicals) (2006/1907/EC) adopted by the European Commission on 1 June 2007, applies a new integrated strategy on the regulation of the use of chemical substances in Europe. The aims of this new strategy were: *to improve protection of human health and the environment from the risk of chemicals while enhancing the competitiveness of the UE chemical industry*. This new

framework regulation tries to balance the weakness of the pre-REACH regulations which established different rules in the regulation for *existing* and *new* chemicals and proved not to provide sufficient information about the effects of the majority of existing chemicals on human health and the environment. Below these old regulations, the identification and assessment of risk of new chemicals were made by authorities and proved to be slow and inefficient. The new REACH regulation places responsibility on risk assessment of substances on industry itself, and authorities are responsible to ensure that industry is meeting its obligations and to take action on substances of very high concern. A single system is established for both existing and new substances with the following characteristics: All substances are covered by REACH unless they are explicitly exempted (basically the pharmaceutical products). Registration of substances requires obtaining relevant information of the substance and better information on hazard and risk. Substances with properties of very high concern will be made subject of authorization, restriction or prohibition. In addition, key aspects of REACH is to reduce vertebrate animal testing, the development of new *in-vitro* test, the used of (Q)SAR (Quantitative structure-activity relationship) models and the look for safer alternatives for dangerous substances. A critical factor defining the information requirements on toxicity and ecotoxicity information is the tonnage of production or import of the substance as direct indicator of exposure of population and the environment. The general requirements of REACH are developed by a series of Technical Guidance Documents with will be periodically revised. As in the **Directive 2001/83/EC**, emphasis is made in the requirements on both exposure assessments and toxicological effects of substances. A whole network on PBT (persistence, bioaccumulation and toxicity) and vPvB (very persistent and very bioaccumulative) assessment is developed with very detailed technical sections on how to integrate existing information, identify gaps and how to generate new quality data to fulfill the information requirements (ECHA, 2008).

So that, we can conclude that in the last decade, a great effort has been made in regulatory networks to improve the protection of human health and the environment in several ways: Firstly, more integrated regulations have been developed in order to minimize gaps, overlapping and confusion in producers, users and monitoring entities. Secondly, environment and human health are increasingly considered as a whole, so that a good control of human health risk is not possible without a good control of environmental risk. Thirdly, environment is also considered as a whole, and a good quality of water bodies and ecosystems in general, implies not only a good physicochemical quality but a good ecological status. And finally, legislations are endowed with extensive technical reports and guidance in order to assist industry and regulatory entities to fulfill the requirements established in legislation. This guidance and technical reports are evaluated and periodically revised by expert scientific work groups designated by the

European Commission and the Parliament in order to take into account the latest scientifically consolidated techniques and methods.

### *2. Priority and emerging pollutants*

A result of the regulatory and scientific efforts made in the last three decades to identify possible dangerous substances for human health and the environment is the increasing number of substances which have been tested for adverse effects and those which have been finally classified as hazardous to human health and the environment. Some of them have even been regulated through international instruments as those incorporated in the global Stockholm Convention on POPs (UNEP, 2009).

Besides the Water Framework Directive (2000/60/EC) in Europe, a list of 33 priority substances was recently consolidated by the Directive 2008/105/EC. This Directive established quality standards (EQS) for each individual substance as MACs and AA-EQs (Annual Average values) in order to protect aquatic organisms against acute and chronic effects. The list is integrated principally by organic contaminants such as Polycyclic Aromatic Hydrocarbons (PAHs), brominated and organochloride compounds, organic solvents; some POPs which are susceptible to appear in water and sediments as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), cyclodiene pesticides, hexaclorobenzene and also tributyltintin compounds and four heavy metals (Hg, Cd, Zn and Pb). Although this regulation marks a beginning in the real application of the WFD in the line of protecting ecosystems from the pressure of chemical contamination, its actual field of application is limited to well known and established contaminants. However, together with these recognized contaminants, an increasing number of a variety of chemical compounds reaches the aquatic systems via the STPs. These chemicals come from industrial but also from domestic activities such as pharmaceutical and cosmetic care products, cleaning agents, paints and solvents. The attention paid by the scientific community to these chemicals has increased with the development of new analytical techniques such as liquid chromatography (LC) coupled with mass spectrometry (MS) and tandem MS ($MS^2$)-, which allow to detect an increasing number of pollutants at trace levels (Petrovic, 2010; Rosal et al, 2010). These new chemicals have been called “emerging pollutants” and shared some general characteristics: they are not covered by the existing water quality regulations, they have not been studied before, or their pharmacological mode of action is well documented but their toxicological effect is unknown, and are considered as potential threats to environmental ecosystems (Cleuvers et al.2003; Farré et al, 2009; Murray, 2010). In general they have low rates of elimination in conventional STPs (Rosal et al, 2010), in some cases they can be

considered as persistent due to their low biodegradability, or pseudo-persistent due to their continuous release (Smital, 2008). A review in literature showed that emerging pollutants receiving the most attention by researchers belong to two broad groups: industrials and pharmaceuticals and personal care products (PPCPs) (Murray et al, 2010). Between industrials, anti-oxidants, perfluorated compounds, phenols (bisphenols and alkilphenols), phthalates, polybrominated diphenilethers (PBDEs) and triazoles, receive special concern because their multiple applications in industrial and consumer products, and their capacity to bioaccumulate, their endocrine disrupting properties, or other dangerous properties as developmental toxicity (Murray et al, 2010). PPCPs frequently detected in freshwater environment include a variety of analgesics, anti-epileptic drugs, antimicrobials, lipid regulators, polycyclic musks and synthetic hormones; their concern is related to their capacity to reach drinking water reservoirs and the proliferation of microbial resistances and their unknown effects on non-target organisms in the aquatic environments (Halling-Sørensen et al, 1998; Murray et al, 2010).

Thorough the present thesis, the toxicity of a selection of different priority and emerging pollutants has been investigated. Between the priority pollutants, the major work was made with heavy metals: $Hg^{2+}$, $Cd^{2+}$, $Zn^{2+}$, $Pb^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Ag^{+}$, $Mn^{2+}$ and the metalloids $AsO_4^{3-}$, $CrO_4^{2-}$, $VO_3^{-}$ and $MoO_4^{2-}$. Heavy metal contamination is one of the most extensive, complex and dangerous situations around the world (Ritter et al, 2002; Counter and Buchanan, 2004; Doop et al, 2010). Many of heavy metals are essential elements for life at trace concentrations but at high concentrations they can be very toxic to human life and the environment ( Hartwig, 2002; Moulis et al. 2010). One of the largest problems associated with heavy metals is their persistence due to the fact that they cannot be degraded like organic pollutants but only transformed. They can remain dormant in a non bioavailable form during decades but a change in the physicochemical conditions (as a drought) can make them bioavailable (Peijnenburg et al. 2003) Also of concern is their potential for bioaccumulation and biomagnification through the food webs causing problems in natural environment, agricultural fields and their water-receiving streams (Agusa el al, 2010). Heavy metal pollution can be point specific or diffuse. In highly contaminated areas it commonly arises from mining or the purification of metals, (Ritter et al, 2002), but also from natural contaminated soils and ground waters (as in Almadén in Spain or Bangladesh) (Brinkel el al, 2009; Berzas Nevado et al, 2009). Other sources of heavy metal contamination were lead-containing hunting material, soil organic amenders from composts and sewage sludge (Smith et al. 2009) and atmospheric deposition from residues and thermoelectric incinerators.Within the European community the eleven elements of highest concern are As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sn, and Tl (EC/1272/2008; EC/105/2008).

Among emerging pollutants, we chose three interesting but very different group of substances: The first group was fibrates (Figure 1), which are pharmaceutical products used to reduce plasma triglycerides and raise the level of high-density-lipoproteins for reducing plasma cholesterol. They have a very specific and well characterized mode of action as they exert their biological effects by binding the peroxisome proliferator-activated receptor α (PPAR α); however their mechanism of toxic action on non-target organisms is scarcely unknown. Fibrates have been stated to be excreted unmodified (Isidori et al. 2007) and have been detected from freshwaters to ground water and even in drinking waters (Andreozzi, 2003; Fent et al, 2006). They are candidates to be catalogued as persistent pollutants as they have high log octanol-water partition coefficients (log $K_{ow}$) (close to 5) and very low efficiencies of elimination in conventional STPs being detected in their effluents at concentrations from hundreds of ng/L to up to 500 µg/L (Stumf et al. 1999; Matcalfe et al. 2003; Fent el al. 2006).

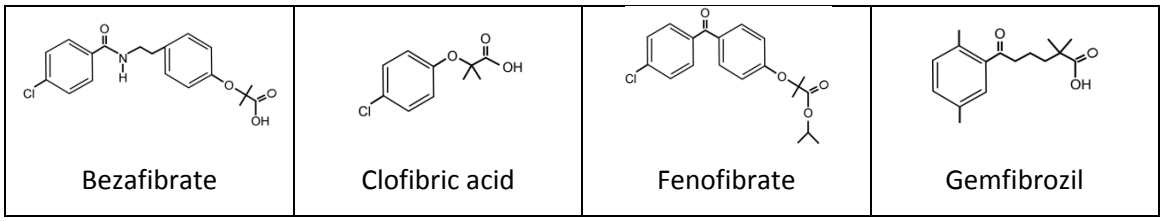

Figure 1. Chemical structure of the four fibrates used in the present thesis.

The second group of emerging pollutants was perfluorinated surfactants (PFCs) and some of their possible environmentally friendly substitutes. A perfluorinated compound (PFC) is an organofluorine compound with all hydrogens replaced by fluorine on a carbon chain—but the molecule also contains at least one different atom or functional group. PFCs have properties similar to fluorocarbons but they have unique properties to make materials stain, oil, and water resistant, and are widely used in diverse applications (Lehmler, 2005; OECD, 2006). The strong covalent bond between the fluor and carbon ions makes PFCs thermally and chemically stable and makes them highly resistant to both chemical and biological degradation. There are many PFCs, but the two most studied are: PFOA or perfluorooctanoic acid, used to make fluoropolymers such as Teflon, among other applications, and PFOS or perfluorooctanesulfonic acid (Figure 2), used in the semiconductor industry, 3M's former Scotchgard formulation, and 3M's former fire-fighting foam mixture (Lehmler, 2005; OECD, 2006). Due to their resistance to abiotic and biotic degradation, PFCs, particularly PFOS and PFOA, are ubiquitous contaminants widely distributed in the global environment; they have been found in air, surface waters, and drinking waters at concentrations gradients of up to several orders of magnitude (pg/L up to µg/L) (Nakayama et al. 2008; Ericson et al., 2009; Loos et al, 2010; Murray et al., 2010); accidental spills form fire training sites or fluorochemical production plants may

introduce PFCs at high concentrations (maximum of 2,210 µg/L) (Moody et al., 2002). In human and animal tissues their concentration may also reach elevated levels (up to 30,000 µg/L) in industrialized areas (Houde et al., 2006). The concerns about possible toxic effect of PFCs has increased due to their global occurrence, persistence and reported toxic effects (Beach, 2006; Hu and Hu, 2009; Eriksen et al., 2010); finally, in 2000, the US-EPA declared PFOS and PFOA withdrawal to avoid environmental pollution and potential health risks and the OECD in 2002 declared these substances as persistent, bioaccumulative and toxic to mammalians. PFOS was banned in Europe by the directive 2006/122/EC and was recently included to the Annex B of the Stockholm Convention on Persistent Organic Pollutants.

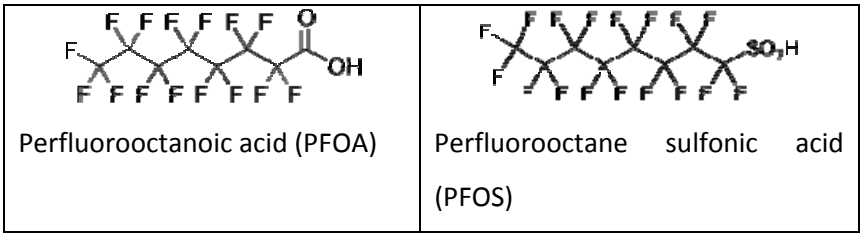


Figure 2. Chemical structure of PFOA and PFOS

The last group of emerging pollutants whose toxicity has been investigated in the present thesis was engineered nanoparticles, specifically different types of $CeO_2$ (ceria) nanoparticles (Figure 3). Nanosized particles have always been present in nature, but the accelerating penetration of engineered nanoparticles (ENP) in the market is raising serious concerns over their potential impact on the environment. In nanotechnology, a particle is defined as a small object that behaves as a whole unit in terms of its transport and properties (Evans et al, 1999). Nanoparticles are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nano-scale, size-dependent properties are often observed and are the base of the special applications of nanoparticles in biomedical, optical and electronic fields (Kami et al, 2011; Mahmoudi et al, 2011). Cerium oxide nanoparticles have a wide range of applications, including their use as additive in diesel fuel in which ceria acts as storage unit for oxygen, taking it from nitrogen oxides to oxidize carbon monoxide and unburnt hydrocarbons (Bumajdad et al, 2009) Though it received considerable attention, the commercial success of this application is limited and the main use of nanoceria is the formulation of slurries for the chemomechanical planarization (CMP) of silicon wafers in the production of integrated circuits. CMP has been the fastest-growing semiconductor operation in the last decade and nanoslurries for CMP take some 50% of the worldwide market for nanopowders.

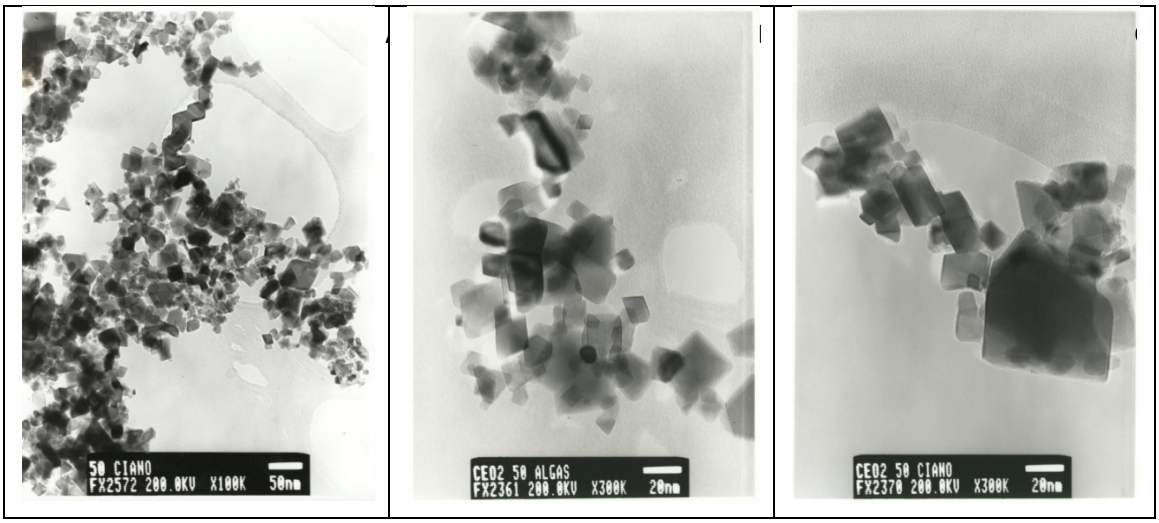

Figure 3. 100 mg/L $CeO_2$ nanoparticles dispersed in pure (A) water, OCDE (B) *P. subcapitata* culture medium and (C) AA/8 N (cyanobacterial culture medium).

Nanoparticles in solution form colloidal suspensions, which are mixtures in which one substance is divided into minute particles and dispersed throughout a second substance. There are no strict boundaries on the size of colloidal particles, but they tend to vary between $10^{-9}$ m to $10^{-6}$ m in size. A colloidal system is not a true solution nor a suspension either because it does not settle out like a suspension will over time. One of the most important characteristics of colloids in suspension is their net electric charge (Evans et al, 1999; Kuhn et al, 2009). In raw water, colloids invariably carry a negative charge (imperfections in the crystalline structure, ionization of peripheral chemical groups, etc.). In order to neutralize this negative surface charge, positive ions, which are present in the raw water or are introduced into it, come together to form a layer around the colloid. Thus an electrical double layer exists around each particle (Evans et al, 1999; Kuhn et al, 2009) (see Figure 4a). The liquid layer surrounding the particle exists as two parts; an inner region, called the Stern layer (fixed part of the double layer), where the ions are strongly bound, and an outer, diffuse, region where they are less firmly attached (diffuse part of the double layer). When a particle moves (e.g. due to gravity), ions within the Stern layer move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear (Evans et al, 1999; Kuhn et al, 2009). As we move away from the surface, the potential drops off roughly linearly within the Stern layer to decay exponentially through the diffuse layer and approaching zero at the imaginary boundary of the double layer.

The potential that exists at this boundary is known as the Zeta potential (ζ-potential) and determines the moving of the colloids and their interaction and aggregation with other particles. A charged particle will move with a fixed velocity in a voltage field. This phenomenon is called electrophoresis and measured by Electrophoretic Light Scattering (ELS). Zeta potential is usually

measured by (ELS), taking advantage of the in the laser scattered by particles under motion when particles are subjected to an electric field. Particle size distribution is usually measured by Dynamic Light Scattering (DLS) based on changes in laser scattered by particles under Brownian motion (Gittings and Saville, 1998; Kuhn et al, 2009; Moore and Carasoly, 2010). The most important factor which affects zeta potential and consequently the aggregation state of colloidal dispersions of metal oxide nanoparticles is pH. In the case of metal oxides, they present surface hydroxyl groups which govern their ion exchange properties and which may exist in dissociated or protonated forms depending on the pH value of the solution. (Kasprzyk-Hordern et al. 2003). A zeta potential value on its own without a quoted pH is a virtually meaningless number.

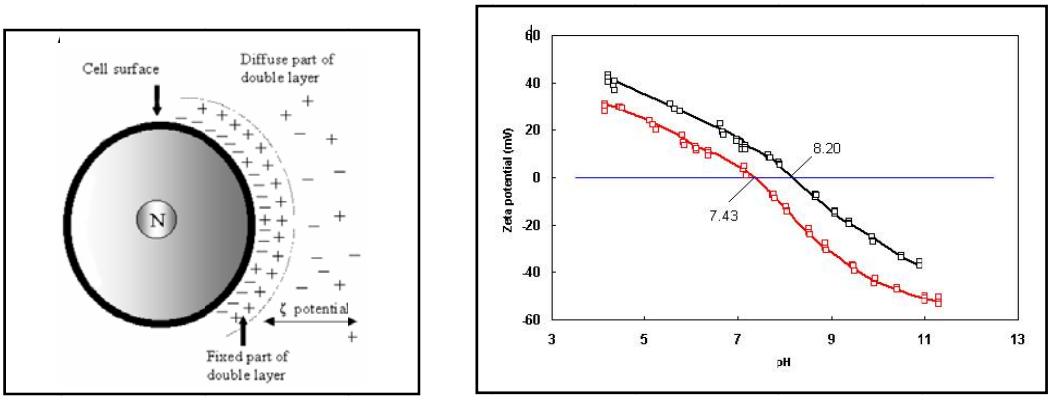


Figure 4: (A) Schematic representation of a nanoparticle dispersed in a fluid. (B) a typical ζ-potential *vs* pH plot.

Although their promising technical applications, nanoparticles present concerns both for human health and the environment (Song et al, 2011; Burnett et al. 2011; Wang, 2010). Due to their special physicochemical characteristics related with their high surface to volume ratio, they can be very reactive or catalytic (Farré et al, 2009). Their small particle size, make them also able to pass through cell membranes (Wang, 2010), and their interactions with biological systems doe to their variable net charge are relatively unknown (Menard et al. 2011). Key aspects for the ecotoxicity assessment of ENP are the goodness of existing dose metrics and ecotoxicity test to define properly the dose and the effect of nanoparticles; the need to address their physicochemical behavior in biologically-relevant media (particle size distribution, ζ-potential, surface area, etc.) (Wang, 2010; Menard et al. 2011); the importance of particle size and surface chemistry in the observed toxicological effects; and the need to define whether the internalization of particles is relevant to the induction of intracellular effects, or whether toxicity may be due to the adsorption of nanoparticles on the cellular membrane.

***3. Assessment of mixture toxicity***

With the increasing number of chemical compounds used in industrial, agricultural and domestic activities, aquatic ecosystems are exposed to an increasing number of substances. In

the same way, the number and complexity of the possible polluted scenarios is also increasing: from a single chemical or groups of chemicals dominated systems (like mining areas and downstream pulp paper facilities) to low-dose multicomponent exposures (as STP-dominated rivers). In addition to chemical pressure, other factors can alter ecosystems and join to the overall factors producing mortality or toxic pressure (Spijker et al, 2011) as changes in water flow, nutrient bioavailability, water, irradiation, Tº and $O_2$. However, due to simplicity and the difficulty to transfer these complex scenarios to laboratory, most ecotoxicity studies and risk assessment strategies on environmental and health risk regulations focus on hazard of individual chemicals and may underestimate the risk associated with the toxic action of mixtures (Kortencamp et al, 2009). In fact, in the analysis of the EC legislation made on the *State of the Art Report on Mixture Toxicity* (Kortencamp et al, 2009) commissioned by the EC to a panel of experts on toxicology and ecotoxicology, they found that the risk assessment in EC legislation framework relies principally on individual chemical risk assessment. They state that this approach could be justified if in typical scenarios only one chemical was toxic, or if the joint action of several chemicals was typically not larger than the most toxic agent in the mixture. However, evidences from experimental studies shows that this is not normally the case (Kortencamp et al, 2009; Kortencamp, 2011). However, these authors point out some noteworthy exceptions in EC legislation with showed an attempt to include risk assessment of mixture in legislation from the last 10 years. These are the Regulation EC Nº 396/2005 *on maximum residue levels of pesticides in or on food of plant and animal origin*, the REACH regulation (EC/1907/2006) which although mainly focused on individual substances, established that substances which are in fact mixtures (isomer mixtures or multiconstituent substances) must be assessed as a whole mixture on their PBT/vPvB properties. In addition, the Regulation EC 1272/2008 *on classification, labeling and packaging of substances and mixtures* derived from REACH, and depending on the principles established in *The UN Globally Harmonised System for Classification and Labeling of Chemicals* (GHS), described a detailed approach for the toxicological assessment of commercial mixtures establishing three methods to assess mixture toxicity: (a) whole mixture testing (b) *dose (concentration) addition* (CA), (c) the summation method, which is the toxicity-weighted summation of the relevant mixture components and the subsequent analysis whether or not the relative amount of relevant components is above or below a pre-defined threshold (Kortencamp et al, 2009). In general these recent regulations based on the latest scientific knowledge recommend adopting *dose (concentration) addition* models based on additivity concepts if there is no evidence for interactions between the components of the mixture, that is, synergisms or antagonisms (Kortencamp et al, 2009; WHO, 2009).

### *3.1 Additivity vs interactions*

One of the key aspirations of mixture (eco)toxicology has been to anticipate quantitatively the effects of mixtures of chemicals from knowledge about the toxicity of their individual components. This can be achieved by making the assumption that the chemicals in the mixture act in concert by exerting their effects without diminishing or enhancing each other toxicity, the so-called non-interaction or additivity assumption (Kortencamp et al, 2009). Dose (concentration) addition and independent action are the two concepts available for formulating the null hypothesis of additivity. Simple similar action refers to chemicals that cause toxicity through a common toxic mode of action (MOA) and are thus evaluated using *dose addition* approaches, also known as *concentration addition* (CA) (Feron & Groten, 2002; Faust et al. 2003). The other way to evaluate defined mixtures using additivity is simple dissimilar action or *independent action* (IA) which is assumed when chemicals cause a common health effect, but by a different toxic MOA (Faust et al. 2003; Teuschler, 2009). In this case, the toxic responses are thought of as biologically and statistically independent events (Teuschler, 2009). However, a similar/dissimilar MOA assumption may require an extensive database and harmonisation on the assignments of MOAs to chemicals and in (eco) toxicology may be misleading since compounds may exert their toxicity via different mechanisms of action in different organisms (Teuchler, 2007). To solve this, in ecotoxicology, the window of predicted toxicity between these two additivity models is assumed to be a safety window of predicted toxicity of chemical mixtures for risk assessment strategies (Kortencamp et al, 2009). Nevertheless, interactions of chemicals leading to departures from additivity are still an issue of concern in ecotoxicology as is commented in the *State of the Art Report on Mixture Toxicity* (Kortencamp et al, 2009): *…Although dose (concentration) addition (and, to a limited extent, independent action) have proven surprisingly powerful in predicting and assessing mixture toxicities, there are also clear cases of synergisms (i.e. higher than expected mixture toxicities)…* and continued *…There is an urgent need to define the conditions that might lead to synergistic mixture toxicities, and to establish how large synergisms are likely to be.*

But what is additivity and what is interaction?. There has been a lot of controversy on this issue from the first works on combined effect of drugs of Bliss (1939) and Loewe (1953) and other authors due to the misleading nature of the issue. Berembaum (1989) tried to compile the different proposed terminologies and definitions and harmonise it; but the issue is extremely hard specially if considerations over MOA is take into account and because several related scientific disciplines as pharmacology, toxicology and ecotoxicology work in related applications based on these concepts but using slightly different terminology. If we take CA and

IA definitions of additivity them synergisms or antagonisms can then be defined in relation to this additivity assumption as upwards or downwards deviations, respectively (Kortencamp et al, 2009), but it implies two different definitions of synergism and antagonism making the issue even more complex. Going back to the origin of the definition of additivity (Loewe additivity), it is based on the classical isobologram (Berembaum, 1989; Chou and Talalay, 1984; Chou, 2006) which is independent in principle, of any consideration on MOA, and may be for instance an harmonised definition of additivity (Bovill, 1998). Isobologram is a graphical representation depicting doses of two drugs in the *x* and *y* axes, and a surface of equipotency as the area between the two axes, a straight line (*additivity line*) connect the concentrations of the two drugs at all the possible ratios exerting additive effect. The experimentally determined effect of each ratio can be added to the plot to identify if the drugs really act additively (experimental points fall on the additivity line) or there are departures from additivity, that is, synergism or antagonism (see Figure 5).

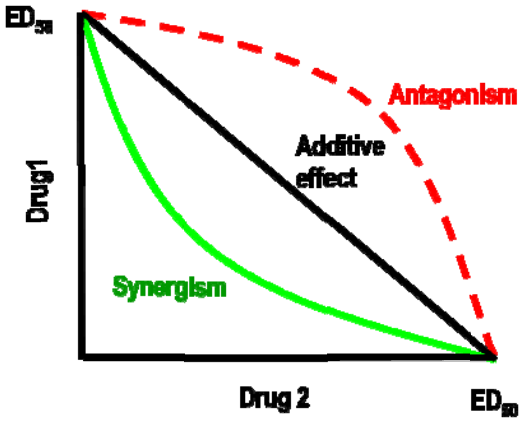


Figure 5: Isobologram depicting additivity line (black straight line), Synergism (green line) and antagonism (red broken line).

The additivity under isobologram graphical representation can be expressed mathematically by the following equation in their most simple definition for two chemicals:

$$\frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} = 1 \qquad (1)$$

Where $(D)_1$ and $(D)_2$ are the doses of chemicals 1 and 2 that in combination produce some specified effect (i. e. 50% of effect on any measured parameter or $EC_{50}$) and $(Dm)_1$ and $(Dm)_2$ are the doses of the chemicals that when applied singly also have the same effect (50% effect; $EC_{50}$). This definition of additivity is in fact the Loewe definition of additivity and is formerly independent of any consideration on the mechanism of action (Berembaum, 1989; Chou, 2006). A practical advantage of taking this definition of additivity as the harmonised one is that in general, predictions of additivity toxicity under CA model are more conservative (a higher

toxicity is usually predicted) that under IA, so that a supposed synergism under IA additivity assumption can be an antagonism or additivity under CA model, but this does not happen in the other way: a synergism under CA assumption will always be a synergism also under IA model.

### *3.2 Combination Index (CI) as a method to measure interactions between pollutants in a mixture*

The Combination Index-Isobologram Equation (Chou and Talalay, 1984) generalized the isobologram equation for *n* drugs and is independent of the mechanism of action of the drugs or the route of exposure (Chou, 2006). It takes into account both the potency and the shapes of the dose-effect curves (the potency parameter is represented by *Dm* and the shape parameter is represented by *m*, that is, the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curve, respectively) by means of the median effect equation (see Figure 6 and 7), and based on the single and combined dose-response curves of the chemical compounds under study, allows the quantification of the degree of deviation from the additivity of a mixture from two to *n* chemicals.

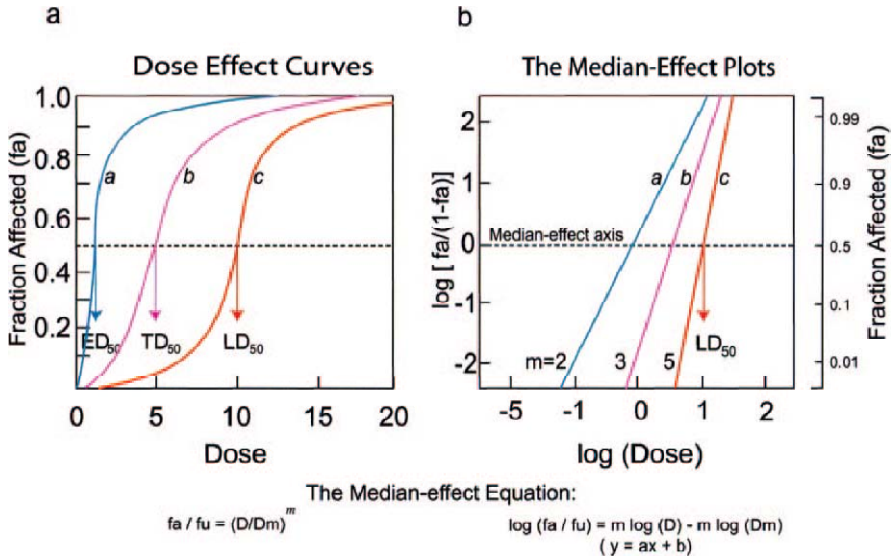


Figure 6: Dose effect curves (a), and their linear equivalence in the median-effect plots (b). In figure b, the potency parameter is represented by *Dm* ($LD_{50}$), and the shape parameter is represented by *m*. the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curve, respectively. Taken from Chou. 2006. *Pharmacol Rev* 58(3), 621-681.

By using the generalized combination index equation for *n*-chemicals (eq. 2) allows to quantitatively measure the degree of deviation from additivity of the mixture at any level of effect (*fa* or $EC_x$) exerted on the organism.

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{[D]_j \Big/ \sum_{1}^{n}[D]\right\}}{(D_m)_j \left\{(f_{ax})_j \Big/ \left[1-(f_{ax})_j\right]\right\}^{1/mj}} \qquad (2)$$

where $^{n}(CI)_x$ is the combination index for *n* chemicals at *x*% of effect; $(D_x)_{1-n}$ is the sum of the dose of *n* chemicals that exerts *x*% of effect in combination, $\{[D_j]/\sum^{n}[D]\}$ is the proportionality of the dose of each of *n* chemicals that exerts *x*% of effect in combination; and $(D_m)_j$ $\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts *x*% of effect. From equation 3, CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, respectively.

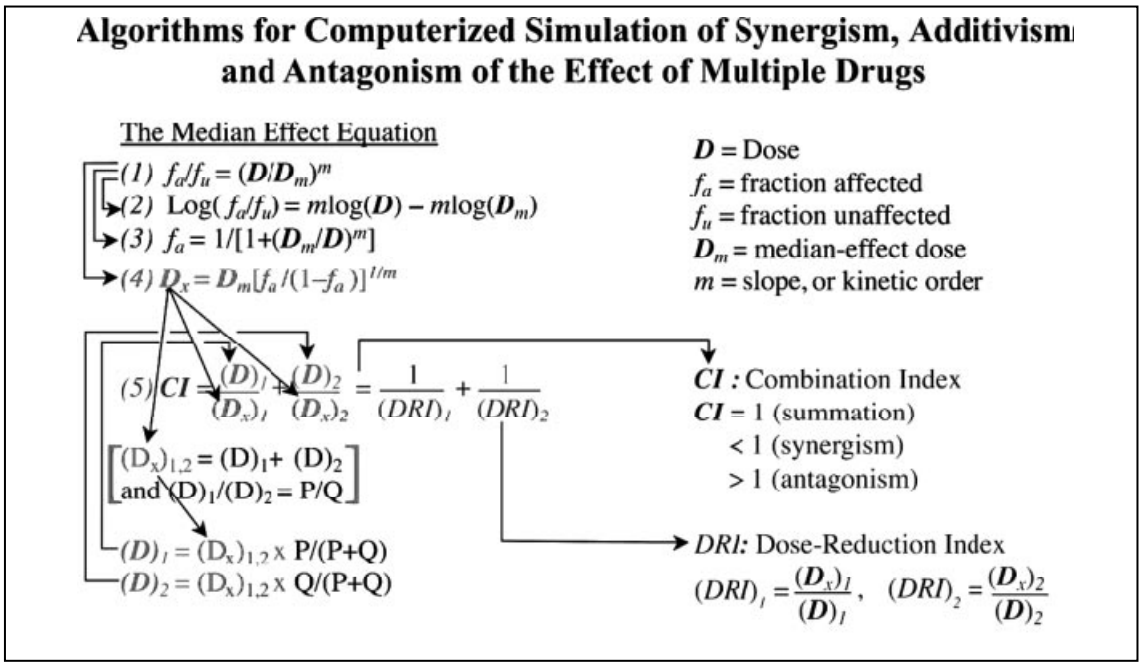


Figure 7. Schematic representation of the algorithms used in CI method in the computerized simulation to measure the interaction between chemicals in a mixture. Taken from Chou. 2006. *Pharmacol Rev* 58 (3), 621-681.

The base of the generalized combination index equation for *n*-chemicals is the same of that of Berembaum (1989) and is equal to CA model generalized equation (Faust et al. 2003) when CI = 1. The main difference is that the assumption of additivity (CI = 1) is not made and the degree of the departure from additivity as CI values can be readily calculated in order to determine the degree of interaction (if any) of the mixture components (Chou, 2006). Combination index method and its generalized equation are actually oriented to identify departures from additivity. Based on the information provided by this equation Chou and Talalay (1984) developed the *fa*-CI plot which is an effect-oriented plot specifically generated to represent the evolution of the degree of the interaction of chemicals along the effect levels elicited on an organism (Chou,

2006). This method is widely used in pharmacology and enzimology and had proven very useful to study drug interactions (Chou, 2006; Jia et al., 2009).

In the present thesis, CI analysis has been applied by the first time to mixtures of environmental pollutants in order to study whether the CI method can be applied to identify deviations from additivity in the combined effect of mixtures of pollutants. Studies will be presented on assessing the combined effect of priority and emerging pollutants in several aquatic organisms. In the selection of pollutants, there are chemicals with the same MOA (heavy metals), same pharmacological MOA but unknown effect on non-target organisms (fibrates), unknown but supposed dissimilar MOAs (as ducusate and chlorinated pollutants) and mixtures of similar, dissimilar and unknown MOAs (perfluorinated surfactants, pesticides, heavy metals and pharmaceutical products).

### *4. Toxicity and ecotoxicity tests*

Ecotoxicology is a multidisciplinary field of science concerned with the study of the harmful effects of various chemical, biological and physical agents on living organisms. It is a very young discipline initially derived from human toxicology but which has become a highly multidisciplinary field due to the amplitude of its scope. It takes advantage of molecular biology, biochemistry, environmental chemistry, geochemistry, social sciences, computer science, etc. to determine the fate, effects, and risks of toxicants on the environment and wildlife (Wright and Welbourn, 2002).

One of the primary tools of environmental toxicology is the use of bioassays in order to measure the effects of substances on living organisms. Bioassays are procedures by which the potency or the nature of a substance is estimated by studying its effects on living matter and whose principal goal is the establishment of causality relationships (Wright and Welbourn, 2002). In toxicology and ecotoxicology the issues under study are generally related with adverse effects on organisms and are primarily measured as “toxicity”. The initially developed toxicity tests were based on end-points related with general or *raw* toxicity such as mortality, citotoxicity, growth inhibition or inhibition of reproduction, etc. But now there are many bioassays dealing with specific toxic effects as genotoxicity, carcinogenicity, mutagenicity, endocrine disrupting capability, neurotoxicity, developmental toxicity, ROS (Reactive Oxygen Species) induction, etc. (EC/2006/1907; Wright and Welbourn, 2002). Regardless of the end point measured, there are basically to types of bioassays: quantal and graded assays. A quantal assay involves an "all or none response" while graded assays are based on the observation that there is a proportionate

increase (or decrease) in the observed response following an increase in the concentration or dose of the pollutants. The graded assay is perhaps the one from which the most useful information can be obtained since it allows to establish an experimental dose-response relationship and to calculate relevant parameters related with the threshold level of the substance as *No Observed Effect Concentrations* (NOECs) with is the highest concentration of a substance at which no statistically significant effects with respect to the control are observed; or parameters related with the potency of the substance as, *Effective Concentrations* (ECx) or *Lethal Concentrations* ($LC_x$) which are the concentration of a substance which produced an *x* percent of the measure effect on the studied parameter with respect to the control. The most used Effective Concentrations are those producing 50% of effect, named as $EC_{50}$ and $LC_{50}$ (EC/2006/1907; Wright and Welbourn, 2002; Eggen et al, 2004).

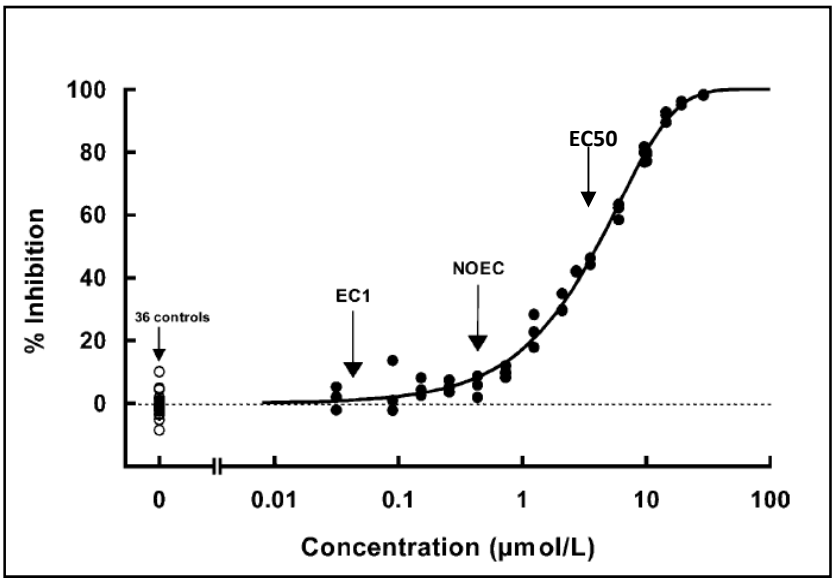


Figure 8. A typical plot depicting effect (% inhibition) *vs* concentration. Modified from Faust et al. 2003. *Aquatic Toxicology* 63, 43- 63.

These two basic classes of bioassays and estimated parameters can be applied at any level of organization under study, from molecular level (as in enzimology or gene expression) to the whole organism. It is above the organisms level, where the highest divergences can be found between human toxicology and ecotoxicology in their goals and methods for two main reasons: (a) human toxicology tries to protect individual human beings while environmental toxicology tries to protect ecological functions (Wright and Welbourn, 2002; Chapman, 2002), and (b), in environmental toxicology, the highest levels of organization (population, biocenosis and ecosystem) are those of the highest relevance (Wright and Welbourn, 2002; EC/2000/60; EC/2006/1907). Specific experimental designs have been developed to study the effects at these levels of organization as microcosms, mesocosms, and in-situ experiments gaining in ecological

relevance (Sabater et al, 2009; Ricart et al, 2009; Geiszinger et al. 2009). But however being those of the highest ecological relevance, the increasing complexity of the systems, make them to have important disadvantages: technical and logistic difficulties, increase of the time consumed (from days to months and even years) and a reduction in control capacity and replication (Wright and Welbourn, 2002). On the other hand, the use of single species exposed to increasing toxicant concentrations based on classical taxa (algae, macroinvertebrates and fish) in standard assay conditions (time of exposure, nutrient concentration, $O_2$, Tº etc.) although having less ecological realism can provide strong evidence of the direct effects of pollutants and allow research on mechanisms of action under controlled conditions (Wright and Welbourn, 2002). In the existing regulations dealing with environmental risk assessment to the aquatic environment (2000/60/EC) or the evaluation of the toxicity of substances (EC/2006/1907; EMEA, 2005), the most usual recommendation to characterize the environmental hazard to the aquatic environment are the acute and chronic toxicity data derived from standard toxicity test (OECD methods) and generally using at least one taxa from three representative trophic levels: green algae (as *Pseudokirchneriella subcapitata* or *Clorella* sp.) or macrophytes (as *Lemna minor*), macroinvertebrate (as *Daphnia magna*), and fish (as *Pimephales promelas or Danio rerio*) as being the easier data to be obtained and the most reliable from the point of view of reproducibility and causation (Wright and Welbourn, 2002).

Figure 9. Micrographs of commonly used species of three relevant taxa: alga (*Pseudokirchneriella subcapitata);* macroinvertebrate *(Daphnia magna);* and fish *(Danio Rerio).*

Nevertheless, information provided from experiments involving higher levels of organization are recommended in order to validate Environmental Quality Standard (EQs) derived under the WFD (2000/60/EC), and to be incorporated in the Weight of Evidence in the assignment of PBT or vPvB characteristics in the PBT assessment (ECHA, 2008b). Other key point gaining relevance in the regulation on the characterization of the hazard of chemicals is the use of reliable data from non-standard toxicity tests (ECHA, 2008a) since sometimes the used of taxa different from the standard ones may provide extra toxicological information about possible harmful effects not detected with standard tests and more information about MOA of chemicals can be derived (Kirkland et al, 2007; Kasper et al, 2007; ECHA, 2008b; Henning-de Jong et al.

2009). This line of thought has been incorporated as a basic principle of REACH so that if a gap in the information requirements is found, a testing strategy must be proposed to be accepted by the European Chemicals Agency (ECHA), including new tests and avoiding if possible the use animal testing (Ahlers, 2008; ECHA, 2008a).

### *5. Microbial bioreporters*

Microbial bioreporters are intact, living microbial cells that have been genetically engineered to produce a measurable signal in response to a specific chemical or physical agent in their environment (Harms et al, 2006; Van der Meer and Belkin 2010). Bioreporters were originally developed for basic research on factors affecting gene expression (Fernández-Piñas et al, 2000; Kondo, 1993; Waidmann, 2011) and were early applied for detecting environmental contaminants, in medical diagnostics or food safety control (Griffiths, 2000;Harms et al, 2006;Van der Meer and Belkin 2010). The genetic modification consists in the introduction of a reporter system in the organism via a genetic vector. The vectors can be replicative plasmids or integrative plasmids which carry a fusion of a gene promoter sequence and a reporter gene. The reporter gene typically codes for a reporter protein that ultimately generates a detectable signal (Harms et al, 2006; Van der Meer and Belkin 2010). The gene promoter typically selected for developing microbial bioreporters are those which are able to respond to some stress, pollutant or change in the environment; When the promoter is fused to the reporter gene, turning on or off the promoter gene causes the reporter gene to be turned on (*lights-on* bioreporters) or off (*lights-off* bioreporters); the terms lights-on and lights-off come from the field of bioluminescent bioreporters but the concept may be applied to other types of reporter systems (Bachman, 2003; Sorensen et al, 2006; Van der Meer and Belkin 2010).


Figure 10: Scheme of the performance of lights-off and lights-on biorreporters. Modified from Sørensen et al. 2006. *Current opinion in biotechnology* 17, 1-6.

Microorganisms, and particularly bacteria, have a very advantageous position in the field of whole-cell bioreporters due to their rapid growth rates, low cost, easy maintenance/preservation and the possibility of genetic manipulation (Van der Meer and Belkin 2010; Belkin, 2007; Ron, 2007; Harms et al, 2006; Sorensen et al, 2006). From the first applications of microbial

bioreporters in the 1970-80s such as the Ames test, *umu* test, and Microtox® (not genetically engineered but based on the naturally bioluminescent marine bacterium *Vibrio fischeri*) (Van der Meer and Belkin 2010); whole-cell bioreporters have been used successfully for the assessment of single compounds, global parameters such as biochemical oxygen demand (BOD) or to provide information on the toxicity (ecotoxicity, genotoxicity, endocrine disrupters) and spatial heterogeneity and dynamics of contaminant bioavailability/ speciation of the toxic compound (Harms et al, 2006, Sorensen et al, 2006; Van der Meer and Belkin 2010; Palmer, 2011). Their success and future perspectives rely on (a) their versatility due to the fact that there exists a large number of reporter gene systems which can generate a variety of measurable signals (b), the number of gene promoters systems continue to grow since basic research on gene regulation is of the highest relevance and genome sequenciation technology is becoming faster and cheaper, (c) reporter genes can be inserted in a wide variety of organisms such as bacterial, yeast, plant, and mammalian cells providing considerable functionality and (d) microbial bioreporters are able to be integrated in miniaturized transducing systems generating *whole cell biosensors*. Whole cell biosensors can be miniaturized up to single-cell array configurations (Van der Meer and Belkin 2010) or automatized as real-time on-site reading systems, which make them a promising tool in environmental monitoring and other fields of application (Harms et al, 2006, Sorensen et al, 2006; Belkin, 2007, Ron, 2007; Van der Meer and Belkin 2010; Palmer, 2011). The use of whole cells rather than cellular elements for the construction of the biosensor has the major advantage that an intact cell can detect complex reactions which can only exist in an intact, metabolically active cell (Van der Meer and Belkin 2010; Sorensen et al, 2006). Thus global parameters can be evaluated such as the bioavailability of contaminants, usually closely associated with their toxicity (Kohler et al, 2000).

### 5.1 Specific, group specific and general bioreporters

The most important property of gene promoters used in environmental applications is that normally, a dose-dependent response of the promoters to a more or less specific compound or change in the environment exists. The degree of specificity goes from single-compound specific bioreporters to unspecific bioreporters or general toxicity bioreporters. (Kohler et al, 2000, Belkin, 2003, Van der Meer et al., 2004, Sorensen et al., 2006; Belkin, 2007; Yagi, 2007; Van der Meer and Belkin 2010; Palmer, 2011). In general, authors have established three groups of bioreporters based on the nature and the width of their specificity: specific, group-specific and general bioreporters. In the group of specific bioreporters, good examples are the *merR*-based bioreporters for the specific detection of $Hg^{2+}$ with a limit of detection of 1nM. *MerR* promoter seems to be induced only in the presence of $Hg^{2+}$ (Selifonova, 1993). Semi-specific bioreporters

include those responding to similar chemicals or those sensing specific effects on organisms which can be elicited by different types of compounds. Between the first one, a remarkable example are those gene promoters belonging to the ArsR-SmtB and MerR transcriptional factor super families which in general respond to several related heavy metals (Osman and Cavet, 2010; Van der Meer and Belkin, 2010; Jounneau et al, 2011); examples are the *arsR*-based bioreporters which respond to As (III/V) and in a lesser extent to Sb (III/V) and which were successfully applied to identify arsenic content above WHO guidelines with more than 90% of correct determinations in 200 ground water samples from Vietnam (Harms, 2006). In the second group, highly relevant and readily applied systems are included, as those microbial bioreporters based on SOS system (global cell response to DNA damage) promoters such as *umu* test (Oda et al., 1985) which detects mutagenic compounds. This test has been standardized by ISO (ISO/CD 13829) and has been found to be very representative in rodent carcinogenicity (Reifferscheid and Hell., 1996). The latest commercially successful semispecific bioreporters are the CALUX® systems integrated in human cell lines. In DR CALUX® expression of luciferase is mediated by activation of the arylhydrocarbon receptor (AhR) and is induced in the presence of dioxin-like compounds. ER CALUX® and AR CALUX® are responsive to estrogens/estrogenic compounds, and androgen/androgenic compounds respectively (Browers et al., 2010). Finally, non-specific bioreporters or general toxicity bioreporters are those in which a constitutively expressed promoter is fused to the reporter genes. Upon exposure to a toxic chemical, either the cell dies or its metabolic activity is inhibited, leading to a decrease in bioluminescent levels. The most widely used member of this group of bioreporters is the classical *lux* based Microtox® (Bulich and Isenberg., 1981; ISO 11348). A summary of the different gene promoter used in microbial bioreporters and their target compound/stress are presented in Table 1.

**Table 1.** Sensor proteins**,** hosts, promoter fusions, chemical targets and detection sensitivity used for bacterial biorreporter construction. Taken from Van der Meer and Belkin, 2010. *Nature Reviews Microbiology* 8, 511-522.

| Sensor proteins | Host chassis | Promoter–reporter fusion | Chemical targets | Detection sensitivity |
|---|---|---|---|---|
| XylR of *Pseudomonas putida* | *Escherichia coli* | *Pu*–lucFF* | Benzene, toluene and xylene | 40 μM |
| DmpR of *P. putida* | *P. putida* | *Po[‡]–luxAB* | Phenol | 3 μM |
| TbuT of *Ralstonia pickettii* | *E. coli* | *tbuA1p–luxAB* | Benzene, toluene and xylene | 0.24 μM |
| HbpR of *Pseudomonas nitroreducens* | *E. coli* | *hbpCp–luxAB* | Hydroxylated biphenyls | 0.4 μM |
| PhnR of *Burkholderia sartisoli* | *B. sartisoli* | *phnSp–luxAB* | Naphthalene and phenanthrene | 0.17 μM |
| IbpR of *P. putida* | *E. coli* | *ibpAp–luxCDABE* | Various aromatics | 1 μM |
| NahR of *P. putida* | *P. putida* | *nahGp–luxAB* | Naphthalene and salicylate | 10 nM |
| AlkS of *Pseudomonas oleovorans* | *E. coli* | *alkBp–luxAB* | $C_6$–$C_{10}$ alkanes | 10 nM |
| TodST of *P. putida* | *P. putida* str. F1 | *todXp–luxCDABE* | Toluene, benzene, phenol, *p*-xylene, *m*-xylene and trichloroethene | 0.3 μM |
| SepR of *P. putida* | *P. putida* str. F1 | *sepAp–luxCDABE* | Solvents | ~0.5 mM |
| FruR of *Erwinia herbicola* | *E. herbicola* | *fruBp–gfp[AAV][§]* | Fructose and sucrose | ~2 μM |
| AraC of *E. coli* | *E. coli* | *pBAD–gfpuv[‖]* | L-Arabinose | 0.5 μM |
| ArsR of *E. coli* | *E. coli* | *arsRp–luxAB* | Arsenite and antimonite | 5 nM |
| MerR of *E. coli* | *E. coli* | *merTp–luxCDABE* | $Hg^{2+}$ | 1 nM |
| CadC of *Staphylococcus aureus* | *Bacillus subtilis* | *cadCp–lucFF* | $Cd^{2+}$, Pb, Sn and Zn | 3 nM |
| ZntR of *E. coli* | *E. coli* | *zntAp–luxCDABE* | Zn, Pb and Cd | 5 μM , 0.7 μM and 10 nM, respectively |
| TetR of *E. coli* | *E. coli* | *tetAp–luxCDABE* | Tetracyclines | 45 nM |
| MphR of *E. coli* | *E. coli* | *mphAp–lacZ* | Macrolides (such as erythromycin) | ~10 μM |
| SOS response proteins of *B. subtilis* | *B. subtilis* | *yorBp–lucFF* | Various antibiotics (for example, ciprofloxacin) | 60 nM |
| SpoIIID and $\sigma^E$ of *B. subtilis* | *B. subtilis* | *yheI–lucFF* | Various antibiotics (for example, linezolid) | 0.1 μM |
| NisRK of *Lactococcus lactis* | *L. lactis* | *nisAp–gfpuv[‖]* | Nisin | 10 ng $l^{-1}$ (3 pM) in culture supernatant and 0.2 μg $l^{-1}$ (60 pM) in milk |
| LuxR of *Aliivibrio fischeri* | *E. coli* | *luxIp–gfp[ASV][§]* | *N*-Acyl homoserine lactones | 1–10 nM |
| Ada of *E. coli* | *E. coli* | *alkAp–luxCDABE* | DNA-alkylating agents | 70 nM *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, for example |
| DnaK and $\sigma^{32}$ of *E. coli* | *E. coli* | *dnaKp–luxCDABE* | An increase in the level of intracellular misfolded proteins | 0.25 M methanol, for example |
| Crp–cAMP transcriptional dual regulator of *E. coli* | *E. coli* | *grpEp–luxCDABE* | An increase in the level of intracellular misfolded proteins | 0.14 μM pentachlorophenol, for example |
| OxyR of *E. coli* | *E. coli* | *katGp–luxCDABE* | Intracellular production of oxygen radicals | 3 μM $H_2O_2$, for example |
| SoxRS of *E. coli* | *E. coli* | ***micFp– luxCDABE*** | Intracellular production of oxygen radicals | Detection sensitivity not indicated |
| RecA–LexA of *E. coli* | *E. coli* | *cdap–gfp* | Single-stranded DNA that arises as a consequence of inhibition of DNA replication | 5 nM *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, for example |
| | | *sfiA–lacZ* | Single-stranded DNA that arises as a consequence of inhibition of DNA replication | 4 nM mitomycin C |
| | | *recAp–luxCDABE* | Single-stranded DNA that arises as a consequence of inhibition of DNA replication | 0.2 nM mitomycin C |
| RecA–LexA of *Salmonella enterica* subsp. *enterica* serovar Typhimurium | S. Typhimurium | *recNp–luxCDABE* | Single-stranded DNA that arises as a consequence of inhibition of DNA replication | 46 nM mitomycin C |
| | | *umuDp–lacZ* | Single-stranded DNA that arises as a consequence of inhibition of DNA replication | 10 nM mitomycinC |

AraC, arabinose operon regulatory protein; *cdap*, promoter of the colicin D gene; cAMP, cyclic AMP; Crp, cAMP regulatory protein; *katGp*, promoter of the catalase–peroxidase gene; *lacZ*, β-galactosidase gene; *lucFF*, firefly luciferase gene; *lux*, bacterial luciferase biosynthesis gene; Rec, recombination and repair; *sfiA*, SOS cell division inhibitor gene (also known as *sulA*); *tbuA1p*, promoter of the toluene monooxygenase α-subunit gene. *A XylR-responsive promoter of *P. put*
[‡]A DmpR-responsive promoter of *P. putida*. [§] Unstable variants of GFP. [‖] A GFP variant that is optimized for maximal fluorescence when excited by ultraviolet light.

### 5.2 Reporter genes

The use of reporter genes (*lux* genes, *luc* genes, *lacZ* operon, green fluorescent protein, aequorin, *crtA* and urogen III methyltransferase) has allowed the construction of sensitive bioreporter strains. With some reporter genes, an exogenously added substrate/prosthetic group is needed to be added to the bioassay so that the signal can be generated (i.e.; *Luc* and aequorin). For other bioreporters, the signal must be activated by an external light source (GFP and UMT), and for a select few bioreporters, the signal is completely self-induced, with no exogenous substrate or external activation being required (i.e. *luxCDABE*). (Kohler et al, 2000; Belkin, 2003; Van der Meer et al, 2004; Sorensen et al, 2006; Yagi, 2007; Van der Meer and Belkin, 2010).

### 5.3 Colored-protein based bioreporters

*LacZ* encodes β-galactosidase (LacZ), an intracellular enzyme that cleaves the disaccharide lactose into glucose and galactose. Isopropyl-β-D-thio-galactoside (IPTG) binds the *lactose repressor* and makes the expression of *lacZ* dependent only on its own promoter. By using some lactose analogues such as X-Gal (bromo-chloro-indolyl-galactopyranoside) the activity of β-galactosidase produces a blue residue. In gene cloning, X-gal is used in a technique called blue/white screening, and it has been used also to generate bioreporters by fusing a gene promoter of interest to *lacZ* (Belkin, 2007; Van der Meer and Belkin, 2010). *Crt*A gene is responsible for carotenoid synthesis in purple bacteria; *CrtA* in these bacteria is responsible for changing the color of the cultures from yellow to red due to O-metilation of spheroidene to spheroidenone (Fujimoto et al., 2006). Due to the inexpensiveness of the detection technique (it can be identified "*de visu*" or quantitatively by spectrophotometry) these reporter genes have been successfully used to develop several kinds of microbial bioreporters including arsenic bioreporters for wide-range screening applications in developing countries (Harms, 2006).

### 5.4 Photoprotein bioreporters

Aequorin is a photoprotein isolated from the jellyfish *Aequorea victoria*. Upon addition of calcium ions ($Ca^{2+}$) and its prosthetic group, coelenterazine, a reaction occurs whose end result is the generation of blue light in the 460 - 470 nm range. Aequorin has been incorporated into human B cell lines for the detection of pathogenic bacteria and viruses in the CANARY assay (Cellular Analysis and Notification of Antigen Risks and Yields) (Rider, 2003). The B cells are genetically engineered to produce aequorin. Upon exposure to antigens of different pathogens, the recombinant B cells emit light as a result of activation of an intracellular signaling cascade

that releases calcium ions inside the cell. Aequorin has also being cloned in many organisms including plant, animal fungi and bacteria (Rider, 2003; Kodlova, 2005;Knight, 1991; Kudla, 2010;), and recently it has been used by our group to record specific calcium signatures induced by different stresses and pollutants in a recombinant aequorin-bioreporter cyanobacterial strain (Torrecilla, et al, 2000; Torrecilla, et al, 2001; Leganes et al, 2009; Barrán-Berdón et al., 2011).

### 5.5 Fluorescent bioreporters

Green fluorescent protein (GFP) was originally isolated and cloned also from the jellyfish *Aequorea Victoria,* it forms a dual system with the photoprotein aequorin in this organism (Stepanenko, 2008). Variants have also been isolated from the sea pansy *Renilla reniformis*. GFP emits green light when exposed to blue light. GFP does not need exogenous cofactors but it requires oxygen for formation and stabilization of the fluor**.** This ability to fluoresce makes GFP highly useful in biosensing assays since it can be used on-line and in real-time to monitor intact, living cells. Additionally, the ability to mutate the *gfp* gene to code for GFPs with emissions other than blue (i.e., cyan, red, and yellow) allows it to be used as a multianalyte detector (Stepanenko, 2008). Consequently, GFP has been used extensively in bioreporter constructs within bacterial, yeast, plant, and mammalian hosts. (Sorensen et al, 2006; Yagi, 2007; Van der Meer and Belkin; 2010; Palmer, 2011). Uroporphyrinogen (urogen) III methyltransferase (UMT) catalyzes a reaction that yields two fluorescent products which produce red-orange fluorescence in the 590 - 770 nm range when illuminated with ultraviolet light (Margesin, 2005; Feliciano, 2006). So as with GFP, no addition of exogenous substrates is required. UMT has been used as a bioreporter for the selection of recombinant plasmids, as a marker for gene transcription in bacterial, yeast, and mammalian cells, and for the detection of toxic salts such as arsenite and antimonite (Roessner, 1995; Wildt and Deuschle, 1999; Margesin, 2005).

### 5.6 Bioluminescent bioreporters

Bioluminescent bioreporters include those based on luciferases. Luciferase is a generic name for an enzyme that catalyzes a light-emitting reaction and can be found in bacteria, algae, fungi, jellyfish, insects, shrimp, and squid (Greer, 2002; Van der Meer and Belkin; 2010). Firefly luciferase (*luc* genes) catalyzes a reaction that produces visible light in the 550 – 575 nm range. A click-beetle luciferase is also available that produces light at a peak closer to 595 nm. Both luciferases require the addition of an exogenous substrate (luciferin) for the light reaction to occur. In bacteria, the genes responsible for the light-emitting reaction (the *lux* genes) have been

isolated and used extensively in the construction of bioreporters that emit a blue-green light with a maximum intensity at 490 nm. Among all reporter genes, the bacterial bioluminescence is probably the most widely used for its high sensitivity and fast response (Van der Meer and Belkin; 2010; Ron, 2007).

The *lux* genetic system consists of five genes, *luxA, luxB, luxC, luxD, and luxE*. (Meighen, 1991). *LuxAB* encoded bacterial luciferase which catalyzes the oxidation of a long chain aliphatic aldehyde and $FMNH_2$, producing light at 490 nm (Prosser et al., 1996). In naturally luminescent bacteria, the aldehyde substrate of luciferase (tetradecanal) is synthesized by the reductase, transferase and synthetase genes encoded by *luxC, D, E*.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{bacterial luciferase}} FMN + RCOOH + H_2O + \text{light (490 nm)}$$

Depending on the combination of these genes, two types of bioluminescent bioreporters can be constructed: *luxAB* bioreporters and *luxCDABE* bioreporters. In the first configuration, as in *luc* bioreporters, to fully complete the light-emitting reaction, a substrate must be supplied to the cell. Typically, this occurs through the addition of the chemical decanal at some point during the bioassay procedure. Instead of containing only the *luxAB*, bioreporters can contain the complete *lux* operon, thereby allowing for a completely independent light generating system that requires neither exogenous additions of substrate nor any excitation by an external light source. So in this bioassay, the bioreporter is simply exposed to a target analyte and a quantitative increase or decrease in bioluminescence results, often within less than one hour. Its quickness and ease of use, along with the reproducibility of the bioassay in real-time and on-line recordings, make *luxCDABE* bioreporters extremely attractive.

## 6. Cyanobacteria and their applications in environmental monitoring

Cyanobacteria, also known as blue-green algae or Cyanophyta, are a diverse group of gram-negative photosynthetic bacteria (Whitton, 1992). They originated during the Precambrian era ($2.8 \times 10^9$ years ago), and as a group are known to survive a wide spectrum of environmental stresses (Knoll. 2008). The ability of cyanobacteria to perform oxygenic photosynthesis is thought to have converted the early reducing atmosphere into an oxidizing one (Knoll. 2008), which dramatically changed the composition of life forms on Earth by stimulating biodiversity and leading to the near-extinction of oxygen-intolerant organisms. According to endosymbiotic theory, cyanobacterial are the ancestors of chloroplasts in plants and eukaryotic algae which evolved from them via endosymbiosis (Liberton et al. 2008). Cyanobacteria are organisms with

a wide ecological distribution and present variety of strategies that allow them to adapt and alter their physical environment (Whitton, 1992): they can be found in freshwater, marine and soil environments. They can even be found in extreme ecosystems as desserts, hypersaline waters or thermal springs (Yannarell et al., 2006; McGregor and Rasmussen, 2008 García-Pichel et al., 2009). A few are endosymbionts in lichens, plants, various protists, or sponges (Wilkinson y Fray., 1979; Bergman et al, 2008). The only condition in which cyanobacteria are not usually found is in acidic environments (pHs below 5) (Whitton, 1992).

Cyanobacteria show a prokaryotic cellular organization. They have a Gram- type cell envelope with plasma membrane and outer membrane separated by a periplasmic space. However, their cell envelope share some similarities with that of Gram+ bacteria, such as the kind and proportion of lipid components, the thickness of the peptidoglican layer and the degree of crosslinking between the peptidoglican chains (Hoiczyk and Hansel., 2000). Some of them can also present an external surface layer and mucilaginous sheet (Smarda et al., 2002). They present a multicopy circular chromosome and a variable number of plasmids of different length (Schneider et al. 2007), and as prokaryotic organisms do not present organelles; however, they have a complex system of inner membranes called thylakoid membranes where the different components of the photosynthetic apparatus are located (Liberton et al. 2008). The thylakoid membrane hosts both respiratory and photosynthetic electron transport (De Ruyter and Fromme, 2008) while their plasma membrane contains only components of the respiratory chain; it is unclear whether thylakoid membranes are invaginations of the inner cell membrane, or an independent membrane system (Schneider et al. 2007). Attached to thylakoid membrane, phycobilisomes act as light harvesting antennae complexes for the photosystems. As in plants and algae, the oxygenic photosynthesis is accomplished by coupling the activity of two light harvesting systems: photosystem (PS) II and I (Z-scheme). The photosynthetic pigments present in cyanobacterial are Chlorophyl a, Phycoerythrin, Phycocyanin and Alophycocianin; unlike plants, the majority of cyanobacteria lack Chlorophyl b. A few genera of cyanobacteria, however, lack phycobilisomes and have chlorophyll b instead (*Prochloron*, *Prochlorococcus*, *Prochlorothrix*). Thus, the cyanobacteria could be defined as organisms characterized by a prokaryotic cell organization, and a photosynthetic apparatus similar to eukaryotic chloroplasts (De Ruyter and Fromme, 2008). Cyanobacteria include unicellular and filamentous species. Some filamentous strains show the ability to differentiate into several different cell types: vegetative cells, the normal, photosynthetic cells that are formed under favorable growing conditions; akinetes, draught resistant spore-like cells, can be formed when environmental conditions become harsh; hormogonia which are gliding filaments serve as propagation forms, and thick-walled heterocysts, which contain the enzyme nitrogenase, can be differentiated in

poor nitrogen environmental conditions (Whitton, 1992; Meeks et al, 2002; Flores and Herrero, 2011).

Table 2. Classification of cyanobacteria according to Bergey's Manual of Systematic Bacteriology (Boone and Castenholz, 2001). The names in parentheses correspond to the names of taxa according to the ICBN.

| CLASS | SUBSECTION | PART | GENUM |
|---|---|---|---|
| CYANOBACTERIA | I (Chroococcales) | I | *Chamaesiphon, Chroococcus, Cyanobacterium, Cyanobium, Cyanothece, Dactylocopsis, Gloeobacter, Gloecapsa, Gloeothece, Microcystis, Prochlorococcus, Prochloron, Synechococcus, Synechocystis* |
| | II (Pleurocapsales) | I | *Cyanocystis, Dermocarpella, Stanieria, Xenococcus* |
| | | II | *Chroococcidiopsis, Myxosarcina, Pleurocapsa* |
| | III (Oscillatoriales) | I | *Arthrospira, Borzia, Crinalium, Geitlerinema, Halospirulina, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Oscillatoria, Planktothrix, Prochlorothrix, Pseudanabaena, Spirulina, Starria, Symploca, Trichodesmium, Tychonema* |
| | IV (Nostocales) | I | *Anabaena, Anabaenopsis, Aphanizomenon, Cyanospira, Cylindrospermopsis, Cylindrospermum, Nodularia, Nostoc, Scytonema* |
| | | II | *Calothrix, Rivularia, Gloeotrichia, Tolypothrix, Microchaete* |
| | V (Stigonematales) | I | *Chlorogloeopsis, Fischerella, Geitleria, Iyengariella, Nostochopsis, Mastigocladopsis, Westiella, Stigonema, Doliocatella* |

Cyanobacterial taxonomy is still an issue of controversy. Cyanobacteria have been traditionally classified in Botany according to morphology and follow the criteria established in the International Code of Botanical Nomenclature (ICBN) (Greuter et al, 2000). Whit the appearance of the Bacteriological Code (BC) cyanobacteria started to be classified in parallel by these two nomenclatures (Lapage et al, 1992). Presently, the most accepted system of classification for cyanobacteria is the unified system presented in the *Bergey's Manual of Systematic Bacteriology* (Boone and Castenholz, 2001). In this system of classification, cyanobacteria are classified into five sections, referred to by the numerals I-V: Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales. The members of Chroococales

are unicellular and usually aggregate in colonies. In Pleurocapsales, the cells have the ability to form small, spherical reproductive cells through multiple fission termed baeocytes. The rest of the sections include filamentous species. In Oscillatoriales, the cells are uniseriately arranged and do not form specialized cells (akinetes and heterocysts). In Nostocales and Stigonematales the cells have the ability to develop diferenciated cells. Stigonematales, unlike Nostocales, includes species with truly branched trichomes.

However, their phylogeny is actually under revision, specially the tree first sections (Chroococcales, Pleurocapsales and Oscillatoriales) since their classification is not fully phylogenetically consistent (Rajaniemi et al, 2005; Swingley et al, 2008).

Cyanobacteria have a long history of adaptation in the planet and have developed strategies to respond and to adapt to almost any environmental change (Witton, 1992; Yannarell et al., 2006; McGregor and Rasmussen, 2008 García-Pichel et al., 2009) and have been found to respond to a large number of both organic pollutants (different types of pesticides) and heavy metals (Rawson et al, 1989; Bachmann, 2003). As primary producers with a key role in the N and C cycles, they are a dominant component of marine and freshwater phytoplankton and any detrimental effect on this group may have a negative impact in nutrient availability to organisms of higher trophic level; for this, cyanobacteria are well suited to be used in environmental toxicity testing and environmental monitoring (Fernandez-Piñas et al, 1991; Perona et al, 1998; Perona el al, 2004).

Many cyanobacteria are amenable to genetic manipulation and at least 40 cyanobacterial genomes have been sequenced (http://bacteria.kazusa.or.jp/cyano; http://cmr.tigr.org), this greatly facilitates the genetic engineering of strains. As in environmental monitoring, cyanobacteria have already been applied in the field of microbial bioreporters but in a marginal way compared to other microorganisms of less ecological relevance (Bachman, 2003). Most work has been made in the construction of strains genetically engineered to respond to certain heavy metals and limiting nutrients (Erbe et al, 1996; Bachmann, 2003; Peca et al, 2008; Bullerjahn et al. 2010). However, the development of biosensors based on cyanobacteria is still very limited; the majority of the strains constructed so far are based on the unicellular cyanobacteria *Synechococcus* sp. PCC7942 and *Synechocystis* sp. PCC6803 and marked with the *lux*AB or *luc* genes so that they are not self-luminescent. Remarkable exceptions are the aequorin-based *Anabaena sp.* PCC7120 derivative strain developed by Torrecilla et al. (2000; 2001) which, as already described, has been used to monitor calcium signals in response to a variety of environmental stresses and recently to record different calcium signatures induced by

pollutants (Barran-Berdón et al. 2011). In our group Muñoz-Martín et al. (2011) have constructed *Anabaena sp.* PCC7120 derivative strains which are self-luminescent *luxCDABE*-marked bioreporters able to respond to different forms of bioavailable phosphate.

In the field of general toxicity bioreporters, the application of cyanobacteria is surprisingly scarce although their high potential; although the *Vibrio fischeri*-based assay (Microtox®) introduced in the 1980s (Bulich and Isenberg., 1981) is actually standardized (ISO 11348) and generally well accepted in the field of ecotoxicology (Nunes-Halldorson and Duran, 2003), a number of drawbacks have been described when measuring turbid or highly colored solutions. In addition, these marine bacteria operate at salt concentrations only around 2%, which limits the solubility of some compounds and can alter heavy metal speciation (Newman and McCloskey, 1996 Deheyn et al, 2004). These problems raise the question of the ecological relevance of this bioassay and thus, many researchers doubt that the use of these bioluminescent marine organisms is justified for toxicity tests in continental environments (freshwater and soil). This may be one of the reasons why this rapid and sensible test is not usually accepted in environmental legislation as test organism. In the search of systems more ecologically relevant, some microbial bioreporters were designed based on soil heterotrophic bacteria bacteria, mainly *Pseudomonas putida*, *Pseudomonas fluorescens*, *Escherichia coli* and *Rhizobium leguminosarum*, and had been applied for soil toxicity testing (Paton et al 1997, Sorensen et al, 2006). However the use of photosynthetic bacteria as cyanobacteria could have a number of advantages: they are a cheaper and easy to handle alternatives due to their trophic independence and ease of cultivation. These advantages make them particularly suited to detecting contaminants in water in general and especially pollutants that may affect primary producers. Based on all this advantages, Shao et al. (2002) developed a general toxicity cyanobacterial assay based on *Synechococcus* sp. PCC7942 and applied it to study the toxicity of herbicides. However this strain is *luc* marked and does not self-luminescent and the addition of luciferin in the bioassay is required in order to get the light signal.

The aim of the present thesis was to evaluate the applicability of a new general toxicity test to determine the individual and combined toxicity of different classes of environmental pollutants. This new toxicity test is based on a freshwater filamentous cyanobacterium of the Order Nostocales: an *Anabaena* PCC 7120 (Figure 11) derivative strain named CPB4337 with carries a chromosomal integration of *luxCDABE* genes which are expressed constitutively.

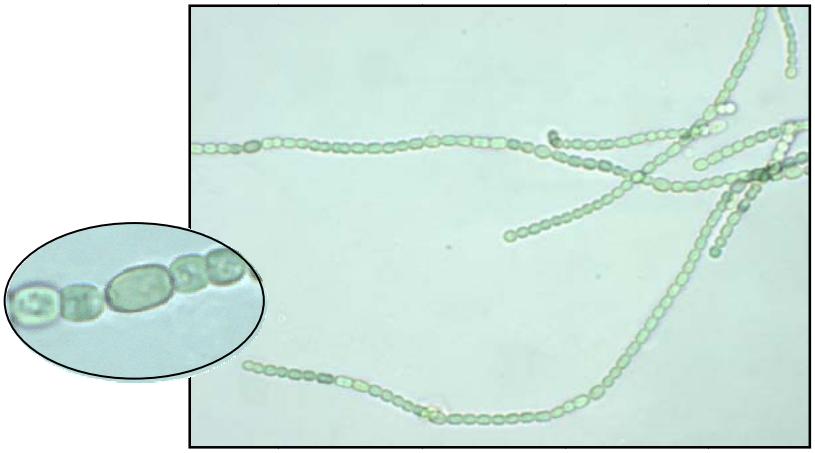

Figure 11. *Anabaena sp*. PCC 7120 as seen by optical microscope.

This strain was obtained by a chromosomal integration of a Tn5 derivative transposon (Tn5-1419) which carried the whole *luxCDABE* operon of the terrestrial luminescent bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*). This cyanobacterial strain has a high self-constitutive luminescence without the need to add exogenous aldehyde. The luminescence is high and stable in a range of temperatures between 20 ºC and 30 °C, in accordance with the characteristics of the luciferase of *Photorhabdus luminescens*, which is the bacterial luciferase with the highest thermal stability (Fernandez Pinas and Wolk, 2000; Szittner and Meighen.1990). Cell viability and growth were not affected by chromosomal integration or by the generation of endogenous aldehyde (Fernandez Pinas and Wolk, 1994). Figure 12 shows bright field and light emission image recorded with a Hamamatsu Photonics System showing the intense light emission from the recombinant cyanobacterium.

Figure 12**:** Micrographs of *Anabaena* sp. PCC 7120 CPB4337; (A) and (B) bright field and light emission image recorded with a Hamamatsu Photonics System of the same filament (courtesy of Dr. Fernandez-Piñas).

The test has been designed to get general toxicity information of a sample as a classical toxicity test but the main difference with these tests is that the measured end-point is not related with growth inhibition, reproduction, death or pigment content but with direct measurement of

bioluminescence inhibition by any toxic compound as in the well established *Vibrio fischeri* Microtox® test. The main advantages over Microtox® are (a) its ecological relevance in continental environments (b) the lack of need to add any salt or substrate to the bioassay avoiding any alteration of the sample´s chemical composition and (c) its thermal stability which makes the be assay suited to be performed at ambient temperature (up to 35 ºC) .

## Objectives

The present Thesis aims to evaluate the applicability of the *lux*-marked cyanobacterial bioreporter *Anabaena* sp. PCC7120 CPB4337 in environmental toxicity studies. To achieve this, firstly, a basic characterization of the bioreporter response under different conditions was performed in order to optimize assay conditions; secondly, the response of the bioreporter to different priority and emerging pollutant as well as real wastewater samples was assessed. A comparison of the response of the novel *lux*-marked cyanobacterial bioreporter with standardized ecotoxicity tests (Microtox®, *Pseudokirchneriella subcapitata* and *Dahpnia magna*) was undertaken in collaboration with the research group of Dr. Roberto Rosal from Universidad de Alcalá de Henares.

Special emphasis was made on the assessment of chemical interaction in pollutant mixtures. These interactions were analyzed by using the Combination index (CI) method, a method widely used in pharmacology to identify drug interactions.

## Specific objectives:

1. To characterize the response of *Anabaena* sp. PCC7120 CPB 4337 in different assay media and times of exposure in order to define standard biotest conditions to perform toxicity assays with *Anabaena* sp. PCC7120 CPB 4337. Optimized and standardized assay conditions are needed in order to obtain reproducible results; in this regard, reference toxicants need to be tested.

2. To characterize the response of *Anabaena* sp. PCC7120 CPB 4337 to different priority, emerging pollutants and real wastewater samples using bioluminescence inhibition as toxicity end-point and to compare its sensitivity (as ECx) with those of standard ecotoxicity tests.

3. To analyze the suitability of *Anabaena* sp. PCC7120 CPB 4337 to respond to the bioavailable fraction of contaminants. To achieve this, the specific issue of heavy metal bioavailability in the presence of different modifying factors (pH, organic chelators and precipitating anions) will be investigated. Chemical modeling (Visual MINTEQ and
4. PHREEQC programs) and correlation analyses will be used in an attempt to link toxicity with metal speciation.

5. To analyze the applicability of the Combination index (CI) method in environmental toxicity studies for the identification and quantification of chemical interactions (departures from additivity) in the toxicity of mixtures of pollutants. To achieve this, the combined toxicity of different priority and emerging pollutants will be assessed from binary to multicomponent mixtures, including pollutant-wastewater mixtures.

6. To explore the potential toxic effect of engineered nanoparticles, specifically $CeO_2$ nanoparticles, on the cyanobacterial bioreporter. A multi end-point toxicity approach will be undertaken; as the physicochemical properties of nanoparticles may determine their interaction with organisms; an extensive physicochemical characterization of the particles will also be performed.

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# CHAPTER II

## Heavy metals: speciation, toxicity and first approach to define toxicant interactions in a mixture: the role of effect levels.

# USE OF *LUX*-MARKED CYANOBACTERIAL BIOREPORTERS FOR ASSESSMENT OF INDIVIDUAL AND COMBINED TOXICITIES OF METALS IN AQUEOUS SAMPLES

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**Handbook on Cyanobacteria: Biochemistry, Biotechnology and Applications.** Editores: Percy M Gault y Harris J Marler. Nova Science Publishers, Inc. New York. USA. ISBN: 978-1-60741-092-8 **(2009)**

# Use of *lux*-marked cyanobacterial bioreporters for assessment of individual and combined toxicities of metals in aqueous samples.

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ABSTRACT

**Available freshwater resources are polluted by industrial effluents, domestic and commercial sewage, as well as mine drainage, agricultural run-off and litter. Among water pollutants, heavy metals are priority toxicants that pose potential risks to human health and the environment. Bacterial bioreporters may complement physical and chemical analytical methods by detecting the bioavailable (potentially hazardous to biological systems) fraction of metals in environmental samples. Most bacterial bioreporters are based on heterotrophic organisms; cyanobacteria, although important primary producers in aquatic ecosystems, are clearly underrepresented. In this chapter, the potential use of self-luminescent cyanobacterial strains for ecotoxicity testing in aqueous samples has been evaluated; for this purpose, a self-luminescent strain of the freshwater cyanobacterium *Anabaena* sp. PCC 7120 which bears in the chromosome a tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) and shows a high constitutive luminescence has been used. The ecotoxicity assay that has been developed is based on the inhibition of bioluminescence caused by biologically available toxic compounds; as a toxicity value, we have used the effective concentration of each tested compound needed to reduce bioluminescence by 50% from that of the control ($EC_{50}$). The bioassay allowed for acute as well as chronic toxicity testing. Cyanobacterial bioluminescence responded sensitively to a wide range of metals; furthermore, the sensitivity of the cyanobacterial bioreporter was competitive with that of published bacterial bioreporters. In contaminated environments, organisms are usually exposed to a mixture of pollutants rather than single pollutants. The toxicity of composite mixtures of metals using the cyanobacterial bioreporter was tested; to understand the toxicity of metal interactions, the combination index CI-isobologram equation, a widely used method for analysis of drug interactions that allows computerized quantitation of synergism, additive effect and antagonism has been used. Finally, this study indicates that cyanobacterial-based bioreporters may be useful tools for ecotoxicity testing in contaminated environments and that the CI-Isobologram equation can be applied to understand the toxicity of complex mixtures of contaminants in environmental samples.**

## INTRODUCTION

Available freshwater resources are polluted by industrial effluents, domestic and commercial sewage, as well as mine drainage, agricultural run-off and litter. Among water pollutants, heavy metals are priority toxicants that pose potential risks to human health and the environment. The evaluation of heavy metal contamination traditionally relies on highly sensitive and specific physical and chemical techniques such as atomic absorption spectroscopy or mass spectrometry; however, such methods are not able to distinguish between available (potentially hazardous to biological systems) and non-available (potentially non-hazardous) fraction of metals that exist in the environment in inert or complexed forms. Whole-cell bioreporters may complement physical and chemical methods by detecting the toxicity related with bioavailable metals in environmental

samples, effectively integrating the complexity of environmental factors (pH, redox potential, exchangeable cations, biological activity, etc) that contribute to bioavailability (Köhler *et al.* 2000). Bacterial bioluminescence (*lux* genes) has been extensively used in the construction of bioreporters (Belkin, 2003; Fernández-Piñas *et al.* 2000). Bacterial luciferase (*luxAB*) catalyzes the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$) producing visible light at 490 nm as a by-product. In naturally luminescent bacteria, the long-chain fatty aldehyde is synthesized by the reductase, transferase and synthetase encoded by *luxCD,E* (Meighen, 1991). The *lux* genes may be either be fused to promoters involved in the response to a particular toxin ("lights-on" concept) or used to indicate general metabolic status ("lights-off" concept) (Belkin, 2003); in the latter case, general toxicity or metabolic bioreporters are used to assess the overall toxicity of samples; in these "lights-off" bioreporters, the light reaction is directly proportional to the metabolic status of the cell and any inhibition of cellular activity is reflected in a decrease of bioluminescence. There are several general toxicity assays, already commercially available, which use the naturally bioluminescent marine bacterium *Vibrio fischeri* such as Microtox® (Azur Environmental), LUMISTOX® (Beckman instruments) or Toxalert® (Merck). The assays based on *Vibrio fischeri* have been frequently used because they are well introduced and standardized (Steinberg *et al*. 1995); although these tests offer rapid, easy handling and cost effective responses, the use of marine microorganisms for ecotoxicity testing to soil or freshwater ecosystems presents a number of problems, mainly related to the necessity of maintaining high saline concentrations in the analyte under test. Salinity may enhance insolubility of some organic substances (Steinberg *et al*. 1995) and/or affect bioavailability of heavy metals (Riba *et al.* 2003). To solve the issue of ecological relevance for terrestrial environments, the lights-off concept has been expanded to the construction of *lux*-marked bioreporters that are based mainly on soil heterotrophic bacteria (for a review see Belkin, 2007).

Cyanobacteria, as a dominant component of marine and freshwater phytoplankton are well-suited for detecting contaminants in aqueous samples (Bachman, 2003). They could represent an alternative/complement to the use of heterotrophic organisms in the development of bioreporters. So far, some cyanobacterial "lights-on" bioreporters have been reported (Mbeunkui, 2002; Schreiter, 2001; Erbe, 1996), but only one lights-off cyanobacterial bioreporter has been described (Shao *et al*., 2002). It is a *luc*-marked construct of the unicellular *Synechocystis* sp. PCC6803; this strain is not self-luminescent and the luciferase substrate, firefly luciferin, has to be added exogenously.

Contaminated environments are usually polluted by a number of different mixtures of pollutants; the number and concentrations of the different chemicals in these mixtures may be highly variable. Thus, organisms may be exposed to multiple pollutants at the same time; however, most toxicity assays have been done with individual contaminants and the few mixture analyses deal mostly with binary combinations of pollutants (Preston *et al.*, 2000; Fulladosa *et al.*, 2005; Dawson *et al.*, 2006). Due to the difficulty to deal with complex mixtures of contaminants, different empirical (Utgikar *et al.*, 2004; Robers *et al.*, 1990; Newman and McCloskey, 1996) and statistical (Ince *et al*., 1999; 2004; Gennings *et al.*, 2005; Ishaque *et al.*, 2006; Dawson *et al.*, 2006) approaches have been used to determine the nature of the combination effect of a mixture of pollutants. All these authors agree with the difficulty to establish a valid model for interpretation of results. A mixture of pollutants may have an additive, synergistic or antagonistic effect on a given organism (Gennings et al., 2005; Chou, 2006). One model that has proved useful to interpret drug interactions is the combination index CI-isobologram equation (Chou, 2006) that allows quantitative determination of pollutant interactions where $CI < 1$, $= 1$ and $> 1$ indicate synergism, additive effect, and antagonism, respectively.

Finally, one important issue on bioassay development is the difficulty to control biological systems and to standardize assay conditions in order to obtain reproducible results (Sorensen et al. 2006;

Belkin, 2007). In classical toxicological bioassays, (*Dhapnia magna*, *selenastrum capricornutum, Oncorhynchus mykiss* etc.), there are some acceptability criteria for validity of test results that are mainly related to the definition and maintenance of normalized assay conditions, the control of the internal variability of the controls, and the determination of the intra-laboratory precision with a reference toxicant in order to demonstrate the capability of the laboratory to obtain reproducible results (EPA, 1994, 2002).

In this chapter, an application of a cyanobacterial lights-off bioreporter for assessment of acute and chronic toxicity of a range of heavy metals (cations and anions) in aqueous samples is described. $CuSO_4$ was shown to be appropriate as a reference toxicant to be used in any toxicity assay with this cyanobacterial bioreporter and finally, the response of the bioreporter to a combination of three metals that displayed different toxicity: $Cu^{2+}$, $Zn^{2+}$ and $Cd^{2+}$ is shown; the effects of heavy metal interaction were analysed by the combination index CI-isobologram equation.

## DEVELOPMENT OF A TOXICITY ASSAY BASED ON THE BIOLUMINESCENT CYANOBACTERIAL BIOREPORTER: AN APPLICATION TO SINGLE AND COMBINED HEAVY METAL TOXICITY.

### Assay procedures

*Strain and culture conditions*

*Anabaena* sp. PCC 7120 strain CPB4337, which bears in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*), was used in this study to assess the toxicity of a range of cationic and anionic heavy metals. This strain shows a high constitutive self-luminescence with no need to add exogenous aldehyde; also cell viability is not significantly affected by the Tn5 insertion and the endogenous generation of aldehyde (Fernandez-Piñas and Wolk, 1994). Luminescence was shown to be high in this strain in a range of temperatures between 20 and 30º C, in accord with *Photorhabdus luminescens* luciferase having the greatest thermal stability (Fernandez-Piñas *et al.* 2000; Szittner and Meighen, 1990). *Anabaena* sp. PCC 7120 strain CPB4337 was routinely grown at 28ºC in the light, Ca. 65 µmol photons $m^2$ $s^{-1}$ on a rotary shaker in 50 ml AA/8 (Allen and Arnon, 1955) supplemented with nitrate (5mM) in 125 ml Erlenmeyer flasks. The strain was grown in liquid cultures with 10 µg of neomycin sulphate (Nm) per ml.

*Effect of external pH on constitutive luminescence*

Cells grown as indicated above, were washed twice and resuspended in $ddH_2O$ buffered with 2 mM of either MES (2-[N-morpholino] ethanesulfonic acid) for external pHs 4 and 5 or MOPS (3-[N-morpholino] propanesulfonic acid) for external pHs 5.8, 7 and 8. For luminescence measurements, 200 µl of the cell suspension were transferred to wells of an opaque white 96-well microtiter plate (PS white, Porvair Sciences Ltd. Shepperton, UK). Luminescence was continuously recorded for at least three hours in a Centro LB 960 luminometer (Berthold Technologies GmbH and Co.KG, Bald Wilbad, Germany) and was expressed in the instrument′ s arbitrary relative light units (RLU). All measurements were conducted in quadruplicate and were repeated at least twice.

*Metal bioassays for acute and chronic toxicity testing*

Standard metal test solutions in $ddH_2O$ were prepared for Cu ($CuSO_4$), Hg [$HgCl_2$], Pb [$Pb(NO_3)_2$], Ag ($AgSO_4$), Cd ($CdCl_2$), Zn ($ZnCl_2$), Co ($CoCl_2$), Ni ($NiSO_4$), Mn ($MnCl_2$), As ($Na_2HAsO_4$), Cr ($K_2CrO_4$), Mo ($Na_2MoO_4$) and V ($VO_3NH_4$) at a concentration of 125 mg/l and were serially diluted for the toxicity experiments. Serial dilutions of metal solutions were buffered with 2 mM MOPS and adjusted to pH 5.8. The use of most buffers (like Tris or Tricine) might not be appropriate as precipitation and complexation of metals might occur (Fernandez-Piñas *et al*. 1991). The alkylsulfonate derivatives of morpholine, like MOPS, are reported to be non-complexing for metals (Kandegedara and Rorabacher, 1999). The USEPA (1991) recommended MOPS for environmental

studies as it does not change the toxicity of effluents and sediment pore waters. Preliminary experiments in the current study showed that 2 mM MOPS did not affect growth or alter metal sensitivity of the cyanobacterium and was suitable to maintain constant pH during the time of the assay.

*Determination of acute and chronic toxicity of heavy metals*

Five to seven serial dilutions of metals and a control (cells not treated with metal) were tested. 160 µl of the appropriate metal were disposed in an opaque white 96-well microtiter plates. Cells, grown as described, were removed, centrifuged, washed twice, resuspended in $ddH_2O$ buffered with MOPS at pH 5.8 and added to the microtiter plate wells to reach a final cell density of 0.5. Three independent experiments with quadruplicate samples were carried out. Luminescence of each sample was recorded every 5 min for up to 1 h in the Centro LB 960 luminometer. For chronic toxicity testing (24 h), the 96-well microtiter plates were kept at room temperature (28 ºC) at low light (Ca. 30 µmol photons $m^2$ $s^-1$) during 24 h and luminescence was recorded for five minutes. All manipulations were conducted under controlled conditions, avoiding metal and microbial contamination and using disposable polystyrene supplies.

Toxicity response of the cyanobacterium was estimated as $EC_{50}$ values: the median effective concentration (mg/l) of a toxicant that causes a 50% of bioluminescence inhibition with respect to a non-treated control. $EC_{50}$ and its related statistical parameters, standard deviation (SD), coefficient of variation (CV) and confidence intervals (CI) were estimated using the linear interpolation method. (Norberg-King, T.J., 1993; USEPA, 1994; USEPA; 2002).

*Reference toxicant and inter-assay variability calibration*

Copper sulfate ($CuSO_4$) was selected as reference toxicant for calibration in all assays (USEPA, 1994, 2002). The final objective of the calibration was to calculate the mean $EC_{50\text{-}1h}$ and $EC_{50\text{-}24h}$ and the range of sensibility, represented by the upper and lower control limits at 95% of confidence of the bioluminescence inhibition assay when *Anabaena* CPB4337 is exposed to the reference toxicant; to achieve this, five copper dilutions were tested by quadruplicate in control wells of each assay to demonstrate good precision and low inter-assay variation.

*Toxicity of heavy metal combinations*

For metal combination experiments, metal solution of Cu ($CuSO_4$), Cd ($CdCl_2$) and Zn ($ZnCl_2$) and their two (Cu+Cd, Cu+Zn, Cd+Zn) and three (Cu+Zn+Cd) combinations were prepared. A constant ratio design (1:1) based on $EC_{50}$ values was selected as recommended by Chou (2006). Five dilutions (serial dilution factor = 2) of each metal and combination plus a control were tested in replicate in three independent experiments; the experimental design is shown in Table 1. All individual metals and two and three combinations assays were carried out at the same time as recommended by Chou (2006) to maximize computational analysis of data.

*Combination Index CI- Isobologram equation for determining heavy metal interaction effects.*

An application of the multiple effect analysis of Chou and Talalay (1977, 1981, 1984, 1991), which is based on the median-effect principle *(*mass-action law*)* (Chou, 1976) that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition (Chou, 2006), was used to calculate combined metal effects. This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations by using the median effect equation.

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \qquad (1)$$

Where *D* is the dose, *Dm* is the dose for 50% effect (inhibition of bioluminescence), *fa* is the fraction affected by dose *D*, *fu* is the unaffected fraction (*fa* = 1-*fu*), and *m* is the shape of the dose-effect curve. Therefore, the method takes into account both the

potency (*Dm*) and shape (*m*) parameters. The conformity of the data to the median-effect principle can be ready manifested by the linear correlation coefficient (*r*) of the data to the logarithmic form of equation 1 (Chou, 2006).

The combination index CI-isobologram equation (Chou, 1991; Chou and Talalay, 1984):

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \qquad (2)$$

**Table 1. Constant ratio design for two and three heavy metal combination for $Cu^{2+}(D)_1$, $Zn^{2+}(D)_2$ and $Cd^{2+}$ $(D)_3$ based on $EC_{50}$ ratios as proposed by Chou and Talalay (1984) and Chou (1991) for drug combination experimental design.**

| Dose (mg/l) | | | | | | |
|---|---|---|---|---|---|---|
| Single toxicant | | | | Two Toxicant Combo | | |
| Dilutions | $Cu^{2+}$ | $Zn^{2+}$ | $Cd^{2+}$ | $Cu^{2+}$ | $Zn^{2+}$ | $Cd^{2+}$ |
| | $(D)_1$ | | | $(D)_1+(D)_2$ (1:3.6) | | |
| ¼ $(EC_{50})_1$ | 0.00625 | | | 0.00625 | 0.0225 | |
| ½ $(EC_{50})_1$ | 0.0125 | | | 0.0125 | 0.045 | |
| 1 $(EC_{50})_1$ | 0.025 | | | 0.025 | 0.09 | |
| 2 $(EC_{50})_1$ | 0.05 | | | 0.05 | 0.18 | |
| 4 $(EC_{50})_1$ | 0.1 | | | 0.1 | 0.36 | |
| | | $(D)_2$ | | $(D)_2+(D)_3$ (1:1.82) | | |
| ¼ $(EC_{50})_2$ | | 0.0225 | | | 0.0225 | 0.041 |
| ½ $(EC_{50})_2$ | | 0.045 | | | 0.045 | 0.083 |
| 1 $(EC_{50})_2$ | | 0.09 | | | 0.09 | 0.167 |
| 2 $(EC_{50})_2$ | | 0.18 | | | 0.18 | 0.334 |
| 4 $(EC_{50})_2$ | | 0.36 | | | 0.36 | 0.668 |
| | | | $(D)_3$ | $(D)_1+(D)_3$ (1:6.56) | | |
| ¼ $(EC_{50})_3$ | | | 0.041 | 0.00625 | | 0.041 |
| ½ $(EC_{50})_3$ | | | 0.083 | 0.0125 | | 0.083 |
| 1 $(EC_{50})_3$ | | | 0.167 | 0.025 | | 0.167 |
| 2 $(EC_{50})_3$ | | | 0.334 | 0.05 | | 0.334 |
| 4 $(EC_{50})_3$ | | | 0.668 | 0.1 | | 0.668 |
| | | | | Three Toxicant combo | | |
| | | | | $(D)_1+(D)_2+(D)_3$ (1:1.82:6.56) | | |
| | | | | 0.00625 | 0.0225 | 0.041 |
| | | | | 0.0125 | 0.045 | 0.083 |
| | | | | 0.025 | 0.09 | 0.167 |
| | | | | 0.05 | 0.18 | 0.334 |
| | | | | 0.1 | 0.36 | 0.668 |

**$EC_{50}$ is the effective concentration of a toxicant witch caused a 50% of bioluminescence inhibition.**

has been used for data analysis of two-metal combinations. For three metal combinations a third term, $(D)_3/(Dx)_3$, is added (Chou, 1991). From equation 2, CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, respectively (Chou, 2006). Equation 2 dictates that metal 1, i.e, $(D)_1$, and metal 2, i.e, $(D)_2$ in the numerators in combination inhibits luminescence by *x%*, $(Dx)_1$ and $(Dx)_2$ in the denominators of equation 2 are the doses of metal 1 and 2 alone, respectively, that also inhibit bioluminescence by *x%*. *Dx* can be readily calculated from equation 1, where *D* is designated for *x%* luminescence inhibition. When equation 2 equals 1 (i.e, CI = 1), it represents the classical isobologram equation. (Chou, 2006).

Data where also evaluated by the isobologram technique (Chou, 1991, Chou and Talalay, 1984), a dose-oriented geometric method of assessing chemical interactions. This method yields conclusions quantitatively identical to those of the effect-oriented CI method described above. Computer program CompuSyn (Chou and Martin, 2005, Combosyn.inc) was used for calculation of dose-effect curve parameters, CI values, conventional isobolograms, *fa*-CI plot (plot representing CI versus *fa*, the fraction affected by a particular dose; see equation 1) and Polygonograms (a polygonal graphic representation depicting synergism, additive effect and antagonism for three or more drug combinations).

**Effect of pH on constitutive self-luminescence**

The bioluminescence response of strain CPB4337 was investigated across a wide range of pH values. Cells were washed and resuspended in $ddH_2O$ buffered with either MES or MOPS to a range of pH values between 4 and 8 and their luminescence recorded as a function of time (Fig. 1).

Self-luminescence remained high and stable during at least 3 h at pH values of 5.8, 7 and 8 with an optimum at pH 5.8 reaching luminescence levels above 30000 RLUs at 1 h. Luminescence significantly decreased to levels around 5000-4000 RLUs at pHs 5 and 4; at pH 5 luminescence remained quite stable during the time of measurement; at pH 4, however, luminescence was stable during 40-60 min but showed a steady decrease thereafter. The data indicated that this strain was able to emit high and steady levels of

luminescence in a wide pH range. The optimum pH of luminescence, 5.8, was therefore chosen for the standard metal toxicity assays; besides, at this pH value, most heavy metals are biologically available as the free ion species and should be fully toxic to the cyanobacterial strain.

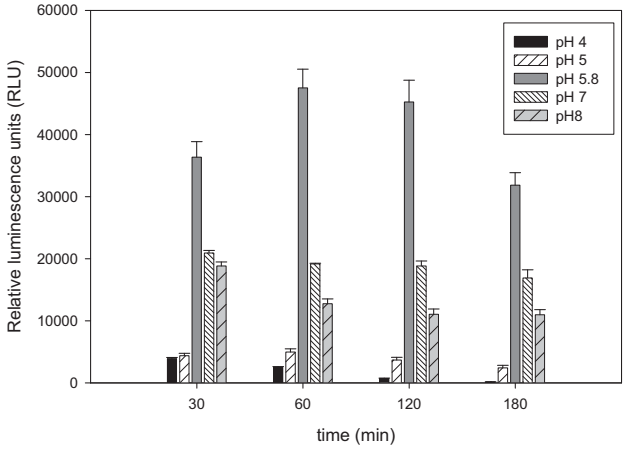


**Figure 1. Effect of external pH on luminescence (expressed as Relative Light Units, RLU) of *Anabaena* sp. PCC 7120 strain strain CPB4337. Cells were resuspended in dd$H_2O$ buffered with either 2 mM MES or 2 mM MOPS at the desired pH at an $OD_{750}$: 0.5. Luminescence was recorded continuously during 3 h. Error bars represent standard error of the means of at least three independent experiments with quadruplicate samples.**

## Toxicity of cationic and anionic heavy metals to strain CPB4337

Table 2 depicts the sensitivity of the assay to nine cationic (Hg, Cu, Zn, Cd, Ag, Pb, Ni, Co and Mn) and three anionic heavy metals ($CrO_4^{2-}$; $VO^{3-}$ and $MoO_4^{2-}$). The metalloid As (as $AsO_4^{3-}$) was also included in the assay due to its reported toxicity to most organisms and the fact that it is considered as one of the most serious contaminants in drinking waters. As also shown in the table, the developed assay allowed for acute (1 h of exposure) as well as chronic (24 h of exposure) toxicity testing.

**Table 2. Acute toxicity (1 h of exposure) and chronic toxicity (24 h of exposure) response as $EC_{50}$ values to several cationic and anionic compounds of the cyanobacterium *Anabaena* sp. PCC7120 CPB 4337.**

| Acute toxicity (1h) | | | | | |
|---|---|---|---|---|---|
| **Toxic compound** | **$EC_{50}$** | **SD** | **CV(%)** | **$CI_L$ 95%** | **$CI_U$ 95%** |
| **$Hg^{2+}$** | **0.1266** | **0.002** | **1.579** | **0.1199** | **0.1295** |
| **$Cu^{2+}$** | **0.0251** | **0.002** | **7.971** | **0.0207** | **0.0283** |
| **$Zn^{2+}$** | **0.0912** | **0.0027** | **2.960** | **0.0849** | **0.0962** |
| **$Cd^{2+}$** | **0.194** | **0.022** | **11.340** | **0.1203** | **0.241** |
| **$Ag^{+}$** | **0.0363** | **0.002** | **5.509** | **0.032** | **0.0425** |
| **$Pb^{2+}$** | **3.82** | **0.978** | **25.602** | **1.0205** | **5.3605** |
| **$Ni^{2+}$** | **5.99** | **1.50** | **25.04** | **3.81** | **8.89** |
| **$Co^{2+}$** | **7.25** | **1.11** | **15.31** | **6.118** | **9.045** |
| **$Mn^{2+}$** | **4.22** | **1.105** | **26.18** | **2.285** | **6.612** |
| **$AsO_4^{3-}$** | **34.93** | **10.37** | **29.69** | **9.906** | **50.844** |
| **$CrO_4^{2-}$** | **7.53** | **2.14** | **28.42** | **5.5036** | **9.81** |
| **$VO_3^{-}$** | **>100** | **-** | **-** | **-** | **-** |
| **$MoO_4^{2-}$** | **11.1** | **3.1363** | **28.25** | **8.93** | **20.38** |

| Chronic toxicity (24 h) | | | | | |
|---|---|---|---|---|---|
| **Toxic compound** | **$EC_{50}$** | **SD** | **CV(%)** | **$CI_L$ 95%** | **$CI_U$ 95%** |
| **$Hg^{2+}$** | **0.0352** | **0.0013** | **3.69** | **0.0322** | **0.0386** |
| **$Cu^{2+}$** | **0.0056** | **0.0003** | **4.61** | **0.0055** | **0.0076** |
| **$Zn^{2+}$** | **0.0798** | **0.0036** | **4.51** | **0.068** | **0.0921** |
| **$Cd^{2+}$** | **0.187** | **0.011** | **5.88** | **0.01471** | **0.2163** |
| **$Ag^{+}$** | **0.0374** | **0.0034** | **9.09** | **0.0284** | **0.0496** |
| **$Pb^{2+}$** | **0.3152** | **0.0089** | **2.82** | **0.2886** | **0.3391** |
| **$Ni^{2+}$** | **0.0538** | **0.0026** | **4.83** | **0.0478** | **0.0595** |
| **$Co^{2+}$** | **0.2652** | **0.0058** | **2.19** | **0.2519** | **0.2746** |
| **$Mn^{2+}$** | **0.5473** | **0.0711** | **12.99** | **0.431** | **0.6803** |
| **$AsO_4^{3-}$** | **52.18** | **8.66** | **16.60** | **33.2** | **64.71** |
| **$CrO_4^{2-}$** | **0.7** | **0.16** | **22.86** | **0.3876** | **0.937** |
| **$VO_3^{-}$** | **0.6152** | **0.108** | **17.56** | **0.3912** | **0.8204** |
| **$MoO_4^{2-}$** | **0.3953** | **0.028** | **7.08** | **0.3621** | **0.4727** |

**$EC_{50}$ = effective concentration (mg/l) of a toxicant that causes a 50% reduction of the self-luminescence emission of the test organism. SD: Standard deviation. CV%: Percent coefficient of variation, where CV% = (standard deviation x 100/mean). $CI_L$ 95% = Lower 95% Confidence interval $CI_U$ 95%: Upper 95% Confidence interval. $EC_{50}$ and all statistical parameters where calculated using the linear interpolation method (Norberg-King, 1993). 3 independent experiments with 4 replicates where carried out for each compounds. In all experiments 5 different concentrations of the toxicant plus a control were tested and a reference toxicant test experiment was included.**

In the acute toxicity assay, two groups of metals could be discerned by toxicity levels: high toxicity (detection range of 0.025 to 0.2 mg/l): Cu, Ag, Zn, Hg and Cd; and low toxicity (detection range of 3 to > 100 mg/l): Pb, Mn, Ni, Co, Cr, Mo, As and V. The order of sensitivity being Cu > Ag > Zn > Hg > Cd > Pb > Mn > Ni > Co > Cr > Mo > As > V. Except for Zn, Cd and Ag that showed no significant change, the sensitivity of the cationic and anionic metals

increased one (Hg, Cu, Pb, Co, Mn and Cr), two (Ni) or three orders of magnitude (V and Mo) after 24 h of exposure; however, chronic toxicity increased the $EC_{50}$ of As (Table 2). As a general conclusion, the cyanobacterial reporter strain was much more sensitive to cationic than to anionic heavy metals, although the sensitivity to most of them increased with exposure time. The strain was extraordinarily sensitive to Cu, As seemed to be the less toxic of the tested compounds.

**Table 3. Comparison of toxicity values as $EC_{50}$ for several bacterial lights-off bioreporters.**

| Bioreporter | $EC_{50}$ (mg $l^{-1}$) | | |
|---|---|---|---|
| | Cu | Zn | Cd |
| *Anabaena* sp. CPB4337 | 0.03 | 0.10 | 0.16 |
| *Synechocystis* sp. PCC6803 *(luc)*[a2] | 0.24 | 0.88 | - |
| *E. coli* HB101 (*luxCDABE*)[a2] | 1.40 | 0.15 | - |
| *Pseudomonas fluorescens 8866* | 0.30 | 0.10 | - |
| *Pseudomonas putida* | 0.17 | 0.04 | - |
| *Pseudomonas fluorescens* 10586s | 0.09 | 0.09 | 0.17 |
| *Pseudomonas fluorescens* 10586s | 0.76 | 0.89 | 0.98 |
| *Rhizobium trifolii* TA1 Tn5*luxAB*[c1] | 0.78 | 0.48 | 2.14 |
| *Photobacterium phosphoreum* 844[c2] | 2.34 | 3.45 | - |
| Microtox®[c2] | 1.89 | 2.35 | 9.78 |
| Microtox®[d3] | 8.00 | 2.50 | - |
| *Rhizobium trifolii* F6 pUCD607 | 0.42 | 0.94 | 0.06 |
| *ToxScreen*®[f4] | 0.02 | 0.60 | 0.06 |
| *Janthinobacterium lividum* YH9-RC | 10.50 | 1.30 | 1.10 |

[a] Data from Shao *et al*. (2003)
[b] Data from Weitz *et al*. (2001)
[c] Data from Paton *et al.* (1997a)
[d] Data from Bulich and Isenberg (1981)
[e] Data from Paton *et al.* (1997b)
[f] Data from Ulitzur *et al.* (2002)
[g] Data from Cho *et al*. (2004)
[1] $EC_{50}$ calculated after 30 min exposure
[2] $EC_{50}$ calculated after 15-20 min exposure
[3] $EC_{50}$ calculated after 5 min exposure
[4] $EC_{50}$ calculated after 20-30 min exposure
- No data

When comparing the performance of strain CPB4337 with that of other bioreporters (Table 3), the sensitivity of the cyanobacterial strain to selected metals was competitive with that of a range of bioluminescence-based bioreporters. In fact, the *lux*-based assay using strain CPB4337 was more sensitive to Zn, Cu and Cd than the commercially available Microtox assay based on the marine luminescent bacteria *Vibrio fischeri* (Bulich and Isenberg, 1981; Paton *et al.*, 1997a, b) and more sensitive to Zn than the recently commercialized ToxScreen bioassay based on *Photobacterium leiognathi* (Ulitzur et al. 2002). The fact that *Anabaena* sp. PCC 7120 strain CPB4337 has an estimated doubling time of 26 h allowed for the development of the chronic assay that may be particularly useful for chemicals which mainly show long-term toxicity (as demonstrated for Cr, V and Mo; Table. 2); long-term biotests based on bacteria with shorter doubling times may cause problems in toxicity data interpretation as potential cell division and growth may occur during the assay.

**Calibration of the toxicity bioassay with *Anabaena* sp. PCC 7120 using copper as a reference toxicant.**

As in any other analytical instrument, toxicity bioassays require being calibrated against a standard, the reference toxicant. This approach is encouraged by USEPA when performing acute and chronic toxicity tests (USEPA, 1994, 2002). Although the test organism can not be adjusted to an expected response, the calibration allows accepting or refusing a test organism that, when exposed to the reference toxicant, falls in or out of the range determined for the chemical compound. Toxicity test precision is described by the mean, standard deviation, and relative standard deviation (percent coefficient variation, or CV%) of the calculated endpoints (i.e, $EC_{50}$, $LC_{50}$, etc) from the replicated test. A reference toxicant concentration series should be selected that will consistently provided partial bioluminescence inhibition at two or more concentrations, and a control chart should be prepared for each reference toxicant, test organism, condition and endpoint. Toxicity end points from five to six are adequate for established the control chart (US EPA, 2002; US EPA, 1996). In this technique a running plot is maintained for the toxicity values (Xi) from successive tests with a given reference toxicant and end points ($EC_{50}$) are examined to determine if they are within prescribed limits. The mean and upper and lower control limits (±2SD) are recalculated with each successive test. Here, a calibration study that determines the intralaboratory precision and relative

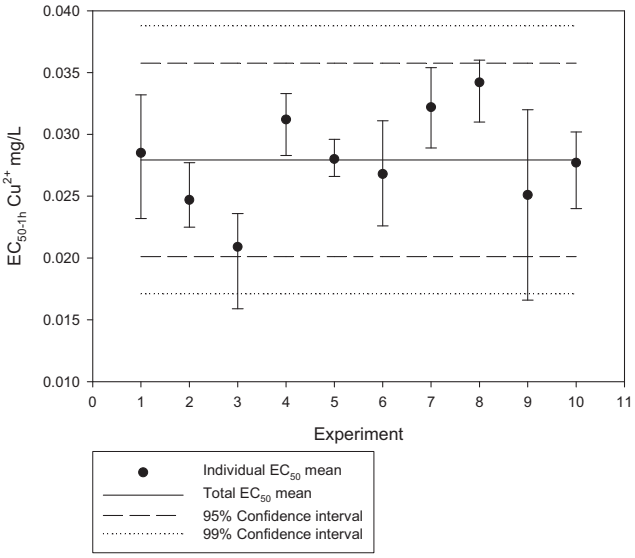


**Figure 2. Control Chart for $Cu^{2+}$ as reference toxicant in acute (1h) toxicity bioassays of *Anabaena sp* PCC7120 CPB 4337. 10 independent experiments with 4 replicates were carried out. The vertical bars indicate 95% confidence intervals for individual $EC_{50}$. Individual $EC_{50}$ and 95% Confidence Intervals where calculated using the linear interpolation method (Norberg-King, 1993). Total $EC_{50}$ mean, 95% and 99% of Confidence interval indicates Mean; Mean ± 2 S.D and Mean ± 3 S.D of all experiments, respectively. S.D: standard deviation of total $EC_{50}$ mean.**

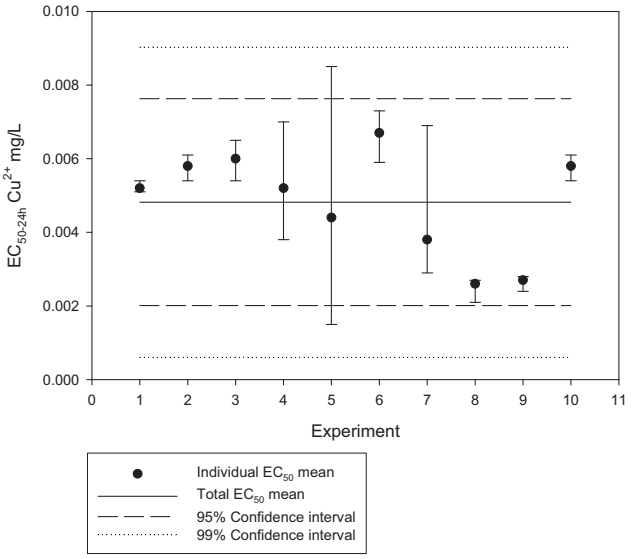


**Figure 3. Control Chart for $Cu^{2+}$ as reference toxicant in Chronic (24 h) toxicity bioassay of *Anabaena sp* PCC7120 CPB 4337. 10 independent experiments with 4 replicates were carried out. The vertical bars indicate 95% confidence intervals for individual $EC_{50}$. Individual $EC_{50}$ and 95% Confidence Intervals where calculated using the linear interpolation method (Norberg-King, 1993). Total $EC_{50}$ mean, 95% and 99% of Confidence interval indicates Mean; Mean ± 2 S.D and Mean ± 3 S.D of all experiments, respectively. S.D: standard deviation of total $EC_{50}$ mean.**

accuracy in performing the acute and chronic toxicity assessment of $Cu^{2+}$ ($CuSO_4$) to *Anabaena* sp. PCC 7120 CPB4337, using 50% bioluminescence inhibition as endpoint, is presented.

The control charts (Fig. 2 and 3) showed the performance of the $EC_{50}$ values of ten independent experiments (with four replicates) of the cyanobacterium *Anabaena* sp. PCC 7120 strain CPB 4337 exposed to the selected reference toxicant ($CuSO_4$) in acute (1 h) and chronic (24 h) toxicity assays. In acute toxicity test (1 h) (Fig. 2), the $EC_{50}$ mean was 0.02793 mg/l of $Cu^{2+}$, standard deviation (SD) was 0.00391, and percent coefficient variation, (CV%) was 14 %. The range of sensitivity represented by the upper and lower 95% confidence limits of the $EC_{50}$ was 0.0201 and 0.0357 mg/l of $Cu^{2+}$, respectively. In the chronic toxicity test (24 h) (Fig. 3), the $EC_{50}$ mean was 0.00482 mg/l of $Cu^{2+}$, standard deviation (SD) was 0.00140, and percent coefficient variation, (CV%) was 29,15 %. Upper and Lower 95% confidence limits of the $EC_{50}$ were 0.00201 and 0.00763 mg/l of $Cu^{2+}$ respectively.

These results indicate that $Cu^{2+}$ is well suited to be used as reference toxicant in toxicity bioassays based on bioluminescence inhibition of the cyanobacterium *Anabaena* sp. PCC 7120 strain CPB4337; the levels of test reproducibility are within those reported by USEPA (2002) from an statistical study on national laboratory performance in which intra-laboratory percent coefficient variations (CV %) ranged from 12.9 % with SDS [Sodium dodecyl (lauryil) sulphate] to 77% with $Cd^{2+}$ as reference toxicants in acute toxicity assays with *Daphnia magna* at 24 h time of exposure (USEPA, 2002), and ranged from 32.1% for *Pimephales promelas* to 58.9% for *Ceriodaphnia dubia* ($LC_{50}$ at 96 h) in chronic toxicity assays with KCl as reference toxicant (USEPA 1994).

## CI-Isobologram analyses of heavy metal interactions

To study heavy metal interactions, three metals that showed different levels of toxicity: $Cu^{2+}$, $Zn^{2+}$ and $Cd^{2+}$ were chosen. These studies were carried out by quantitative analyses of synergism or antagonism at different effect levels (*fa*).

Table 4 showed the dose-effect curve parameters [*Dm* ($EC_{50}$), *m* (Shape of the curve), and *r* (the conformity of data to the mass-action law)] of $Cu^{2+}$, $Zn^{2+}$ and $Cd^{2+}$ and their two and three combinations. *Dm* values are in good agreement with these obtained with the linear interpolation method (Norberg-King, T.J., 1993) (see Table 2). And *r* values ranged from 0.895 to 0.971, indicating good agreement to the mass-action law (Chou, 1994, 2005).

**Table 4. Dose-effect curve parameters for $Cu^{2+}$, $Zn^{2+}$ and $Cd^{2+}$ and their two and three combinations.**

| Metal /Combo | ***Dm*** | ***m*** | ***r*** |
|---|---|---|---|
| Cu | 0.02267 | 2.00007 | 0.89924 |
| Zn | 0.10058 | 2.53390 | 0.96648 |
| Cd | 0.16128 | 1.77381 | 0.89542 |
| Cd + Cu | 0.10227 | 2.71092 | 0.92843 |
| Cu + Zn | 0.06942 | 2.71824 | 0.94201 |
| Cd + Zn | 0.16188 | 2.36976 | 0.97125 |
| Cu + Zn + Cd | 0.12605 | 2.88579 | 0.94537 |

***Dm*: Median-effect dose = $EC_{50}$. *m* is the slope of the dose-effect curve (see equation 1) and *r* is the goodness of fit of data to the mass-action law. 3 independent experiments with two replicates were used for data analysis.**

The *Dm* and *m* values for single metals and for their combination mixtures were used for calculating synergism or antagonism based on the CI equation (2) (Chou, 2006).

*Fa*-CI plot (Fig. 4) depicted the CI (Combination Index values) versus *fa* (fraction of luminescence inhibited by a toxicant with respect to the control) for two (Cu+Cd, Cu+Zn and Zn+Cd) and three (Cu+Zn+Cd) metal combination. Fa-CI plot is an effect-oriented plot (Chou, 2006) that showed the evolution of the kind of interaction (CI<1, CI=1 and CI>1 indicated synergism, additive effect and antagonism, respectively) as a function of the level of the effect of the toxicant on the organism (*fa*, where ECa = *fa* x 100; i.e., $EC_{20}$= $f_{20}$ x100).

As shown in fig. 4, at lower *fa* levels, all tested metal combination showed antagonism; the combination of the three metals showed the strongest antagonism. At *fa* levels near 0.5 for the Cu+Cd combination and near 0.8 for Cu+Zn and Cu+Zn+Cd combinations, CI approached a value of 1, indicating an additive effect. Interestingly, at *fa* levels higher than 0.8 for Cu+Cd combination and higher than 0.95 for the Cu+Zn and Cu+Zn+Cd combinations,CI values were lower than 0.9 indicating that these metal interactions became synergistic.

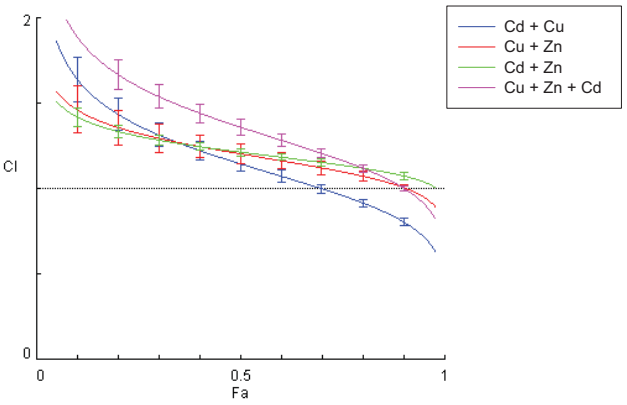


**Figure 4. Combination index Plot (*fa-CI plot*) for a set of 4 heavy metal combinations (Cd+Zn, Cu+Cd, Zn+Cu and Cu+Zn+Cd). CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa*= 0.10 to 0.95. CI< 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively. Three independent experiments with two replicates were used. The vertical bars indicate 95% of confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005). Figure generated by using CompuSyn (Chou and Martin, 2005).**

The Cd+Zn combination was antagonistic at effect levels below 0.9 and became nearly additive above this value. These results showed that the interactions between these three metals were antagonistic or synergistic depending on the effect levels: at low levels, all combination showed antagonism while synergism was found at higher effect levels.

be due to competition for uptake/binding sites in the cell membrane or else suppression of toxic effect by one cation towards the others; in fact, it has been reported than Zn suppressed the toxic effect of Cu (Dirilgen and Inel, 1994). However, when the toxic effect of the metal combination was high, the metal interactions turned to be synergistic, probably due to membrane damage and bulk entry of the three metals inside the cells. In fact, cadmium and copper have been shown to cause membrane damage to microbial cells (Cabral, 1990; Fernández-Piñas et al, 1997; Sharma, 1999). Preston *et al.*, (2000) also found

synergistic interactions between the toxic effect of Zn+Cu and Zn+Cd combinations to a luminescent *Escherichia coli* strain and Cd+Cu combination to a luminescent *Pseudomonas fluorescens* strain. In a similar study, Ince *et al.* (1999) found that binary mixtures of Zn, Cu, Co and Cr were mostly antagonistic to both *Vibrio fischeri* and the duckweed *Lemna minor;* Fulladosa *et al.* (2005) also found an antagonistic effect for Co+Cd, Cd+Zn, Cd+Pb and Cu+Pb combinations and a synergistic effect for Co+Cu and Zn+Pb combinations to *Vibrio fischeri*. The observed differences in metal interaction toxicity between different organisms are probably related with metal specific uptake mechanisms and support the notion of the "battery" concept that relies on the need to use different bioassays based on species of different origin to assess the ecological impacts of pollutant release into natural environments (Ince *et al.,*1999).

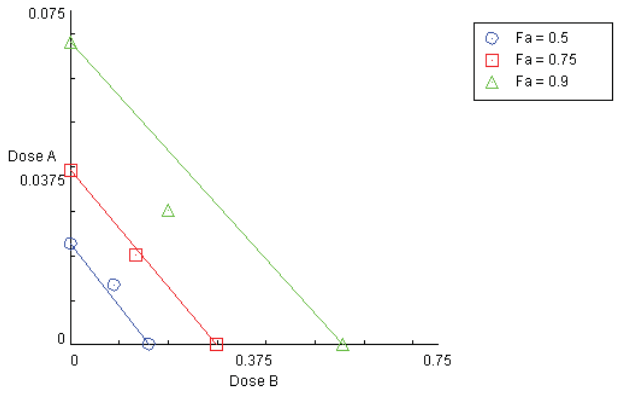


**Figure 5: Classic isobologram for the combination of $Cu^{2+}$ (Dose A) and $Cd^{2+}$ (Dose B) in mg/l as calculated by CompuSyn (Chou and Martin, 2005). Lines indicate simple addition at the *fa* levels of 0.5, 0.75 and 0.9. *Fa* is the fractional inhibition of bioluminescence of the sample. Combination data points on the hypotenuse, on the lower left and on the upper right indicate an additive effect, synergism and antagonism respectively. Figure generated by using CompuSyn (Chou and Martin, 2005).**

The CI plot is an effect-oriented graphic while the isobologram is a dose-oriented graphic but both should yield exactly identical conclusions for metal interactions Chou, 1994, 2006). Figures 5, 6 and 7 showed classic isobolograms for Cu+Cd, Cu+Zn and Zn+Cd combinations, respectively, at *fa* levels of 0.5 ($EC_{50}$), 0.75 ($EC_{75}$) and 0.9 ($EC_{90}$).

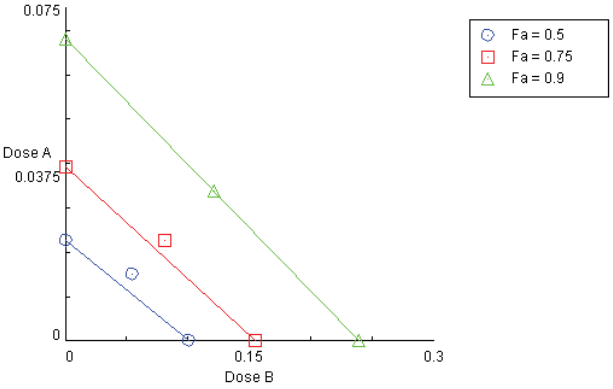


**Figure 6: Classic isobologram for the combination of $Cu^{2+}$ (Dose A) and $Zn^{2+}$ (Dose B) in mg/l as calculated by CompuSyn (Chou and Martin, 2005). Lines indicate simple addition at the *fa* levels of 0.5, 0.75 and 0.9. *Fa* is the fractional inhibition of bioluminescence of the sample. Combination data points on the hypotenuse, on the lower left and on the upper right indicate an additive effect, synergism and antagonism respectively. Figure generated by using CompuSyn (Chou and Martin, 2005).**

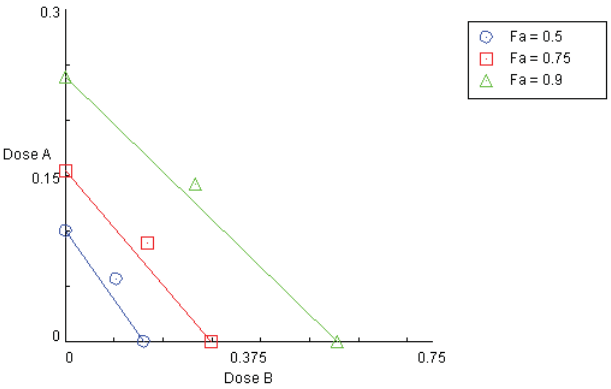


**Figure 7. Classic isobologram for the combination of $Zn^{2+}$ (Dose A) and $Cd^{2+}$ (Dose B) in mg/l as calculated by CompuSyn (Chou and Martin, 2005). Lines indicate simple addition at the *fa* levels of 0.5, 0.75 and 0.9. *Fa* is the fractional inhibition of bioluminescence of the sample. Combination data points on the hypotenuse, on the lower left and on the upper right indicate an additive effect, synergism and antagonism respectively. Figure generated by using CompuSyn (Chou and Martin, 2005).**

Figure 5 showed a classic isobologram for Cu+Cd combination; the relationship of Cu+Cd combination could be appropriately described as antagonistic at *fa*=0.5, additive at *fa*=0.75 and synergistic at *fa*=0.9. Figure 6 showed a classic isobologram for Cu+Zn combinations, their relationship could be appropriately described as antagonistic at *fa*=0.5 and *fa*=0.75 and as additive at *fa*=0.9. Figure 7 showed a classic isobologram for Zn+Cd combination and their relationship could be described as slightly

antagonistic at all effect levels (*fa*=0.5, 0.75 and 0.9). As expected, both the *fa*-CI plot and isobolograms yielded identical results at the shown effect levels.

A polygonogram is a semiquantitative method of representing interactions between three or more compounds at a determined *fa* level. Fig 8 showed polygonograms at *fa*=0.5 and *fa*=0.9 for three metals (Cu, Zn and Cd) in two metal combinations. As already expected, at *fa*=0.5 Zn+Cd and Zn+Cu combination exhibited moderate antagonism and Cu+Cd slight antagonism. Cu+Zn and Zn+Cd combinations showed nearly additive interactions at *fa*=0.9 and Cu+Cd moderate synergism.

In general, the results reported in this chapter show that the mass action low (Chou, 1976) and the CI-Isobologram analyses (Chou and Talalay, 1981) are applicable for toxicity interpretation of single and combined heavy metal toxicities in bioluminescence-inhibition based bioassays. The *fa*-CI plot was useful for a rapid

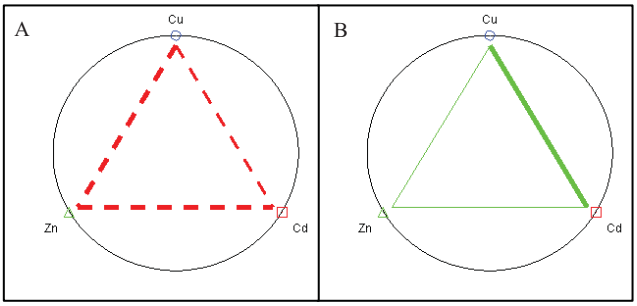


**Figure 8. Polygonogram at a fractional inhibition of bioluminescence, *fa* = 0.5 (A) and *fa*= 0.9 (B) for three heavy metals in two-metal combinations (Cd+Zn, Cd+Cu, Zn+Cu) as calculated by CompuSyn (Chou and Martin, 2005). Solid lines indicate synergisms, broken lines indicate antagonism. The thickness of the line represents the strength of synergism or antagonism. Figure generated by using CompuSyn (Chou and Martin, 2005).**

visual interpretation of general trends of interactions, isobologram offered more partial and clear vision of those interactions but it couldn't represent in two dimensional graphics interactions of more than two components; but, since it is dose-oriented, it allows to represent not only constant ratio interactions but also non-constant ratio interactions in order to know ratios of maximal synergy or antagonism (Chou, 2006; Chou and Martin, 2005). Finally polygonograms offered a whole overview of interactions of three or more compounds and allowed rapid interpretations of interactions.

The CI-isobologram equation is widely used for drug interactions in medicine and pharmacology; this report is probably the first application of this model in environmental toxicology. Most studies in this field are based on models such as the simple additive model in which the combined toxicities of (*A*+*B*) are equal to the sum of the individual toxicities of (*A*)+(B) [directly as percent inhibition or via transformation to toxicity units (TU) where TU=(100/$EC_{50}$)] (Hermens and Leeuwagh 1982; Stratton, 1988; Ribo and Rogers 1990; Pedersen and Petersen 1996), or the fractional product method where the combined effect of *A*+*B* is equal to [1-(1-*fa*)(1-*fb*)] (Webb, 1966) witch takes into account the potency (*f*) but not the shape (*m*) of the dose-effect curve and, according to Chou (2006), these models may have limited validity. More recently, Gennings *et al.*, (2005) have reported an additivity model for assessing toxicological interactions that takes into account the definition of additivity given by Berenbaum (1985; 1989) which is based on the classical isobolograms for the combination of two chemicals as well as changes in the slope of a dose-response curve of a chemical in the presence of another chemical.

## CONCLUSIONS

In this chapter, an application of a general toxicity lights-off bioassay based on self-luminescent filamentous cyanobacteria for the assessment of individual and combined metal toxicity in aqueous samples is reported. The bioassay allowed for acute as well as chronic toxicity testing. The assay is simple, rapid and highly sensitive. Calibration of the assay using copper as reference toxicant has demonstrated that the levels of test reproducibility are within those reported by USEPA. The combination index-isobologram equation has proven to be a suitable method for toxicity assessment of metal interactions in the cyanobacterial bioreporter; in general, the toxicity of binary and ternary combinations of Cd, Cu and Zn was antagonistic at low effect levels but synergistic at high effect levels;

in this context, more systematic studies on toxicity of combinations of pollutants towards a range of organisms are needed as any possible synergistic enhancement in toxicity should be taken into account when establishing environmental safety regulations. Due to its sensitive response, low maintenance cost and ecological relevance, the freshwater luminescent filamentous cyanobacterial strain CPB4337 expand the battery of available bacterial bioreporters and may be particularly well suited to provide information on potential environmental damage of various freshwater environments.

## ACKNOWLEDGEMENTS

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# EFFECT OF PH, EDTA, AND ANIONS ON HEAVY METAL TOXICITY TOWARDS A BIOLUMINESCENT CYANOBACTERIAL BIOREPORTER.

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# Effect of pH, EDTA, and Anions on Heavy Metal Toxicity Toward a Bioluminescent Cyanobacterial Bioreporter

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**Abstract** The bioavailability and therefore toxicity of a metal depends on the chemical species present in a particular environment. We evaluated the effect of a series of factors that could potentially modify metal speciation on the toxicity of Hg, Cu, Zn, and Cd toward a recombinant strain of the freshwater cyanobacterium *Anabaena* sp. PCC 7120 with cloned *lux* operon of luminescent terrestrial bacterium *Photorhabdus luminescens*. The strain, denoted as *Anabaena* CPB4337, showed a high constitutive luminescence with no need to add exogenous aldehyde. The tested factors were pH, EDTA (as organic ligand), and anions $PO_4^{3-}$, $CO_3^{2-}$, and $Cl^-$. Chemical modeling and correlation analyses were used to predict metal speciation and link it with toxicity. In general, metal toxicity significantly correlated to the predicted metal free-ion concentration, although Zn–EDTA complexes and certain Hg chloro-complexes could also exhibit some toxicity to cyanobacteria. An interesting feature of metal toxicity to strain *Anabaena* CPB4337 was that low amounts of $PO_4^{3-}$ and $CO_3^{2-}$ increased metal toxicity; this effect could not be related to significant changes in metal speciation and could be attributed to a modulating effect of these anions on metal/uptake toxicity. The combination of toxicity studies that take into account a range of factors that might modulate metal toxicity with chemical modeling to predict changes in metal speciation might be useful for interpreting complex toxicity data. Finally, this cyanobacterial bioreporter, due to its ecological relevance as a primary producer, could be used as a tool for toxicity assessment in freshwater environments.

Available freshwater resources are polluted by industrial effluents, domestic and commercial sewage, as well as mine drainage, agricultural runoff and litter. Among water pollutants, heavy metals are priority toxicants that pose potential risks to human health and the environment. The evaluation of heavy metal contamination traditionally relies on highly sensitive and specific physical and chemical techniques such as atomic absorption spectroscopy or mass spectrometry; however, such methods are not able to distinguish between available (potentially hazardous to biological systems) and nonavailable (potentially nonhazardous) fractions of metals that exist in the environment in inert or complexed forms. Toxicity bioassays and biosensors might complement physical and chemical methods by detecting the toxicity related with bioavailable metals in environmental samples, effectively integrating the complexity of environmental factors (pH, redox potential, exchangeable cations, biological activity, etc.) that contribute to bioavailability (Köhler et al. 2000). Bioavailability is strongly affected by the speciation of a metal in a particular environment. Water chemistry parameters such as alkalinity, pH, salinity, hardness, phosphates, or ionic strength (Cook et al. 2000; Ho et al.

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1999) might influence metal ion toxicity either directly by lowering free metal ion concentration or indirectly through synergistic or antagonistic effects. Furthermore, bioavailable concentrations of metals in the environment can be altered by chelating substances from natural (humic and fulvic acids) or anthropogenic sources (EDTA or polyphosphates). Most studies have found that toxicity is usually a function of the free metal ion because this species is generally the most bioavailable one (Campbell 1995); however, there are many other reports that showed that the toxic response does not always conform to the free-ion model and that organic complexing agents and/or inorganic hydroxyl or carbonate complexes might also exhibit some toxicity to target organisms (Allen and Hansen 1996; Campbell et al. 2000; Deheyn et al. 2004; Fernandez-Piñas et al. 1991; Parent et al. 1996).

Cyanobacteria are the only prokaryotic organisms carrying out an oxygen-evolving photosynthesis. They originated during the Precambrian era ($2.8 \times 10^9$ years ago), and as a group they are known to survive a wide spectrum of environmental stresses. As primary producers with a key role in the N and C cycles, they are a dominant component of marine and freshwater phytoplankton and any detrimental effect on this group might have a negative impact in nutrient availability to organisms of higher trophic level.

In this study, we report the effect of potential modifying factors on metal speciation such as pH, complexing agent EDTA, and anions $PO_4^{3-}$, $CO_3^{2-}$, and $Cl^-$ on the toxicity of Cu, Zn, Hg, and Cd toward a self-luminescent recombinant strain of the freshwater cyanobacterium *Anabaena* sp. PCC 7120. As luminescence is directly proportional to the metabolic status of the cell and any inhibition of cellular activity is reflected in a decrease of bioluminescence; toxicity was measured as luminescence inhibition caused by biologically available metal species. We used chemical modeling (Visual MINTEQ and PHREEQC programs) and correlation analyses in an attempt to link toxicity with metal speciation.

## Methods and Materials

### Strain and Culture Conditions

*Anabaena* sp. PCC 7120 strain CPB4337 (hereinafter *Anabaena* CPB4337), which bears in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*), was used in this study as a bioreporter of metal toxicity. This strain shows a high constitutive self-luminescence with no need to add exogenous aldehyde; also, cell viability is not significantly affected by the Tn5 insertion and the endogenous generation of aldehyde (Fernandez-Piñas and Wolk 1994). Luminescence was shown to be high in this strain in a range of temperatures between 20°C and 30°C, in accord with *Photorhabdus luminescens* luciferase having the greatest thermal stability (Fernandez-Piñas et al. 2000; Szittner and Meighen 1990). *Anabaena* CPB4337 was routinely grown at 28°C in the light, Ca.65 μmol photons $m^2$/s on a rotary shaker in 50 mL AA/8 medium (Allen and Arnon 1955) supplemented with nitrate (5 mM) in 125-mL Erlenmeyer flasks. The strain was grown in liquid cultures with 10 μg of neomycin sulfate (Nm) per mL.

### Heavy Metal Toxicity Assays

Toxicity response of the cyanobacterium was estimated as $EC_{50}$ values, the median effective concentration of the metal that causes a 50% bioluminescence inhibition with respect to a nontreated control. Standard metal solutions of Hg, Cu, Zn, and Cd were serially diluted (five to eight serial dilutions) for the $EC_{50}$ determinations and were buffered with 2 mM MES [2-(*N*-morpholino) ethanesulfonic acid] and adjusted to pH 5.8. The use of most buffers (like Tris or Tricine) might not be appropriate, as precipitation and complexation of metals might occur (Fernandez-Piñas et al. 1991) and the alkylsulfonate derivatives of morpholine, like MES, are reported to be noncomplexing for metals (Kandegedara and Rorabacher 1999). Previous experiments in the current study showed the following: luminescence of strain *Anabaena* CPB4337 was high in a pH range from 5.8 to 8 (not shown); pH 5.8 was finally chosen for the toxicity bioassays because most metals are biologically available; according to Visual MINTEQ and PHREEQC calculations, the free-ion species of Cu, Zn, and Cd ranged between 95% and 99.99% of the total metal species present at pH 5.8 (Table 1); however, in the case of Hg, chemical modeling predicted that for pH values between 1 and 9, in the range of Hg concentrations used in the study, less than 0.001% was present as free ion $Hg^{2+}$.

Toxicity bioassays were as follows: 160 μL from the serial dilutions of each heavy metal plus a control [double distilled water ($ddH_2O$) buffered with 2 mM MES at pH 5.8] were disposed in an opaque white 96-well microtiter plates. Cells, grown as described, were washed twice and resuspended in $ddH_2O$ buffered with 2 mM MES at pH 5.8 and were added to the microtiter plate wells to reach a final cell density of 0.5 at optical density (OD) 750 nm. Luminescence of each sample was recorded every 5 min in a Centro LB 960 luminometer up to 30 min. Three independent experiments with quadruplicate samples were conducted.

**Table 1** Predicted percentages of the total concentration of metal present as free-ion and dominant forms of metal complexes in the aqueous phase at increasing pH values and increasing phosphate and carbonate concentrations as calculated by Visual MINTEQ

| | Hg | | Cu | | Zn | | Cd | |
|---|---|---|---|---|---|---|---|---|
| pH | | | | | | | | |
| 5.8 | $Hg(OH)_2$ | 93.674 | $Cu^{2+}$ | 98.117 | $Zn^{2+}$ | 98.436 | $Cd^{2+}$ | 99.806 |
| | $HgOH^+$ | 0.148 | $CuSO_4$ | 0.011 | $ZnOH^+$ | 1.558 | $CdCl^+$ | 0.187 |
| | $HgCl_2$ | 1.395 | $CuOH^+$ | 1.227 | | | | |
| | HgClOH | 4.719 | $Cu(OH)_2$ | 0.643 | | | | |
| 7 | $Hg(OH)_2$ | 99.871 | $Cu^{2+}$ | 35.092 | $Zn^{2+}$ | 79.826 | $Cd^{2+}$ | 99.705 |
| | HgClOH | 0.123 | $CuOH^+$ | 6.969 | $ZnOH^+$ | 20.046 | $CdCl^+$ | 0.187 |
| | | | $Cu(OH)_2$ | 57.880 | $Zn(OH)_2$ | 0.123 | $CdOH^+$ | 0.097 |
| | | | $Cu_2(OH)_2^{2+}$ | 0.054 | | | | |
| 7.5 | $Hg(OH)_2$ | 99.959 | $Cu^{2+}$ | 5.514 | $Zn^{2+}$ | 55.228 | $Cd^{2+}$ | 99.474 |
| | HgClOH | 0.039 | $CuOH^+$ | 3.465 | $ZnOH^+$ | 43.912 | $CdCl^+$ | 0.187 |
| | | | $Cu(OH)_2$ | 91.007 | $Zn(OH)_2$ | 0.856 | $CdOH^+$ | 0.305 |
| | | | $Cu_2(OH)_2^{2+}$ | 0.013 | | | | |
| 8 | $Hg(OH)_2$ | 99.987 | $Cu^{2+}$ | 0.595 | $Zn^{2+}$ | 27.222 | $Cd^{2+}$ | 98.749 |
| | HgClOH | 0.012 | $CuOH^+$ | 1.183 | $ZnOH^+$ | 68.548 | $CdCl^+$ | 0.185 |
| | | | $Cu(OH)_2$ | 98.219 | $Zn(OH)_2$ | 4.226 | $CdOH^+$ | 0.959 |
| $PO_4^{3-}$ (mg/L$^1$) | | | | | | | | |
| 0.1 | $Hg(OH)_2$ | 96.180 | $Cu^{2+}$ | 94.943 | $Zn^{2+}$ | 97.530 | $Cd^{2+}$ | 99.792 |
| | $HgCl^+$ | 0.013 | $CuHPO_4$ | 0.012 | $ZnOH^+$ | 2.445 | $CdCl^+$ | 0.187 |
| | $HgCl_2$ | 0.606 | $CuOH^+$ | 2.332 | $ZnHPO_4$ | 0.013 | $CdHPO_4$ | 0.010 |
| | HgClOH | 3.152 | $Cu(OH)_2$ | 2.423 | | | | |
| 1 | $Hg(OH)_2$ | 96.188 | $Cu^{2+}$ | 96.193 | $Zn^{2+}$ | 97.403 | $Cd^{2+}$ | 99.701 |
| | $HgOH^+$ | 0.048 | $CuH_2PO_4^+$ | 0.039 | $ZnOH^+$ | 2.432 | $CdCl^+$ | 0.186 |
| | $HgCl^+$ | 0.013 | $CuHPO_4$ | 0.098 | $ZnH_2PO_4^+$ | 0.038 | $CdHPO_4$ | 0.102 |
| | $HgCl_2$ | 0.604 | $CuOH^+$ | 1.869 | $ZnHPO_4$ | 0.120 | | |
| | HgClOH | 3.146 | $Cu(OH)_2$ | 1.541 | | | | |
| 10 | $Hg(OH)_2$ | 96.226 | $Cu^{2+}$ | 95.173 | $Zn^{2+}$ | 96.083 | $Cd^{2+}$ | 98.840 |
| | $HgCl^+$ | 0.013 | $CuH_2PO_4^+$ | 0.369 | $ZnOH^+$ | 2.340 | $CdCl^+$ | 0.178 |
| | $HgCl_2$ | 0.593 | $CuHPO_4$ | 0.933 | $ZnH_2PO_4^+$ | 0.382 | $CdHPO_4$ | 0.971 |
| | HgClOH | 3.119 | $CuOH^+$ | 1.806 | $ZnHPO_4$ | 1.188 | | |
| | | | $Cu(OH)_2$ | 1.477 | | | | |
| 100 | $Hg(OH)_2$ | 93.950 | $Cu^{2+}$ | 92.786 | $Zn^{2+}$ | 89.383 | $Cd^{2+}$ | 94.679 |
| | $HgCl^+$ | 0.032 | $CuH_2PO_4^+$ | 3.333 | $ZnOH^+$ | 1.257 | $CdCl^+$ | 0.152 |
| | $HgCl_2$ | 1.328 | $CuHPO_4$ | 2.971 | $ZnH_2PO_4^+$ | 3.220 | $CdHPO_4$ | 5.163 |
| | HgClOH | 4.611 | $CuOH^+$ | 0.586 | $ZnHPO_4$ | 6.135 | | |
| | | | $Cu(OH)_2$ | 0.169 | | | | |
| $CO_3^{2-}$ (mg/L) | | | | | | | | |
| 0.1 | $Hg(OH)_2$ | 93.677 | $Cu^{2+}$ | 97.921 | $Zn^{2+}$ | 98.432 | $Cd^{2+}$ | 99.801 |
| | $HgCl_2$ | 1.433 | $CuOH^+$ | 1.205 | $ZnOH^+$ | 1.557 | $CdCl^+$ | 0.187 |
| | HgClOH | 4.783 | $Cu(OH)_2$ | 0.628 | | | | |
| 1 | $Hg(OH)_2$ | 93.692 | $Cu^{2+}$ | 97.838 | $Zn^{2+}$ | 98.396 | $Cd^{2+}$ | 99.759 |
| | $HgOH^+$ | 0.074 | $CuOH^+$ | 0.059 | $ZnOH^+$ | 1.549 | $CdCl^+$ | 0.186 |
| | $HgCl^+$ | 0.032 | $Cu(OH)_2$ | 1.199 | $ZnHCO_3^+$ | 0.046 | $CdHCO_3^+$ | 0.048 |
| | $HgCl_2$ | 1.428 | | | | | | |
| | HgClOH | 4.774 | | | | | | |

**Table 1** continued

| | Hg | | Cu | | Zn | | Cd | |
|---|---|---|---|---|---|---|---|---|
| 10 | $Hg(OH)_2$ | 93.759 | $Cu^{2+}$ | 97.005 | $Zn^{2+}$ | 98.028 | $Cd^{2+}$ | 99.759 |
| | $HgOH^+$ | 0.075 | $CuCO_3$ | 0.566 | $ZnOH^+$ | 1.502 | $CdCl^+$ | 0.186 |
| | $HgCl^+$ | 0.032 | $CuOH^+$ | 1.159 | $ZnHCO_3^+$ | 0.442 | $CdHCO_3^+$ | 0.048 |
| | $HgCl_2$ | 1.402 | $Cu(OH)_2$ | 0.597 | | | | |
| | HgClOH | 4.732 | $Cu(OH)_2^{2+}$ | 0.020 | | | | |
| 100 | $Hg(OH)_2$ | 99.897 | $Cu^{2+}$ | 4.015 | $Zn^{2+}$ | 44.349 | $Cd^{2+}$ | 86.488 |
| | HgClOH | 0.101 | $CuCO_3$ | 39.639 | $ZnOH^+$ | 30.360 | $CdCl^+$ | 0.134 |
| | | | $Cu(CO_3)_2^{2-}$ | 0.147 | $ZnHCO_3^+$ | 7.016 | $CdOH^+$ | 0.073 |
| | | | $CuOH^+$ | 2.144 | $ZnCO_3$ | 16.662 | $CdHCO_3^+$ | 12.734 |

*Note*: Total metal concentration for calculations of each metal is 10 μM

## Effect of Modifying Factors on Metal Toxicity

To investigate the effect of modifying factors on Hg, Cu, Zn, and Cd toxicity, a metal concentration of 10 μM was chosen to elicit a strong toxic response; the $EC_{50}$ values for each of the metals after 30 min of exposure were below this concentration (see the Results section and Fig. 1).

To relate bioavailability and toxicity, the effect of pH, organic ligand (EDTA), phosphate (as $NaH_2PO_4$), carbonate (as $Na_2CO_3$), and chloride (as NaCl) on the toxicity of Hg, Zn, Cu, and Cd to strain *Anabaena* CPB4337 was checked. These potential modifying factors were varied within ranges that might be found in freshwater environments (Perona et al. 1999; Van Dijk et al. 1994): pH from 5.8 to 8; chelate/metal ratios between 0 and 2, phosphate, carbonate, and NaCl concentrations from 0.1 to 10 mg/L; a 10-fold higher phosphate/carbonate/chloride concentration (100 mg $L^{-1}$) was also used. It was considered interesting to check the effect of a much higher salt concentration, 2% NaCl (w/v), on cyanobacterial luminescence and metal toxicity because this high salt content is present in the widely used bioassays based on the marine bioreporter *Vibrio fischeri* and previous studies have reported that it affected metal bioavailability and toxicity (Deheyn et al. 2004; Newman and McCloskey, 1996; Riba et al. 2003).

To check the effect of pH on metal toxicity, 160 μL of the appropriate metal solution buffered with 2 mM MES and adjusted at pHs 5.8, 7, 7.5, and 8 were disposed on the microtiter plates. Cells grown as described were centrifuged, washed, resuspended in $ddH_2O$ buffered with 2 mM MES and adjusted to pH 5.8, 7, 7.5, and 8, and were added to reach a final cell density ($OD_{750\ nm}$) of 0.5; the final pH of the bioassay was checked for each metal concentration.

To investigate the effect of the complexing agent EDTA on metal toxicity, EDTA/metal solutions were prepared to get final chelate/metal molar ratios of 0.5, 1, 1.5, and 2; three controls were included: 0 $M^{2+}$ (untreated control), 10 μM $M^{2+}$ (metal treated control for toxic response), and 20 μM EDTA (EDTA control to monitor any effect of the chelator on cell self-luminescence). The EDTA/metal solutions and controls were buffered with 2 mM MES and adjusted to pH 5.8 and were incubated for at least 48 h at room temperature to ensure metal complexation (Fernandez-Piñas et al. 1991; Riether et al. 2001; Tauriainien et al. 2000).

To investigate the effect of phosphate, carbonate, and chloride on metal toxicity, phosphate/metal, carbonate/metal, and chloride/metal solutions were prepared to get a final metal concentration of 10 μM and final concentrations of 0.1, 1, 10, and 100 mg/L phosphate (as $NaH_2PO_4$), carbonate (as $Na_2CO_3$), or chloride (as NaCl). Then 0 $M^{2+}$ (untreated control), 10 μM $M^{2+}$ (metal treated control for toxic response), 100 mg/L phosphate/carbonate/chloride (controls to monitor any effect of phosphate, carbonate, or choride on cell self-luminescence) were included. Two percent NaCl (w/v) was also used to study its effect on cyanobacterial luminescence and metal toxicity. The metal/phosphate, metal/carbonate, and metal/chloride mixtures were allowed to soak for at least 48 h at room temperature to allow complexes to be formed (Fernandez-Piñas et al. 1991). All solutions were buffered with 2 mM MES and adjusted to pH 5.8, except those containing 100 mg/L carbonate whose pH was adjusted to 7 to avoid equilibrium shifts to bicarbonate and $CO_2$.

The bioassays with the modifying factors EDTA, phosphate, carbonate, and chloride were essentially carried out as for the standard metal assay; luminiscence messurements was recorded every 5 min in the Centro LB 960 luminometer up to 30 min. Three independent experiments with triplicate samples were carried out for each case.

## Modelling of Metal Speciation

Two programs were used to predict metal speciation: Visual MINTEQ and PHREEQC. The chemical

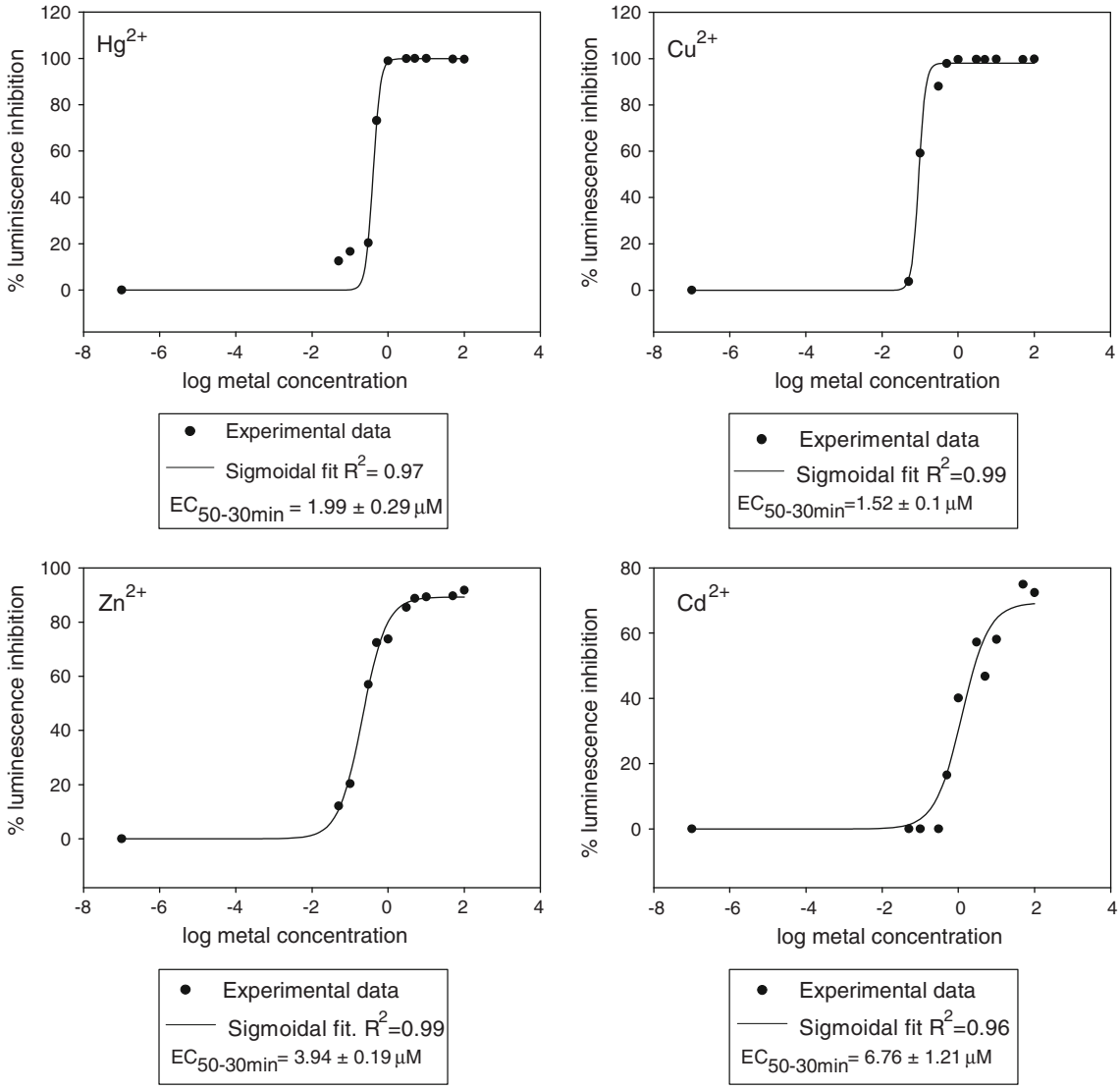


**Fig. 1** Bioluminescence inhibition curves for Hg, Cu, Zn, and Cd. The curves present the percent of bioluminescence inhibition versus logarithm of metal concentration (expressed in µM). $EC_{50}$ values after 30 min of exposure to the metals ($EC_{50-30\ min}$) and standard deviation were calculated from three independent experiments with triplicate samples

equilibrium model Visual MINTEQ (http://www.lwr.kth.se/English/OurSoftware/vminteq/index.htm) is based on the program PC MINTEQA2 version 4.0 (Allison et al. 1991). Assumptions of a fixed pH, fixed potential redox (Eh), closed system, and no precipitation of solid phases were made during computations. The geochemical model PHREEQC version 2 (Parkhurst and Appelo 1999) was used with the aid of the graphical user interface PHREEQCI (version 2) http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqc/index.html). These chemical models have proved very useful for linking speciation to metal toxicity and biosorption processes in a number of organisms (Campbell et al. 2000; Deheyn et al. 2004; Herrero et al. 2005; Newman and McCloskey 1996). Both model calculations were very similar and, to simplify, only dominant metal species for each tested condition as calculated by Visual MINTEQ are shown in Tables 1 and 3.

## Analysis of Results

The toxic response of *Anabaena* CPB4337 as $EC_{50}$ values was estimated by fitting the experimental luminescence inhibition data to a three-parameter logarithmic function:

$$f = a/\{1 + \exp[-(x - x_0)/b]\},$$

where $f$ is the percentage of bioluminescence inhibition, $x$ is the logarithm of metal concentration, $a$, $b$, and $x_0$ are the parameters of the equation estimated by the model.

One-way analyses of variance (ANOVA) and linear regression analyses were computed using MINITAB Release 14 for Windows (Minitab Inc., USA).

## Results

### Toxicity Assays of Heavy Metals to Strain *Anabaena* CPB4337

For each metal treatment, a concentration–response curve could be established from which the $EC_{50}$ values were derived (Fig. 1). The bioluminescent cyanobacterium responded sensitively to the four metals tested; the 30-min $EC_{50}$ values and 95% confidence intervals calculated for each of the metals were as follows: Hg, 1.99 ± 0.29 μM (1.41–2.57); Cu, 1.52 ± 0.10 μM (1.32–1.72); Zn; 3.94 ± 0.19 μM (3.56–4.32); Cd, 6.76 ± 1.21 μM (4.34–9.18). Based on these values, the order of sensitivity of *Anabaena* CPB4337 toward the tested metals was Cu ≥ Hg > Zn > Cd.

### Effect of Modifying Factors on Metal Toxicity

Figure 2 illustrates the effect of increasing pH values (5.8–8) on Hg, Zn, Cu, and Cd toxicity to strain *Anabaena* CPB4337 after 30 min of exposure. A pH increase significantly ameliorated Hg, Zn, and Cu toxicity (ANOVA, $p < 0.05$). Cd toxicity was not significantly remediated at higher pH values (ANOVA, $p < 0.05$). As shown in Table 1, the amelioration of Zn and particularly Cu toxicity at increasing pH values could be explained by the formation of nonavailable or less available metal hydroxides

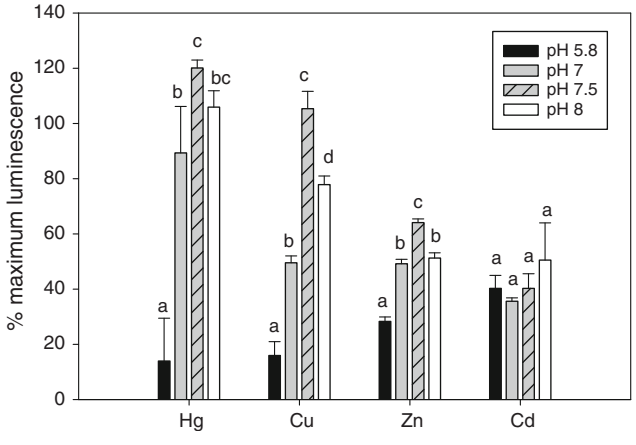


**Fig. 2** Effect of increasing pH on Hg, Zn, Cu, and Cd toxicity to *Anabaena* CPB4337. All measurements were conducted in quadruplicate and were repeated at least twice. Values with the same superscript letter were not significantly different ($p < 0.05$) as determined by ANOVA between the different pH treatments for each metal concentration tested

**Table 2** Correlation analyses between percent maximum luminescence ($y$) and percent free ions for Cu, Zn, and Cd or the logarithm of $Hg^{2+}$ concentration (μM) for Hg ($x$)

| Factor | Free ion | Regression parameters | | | | |
|---|---|---|---|---|---|---|
| | | $x_0$ | $m$ | $r$ | $R^2$ | $p$ |
| pH | $Cu^{2+}$ | 89.44 | −0.78 | −0.92 | 0.84 | <0.1 |
| | $Zn^{2+}$ | 92.30 | −0.67 | −0.82 | 0.68 | <0.05 |
| | $Cd^{2+}$ | – | – | – | – | – |
| | $Hg^{2+}$ | 105.24 | −3.68 | −0.96 | 0.93 | <0.05 |
| EDTA | $Cu^{2+}$ | 132.81 | −1.32 | −0.97 | 0.94 | <0.05 |
| | $Zn^{2+}$ | – | – | – | – | – |
| | $Cd^{2+}$ | 184.15 | −1.82 | −0.98 | 0.97 | <0.01 |
| | $Hg^{2+}$ | – | – | – | – | – |
| $PO_4^{2-}$ | $Cu^{2+}$ | – | – | – | – | – |
| | $Zn^{2+}$ | 207.07 | −1.91 | −0.85 | 0.72 | <0.1 |
| | $Cd^{2+}$ | – | – | – | – | – |
| | $Hg^{2+}$ | −194.47 | −19.26 | −0.81 | 0.65 | <0.1 |
| $CO_3^{2-}$ | $Cu^{2+}$ | 114.80 | −1.07 | −0.98 | 0.95 | <0.05 |
| | $Zn^{2+}$ | 163.06 | −1.56 | −0.99 | 0.99 | <0.01 |
| | $Cd^{2+}$ | 801.61 | −7.85 | −0.99 | 0.98 | <0.01 |
| | $Hg^{2+}$ | −184.29 | −18.19 | −0.99 | 0.99 | <0.01 |

*Note*: Parameters of linear regression equations: $x_0$ (value of $y$ when $x = 0$); $m$ (slope) and $r$ (correlation coefficient) as well as $R^2$ (goodness-of-fit coefficient) and $p$-values are given. Analyses were computed using MINITAB Release 14 for Windows. –: $p$-values > 0.1

(particularly neutral hydroxyl complexes) and the concomitant decrease of the free-ion form; in fact, significant and negative correlations were found between percent maximum luminescence and percent free $Cu^{2+}$ ($r = -0.92$, $p < 0.1$) and between percent maximum luminescence and percent free $Zn^{2+}$ ($r = -0.82$, $p < 0.05$) (Table 2). In the case of Cd, up to pH 8 the free-ion form predominated (Table 1) and no significant correlation was found between percent maximum luminescence and percent free $Cd^{2+}$ (Table 2), which might explain why Cd toxicity was not reduced with increasing pH values. Whereas for Zn, Cu, and Cd, the free-ion form clearly governed toxicity in the tested pH range, Hg essentially appeared as the neutral species, mainly $HgCl_2$, Hg(OH)Cl, and $Hg(OH)_2$ between pH 5.8 and 8 (Table 1).Within this pH range, the predominant Hg-containing ion pair was the dihydroxy species; the neutral dichloro-complex was present below pH 7 and the free-ion $Hg^{2+}$ species was present at extremely low concentrations (from $2.48 \times 10^{-11}$ M at pH 5.8 to $1.05 \times 10^{-15}$ M at pH 8 as calculated by Visual MINTEQ and PHREEQC). The most available and probably toxic Hg species could be the free-ion form even at these very low concentrations and perhaps the neutral dichloro-complex that was not present at the higher pH values (Table 1). A significant negative correlation was found

between percent maximum luminescence and the logarithm of free $Hg^{2+}$ concentration ($r = -0.96$, $p < 0.05$), which could explain most of the observed Hg toxicity variation.

Figure 3 shows the effect of complexing agent EDTA on metal toxicity in strain *Anabaena* CPB4337 after 30 min of exposure. The results showed that EDTA addition had a significant (ANOVA, $p < 0.05$) effect on remediation of all the tested metal toxicities. As calculated by Visual MINTEQ, the chelator markedly decreased the free-ion proportion of Cu, Cd, and Zn: At ratio EDTA/metal = 0.5, only 46% of Cu and Zn and 49% of Cd remained as the free-ion form, at ratios EDTA/metal = 1 and higher, metals were present as EDTA–metal complexes (not shown); for Cu and Cd, the toxic response most probably related to the amount of noncomplexed metal present, suggesting that the metal–EDTA complex was nontoxic to the cyanobacterial strain and that toxicity was governed by the free-ion concentration. This is confirmed by the significant and negative correlations found between percent maximum luminescence and percent free $Cu^{2+}$ ($r = -0.97$, $p < 0.05$) and between percent maximum luminescence and percent free $Cd^{2+}$ ($r = -0.98$, $p < 0.01$) in the EDTA experiment (Table 2). In the case of Hg, Visual MINTEQ and PHREEQC did not predict the formation of Hg–EDTA complexes and the concentration of free-ion $Hg^{2+}$ ($2.5 \times 10^{-11}$ M) was constant through the increasing EDTA/Hg ratios. Furthermore, no correlation existed between percent maximum luminescence and the logarithm of the predicted $Hg^{2+}$ concentration (Table 2). However, Hg toxicity amelioration by EDTA followed the pattern of Cu and Cd toxicity; so, most probably Hg–EDTA complexes were likely to be formed. For Zn, although there was also a decrease in toxicity with increasing EDTA/metal ratios, the results were somewhat different in that for an EDTA/metal ratio of 1:1, bioluminescence was already below 100% maximum luminescence. Additionally, no significant correlation was found between percent maximum luminescence and percent free $Zn^{2+}$ (Table 2); possibly, the Zn–EDTA complexes might be available and toxic to cyanobacteria. It should also be noticed that EDTA itself was not toxic to the cells; in fact, an enhancement of bioluminescence could be seen in the EDTA controls treated with the highest EDTA concentration, 20 μM (Fig. 3).

Figure 4a shows the effect of increasing phosphate concentrations (0.1, 1, 10, and 100 mg/L) on Hg, Zn, Cu, and Cd toxicity to strain *Anabaena* CPB4337. Only Zn toxicity was significantly remediated by 100 mg/L of phosphate in the medium (ANOVA, $p < 0.05$). Hg and Cu toxicity significantly increased with the addition of the lowest phosphate concentration, 0.1 mg/mL, and did not recover with increasing phosphate concentration (ANOVA, $p < 0.05$); this could not be explained by significant

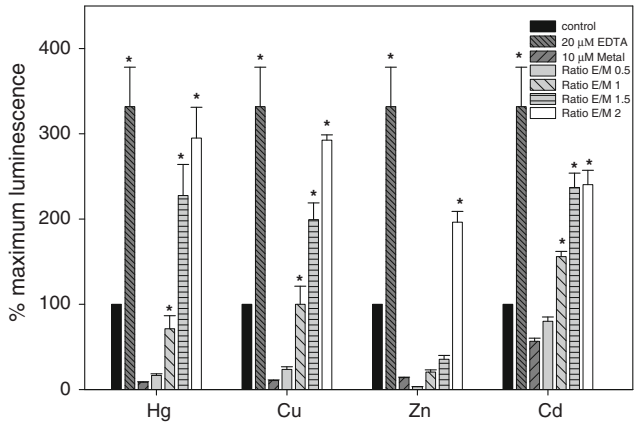


**Fig. 3** Effect of the addition of chelating agent EDTA on Hg, Cu, Zn, and Cd toxicity to *Anabaena* CPB4337. * Statistically significant differences ($p < 0.05$) as determined by ANOVA with respect to the metal-treated control. Error bars represent standard deviation of the means of at least two independent experiments with triplicate samples

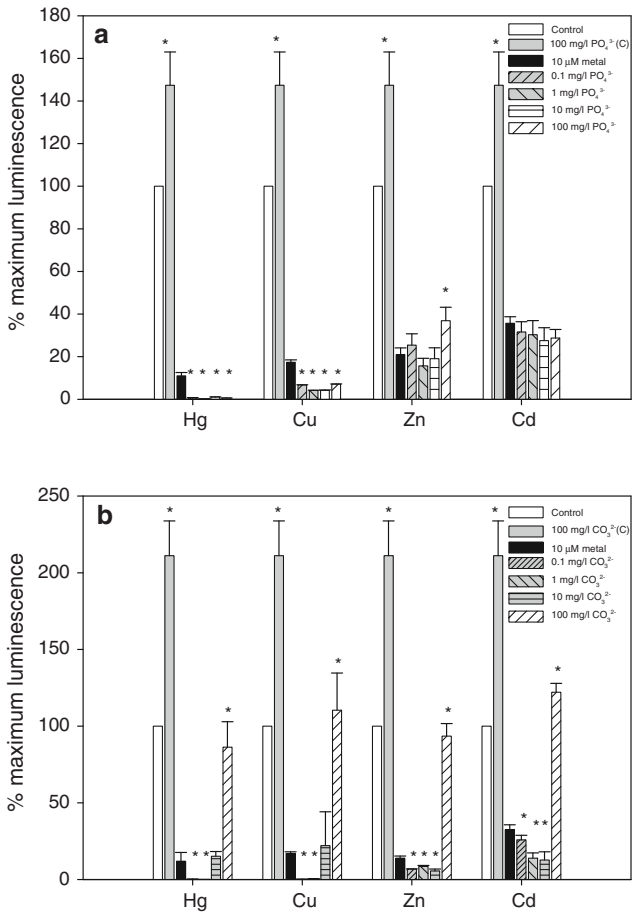


**Fig. 4** Effect of the addition of phosphate and carbonate on Hg, Cu, Zn, and Cd toxicity to *Anabaena* CPB4337. * Statistically significant differences ($p < 0.05$) as determined by ANOVA with respect to the metal-treated control. Error bars represent standard deviation of the means of at least two independent experiments with triplicate samples

changes in metal speciation (Table 1). Cd toxicity did not change with the addition of phosphate at the tested concentrations. The Visual MINTEQ program predicted that the free-ion form predominated for Cu, Zn, and Cd at the four tested phosphate concentrations (Table 1); however, for 100 mg/L of added phosphate, the $Zn^{2+}$ free-ion species was the lowest of the free-ion species of the four metals. Additionally, the program predicted the formation of neutral monohydrogen phosphate and charged dihydrogen phosphate with Cu, Zn, and Cd, but the amount of $ZnHPO_4$ formed accounted for a higher percentage than the monohydrogen phosphates formed with the other three metals; in fact, only for Zn, a significant correlation between percent maximum luminescence and percent free ion ($r = -0.85$, $p < 0.1$) was found (Table 2), which would explain, at least partially, the observed remediation of Zn toxicity by a decrease of free $Zn^{2+}$ ions at 100 mg/L of added phosphate. In the case of Hg, phosphate addition did not substantially changed Hg speciation (Table 1): The neutral dihydroxy species was the predominant form in the tested phosphate range, the free-ion form slightly increased, as calculated by Visual MINTEQ and PHREEQC, from $2.84 \times 10^{-11}$ M at 0 mg/mL phosphate to $7.07 \times 10^{-11}$ M at 100 mg/mL added phosphate; this slight increase along with the increase of the neutral dichloro-complex from 0.6% to 1.32% (Table 1) might partially explain the observed Hg toxicity increase (Fig. 4a). In fact, a negative and significant correlation was found between percent maximum luminescence and the logarithm of predicted $Hg^{2+}$ concentration, ($r = -0.81$, $p < 0.1$) (Table 2). The observed increase of Cu toxicity could not be explained by changes in speciation (Table 1); additionally, no significant correlation was found between percent maximum luminescence and percent free $Cu^{2+}$ (Table 2). Finally, 100 mg/L phosphate, on its own, increased luminescence.

Figure 4b shows the effect of increasing carbonate (0.1, 1, 10, and 100 mg/L) on Hg, Cu, Zn, and Cd toxicity to strain *Anabaena* CPB4337. The addition of 100 mg/L carbonate significantly ameliorated metal toxicity (ANOVA, $p < 0.05$). Similar to the results with phosphate, there was increased toxicity of the four metals with lower carbonate concentrations (up to 10 mg/L); this could not be explained by significant changes in metal speciation in this range of carbonate concentrations (Table 1). In the case of Cu, Zn, and Cd, the chemical modeling program predicted that the free-ion form predominated up to 10 mg/L of added carbonate, whereas at 100 mg/L, the free-ion form significantly decreased due to the formation of bicarbonate and carbonate complexes (Table 1) that might be non-available or less available than the free-ion form and thus less toxic; this might explain the observed significant (ANOVA, $p < 0.05$) remediation of Cu, Zn, and Cd toxicity with 100 mg/L of added carbonate. Regarding Hg, the neutral dihydroxy complex predominated and its proportion increased with increasing carbonate concentrations. The neutral dichloro-complex significantly decreased at 10 mg/L added carbonate and was no longer present at 100 mg/L (Table 1); the free-ion form concentration, as calculated by Visual MINTEQ and PHREEQC, significantly decreased with increasing carbonate concentrations (from $2.84 \times 10^{-11}$ M at 0 mg/L to $1.29 \times 10^{-15}$ M at 100 mg/L added carbonate). The decrease of free-ion $Hg^{2+}$ and $HgCl_2$ at 100 mg/L added carbonate might account for the observed amelioration of Hg toxicity. In agreement with the toxicity results at higher carbonate concentrations, a significant and negative correlation existed between percent maximum luminescence and percent $Cu^{2+}$ ($r = -0.98$, $p < 0.05$), percent $Zn^{2+}$ ($r = -0.99$, $p < 0.01$), percent $Cd^{2+}$ ($r = -0.99$, $p < 0.01$) and the logarithm of predicted $Hg^{2+}$ concentration ($r = -0.99$, $p < 0.01$) (Table 2). Finally, the addition of 100 mg/L carbonate, on its own, increased luminescence significantly (ANOVA, $p < 0.05$).

The addition of NaCl up to 100 mg/L did not have any significant effect on metal toxicity or speciation (not shown); however, the addition of 2% NaCl, the salt concentration needed for *Vibrio fischeri*-based bioassays, had a significant effect on cyanobacterial cell luminescence, metal toxicity, and speciation of the four metals. As shown in Fig. 5, 2% NaCl, on its own, significantly (ANOVA, $p < 0.05$) inhibited cell luminescence (50% inhibition) of the strain *Anabaena* CPB4337; furthermore, high salt induced substantial metal speciation changes (Table 3) and also affected metal toxicity. In the case of Hg, the addition

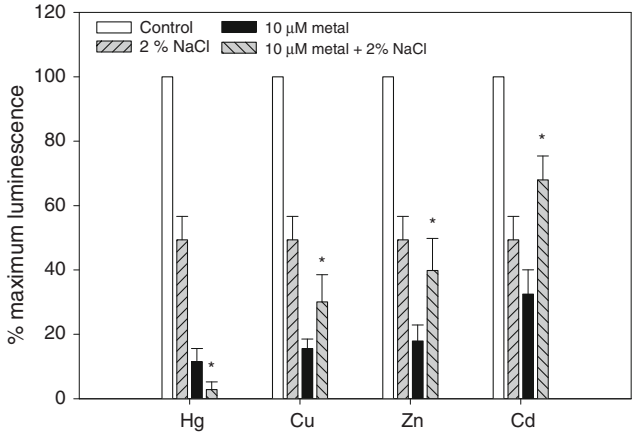


**Fig. 5** Effect of the addition of 2% NaCl on Hg, Cu, Zn, and Cd toxicity to *Anabaena* CPB4337. * Statistically significant differences ($p < 0.05$) as determined by ANOVA with respect to the metal-treated control. The 2% NaCl control was significantly (ANOVA, $p < 0.05$) different with respect to the cell luminescence control. Error bars represent standard deviation of the means of at least two independent experiments with triplicate samples

**Table 3** Predicted percentages of the total concentration of metal present as free-ion and dominant forms of metal complexes in the aqueous phase at 2% NaCl as calculated by Visual MINTEQ

| NaCl | Hg | | Cu | | Zn | | Cd | |
|---|---|---|---|---|---|---|---|---|
| 0% | $Hg(OH)_2$ | 93.674 | $Cu^{2+}$ | 98.117 | $Zn^{2+}$ | 98.436 | $Cd^{2+}$ | 99.806 |
| | $HgOH^+$ | 0.148 | $CuSO_4$ | 0.011 | $ZnOH^+$ | 1.558 | $CdCl^+$ | 0.187 |
| | $HgCl_2$ | 1.395 | $CuOH^+$ | 1.227 | | | | |
| | HgClOH | 4.719 | $Cu(OH)_2$ | 0.643 | | | | |
| 2% | $HgCl_2$ | 12.353 | $Cu^{2+}$ | 77.080 | $Zn^{2+}$ | 75.323 | $Cd^{2+}$ | 5.498 |
| | $HgCl_3^-$ | 34.272 | $CuCl^+$ | 20.585 | $ZnCl^+$ | 21.245 | $CdCl^+$ | 48.748 |
| | $HgCl_4^{2-}$ | 53.373 | $CuCl_2$ | 1.026 | $ZnCl_2$ | 1.277 | $CdCl_2$ | 32.845 |
| | | | $CuOH^+$ | 0.747 | $ZnCl_3^-$ | 1.428 | $CdCl_3^-$ | 7.530 |
| | | | $Cu(OH)_2$ | 0.525 | $ZnCl_4^{2-}$ | 0.443 | $CdCl_4^{2-}$ | 5.364 |

*Note*: Total metal concentration for calculations of each metal is 10 μM

of 2% NaCl significantly (ANOVA, $p < 0.05$) increased toxicity (Fig. 5) and substantially changed speciation (Table 3), the hydroxyl complexes were no longer present, and the charged chloro-complex $HgCl_4^{2-}$ clearly predominated (53.37%); the free-ion form, $Hg^{2+}$, was present at extremely low amounts ($5.33 \times 10^{-18}$ M), as calculated by Visual MINTEQ and PHREEQC. As the free-ion form concentration was negligible, the toxic metal species could be $HgCl_4^{2-}$ and, to some extent, the neutral dichloro-complex $HgCl_2$. On the other hand, 2% NaCl significantly (ANOVA, $p < 0.05$) ameliorated Cu, Zn, and Cd toxicity to the strain *Anabaena* CPB4337 (Fig. 5). This might be explained by changes in metal speciation (Table 3); the addition of 2% NaCl decreased the available and toxic free-ion forms $Cu^{2+}$ and $Zn^{2+}$ by 20% and $Cd^{2+}$ by almost 95%, with the concomitant increase of chloro-complexes that might be nonavailable and nontoxic.

## Discussion

One important characteristic of whole-cell bioreporters is that they reflect the real physiological impact of toxic compounds, as they report on the bioavailable fraction of toxicants. Bioavailability and toxicity of metals depends on their speciation in aqueous environments (Allen and Hansen 1996). Of the possible chemical forms of a metal, the free ion is usually the most toxic one (Campbell 1995); however, that does not necessarily mean that the free metal ion is the only toxic species (Allen and Hansen 1996; Campbell et al. 2000; Fernandez-Piñas et al. 1991).

The study of the effect of modifying factors indicated that, in general, cyanobacterial toxicity correlated with free metal ion concentration (Table 2); chemical modeling predicted that any decrease in the free-ion concentration generally correlated with the corresponding formation of neutral and charged metal hydroxides, metal phosphate, carbonate, and chloride species, and EDTA complexes. The formation of all of these metal complexes, with some exceptions, correlated with toxicity amelioration, implying that they were not toxic probably due to low or no bioavailability to the cyanobacterial cells. One exception was the observed toxicity of the Zn–EDTA complexes at a ratio EDTA/Zn 1:1 (Fig. 3). These complexes could be toxic to the cyanobacteria either by direct uptake or by the ability of the cyanobacterial strains to actively release Zn from the complex, as already pointed out by Paton et al. (1997) in their study with a bioluminescent strain of *Pseudomonas fluorescens*. Campbell et al. (2000) found a similar behavior in the toxicity of Zn and Cd to a bioluminescent construct of *Escherichia coli* in the presence of EDTA and fulvic acid. These authors also reported an stimulatory effect of EDTA on cell luminescence; they suggested that this stimulatory effect could be due to a surface permeability effect or uptake/metabolism of EDTA by *E. coli* cells.

Mercury also represented an exception due to the fact that chemical modeling of Hg speciation predicted that, under almost all the tested conditions, the predominant species (more than 98%) were the neutral $Hg(OH)_2$, Hg(OH)Cl, and $HgCl_2$ while the amount of free $Hg^{2+}$ ion was very low, almost negligible; so, it was not easy to determine which was the toxic mercury species to cyanobacteria. However, the very low concentration of free $Hg^{2+}$ ion decreased even more with increasing pH, phosphate, and carbonate and this correlated with the observed remediation of toxicity (Table 2). If free-ion $Hg^{2+}$ is the main toxic species, the $EC_{50}$ calculated for Hg (Fig. 1) is actually much lower, with a value of $6.27 \times 10^{-12}$ M, as calculated by Visual MINTEQ and PHREEQC; thus, in terms of free-ion concentration, $Hg^{2+}$ appears to be more toxic than $Cu^{2+}$, $Zn^{2+}$, or $Cd^{2+}$ to *Anabaena* CPB4337. However, we cannot discard that the neutral species $HgCl_2$ might also show some toxicity under certain conditions, as

an increase in pH or carbonate concentration clearly diminished toxicity and the concentration of this species; in this regard, Newman and McCloskey (1996) also considered $HgCl_2$ to be bioavailable due to its lipophilicity (Simkiss 1983). Herrero et al. (2005) also found that the macroalga *Cystoseira baccata* accumulated Hg mainly as $HgCl_2$. In addition, 2% NaCl had a significant effect on Hg speciation, with $HgCl_4^{2-}$ as the predominant form, and a further decrease in the concentration of the free-ion form, $Hg^{2+}$, which reached $5.33 \times 10^{-18}$ M—a value almost identical to that reported by Deheyn et al. (2004) in their Microtox assay medium— indicating that our speciation calculations by Visual MINTEQ and PHREEQC were also accurate for Hg. In spite of the negligible free $Hg^{2+}$ concentration, this high salt concentration increased Hg toxicity to the strain *Anabaena* CPB4337, suggesting that the charged chloro-complex $HgCl_4^{2-}$ might also be toxic species to cyanobacteria.

An interesting feature of metal toxicity to the strain *Anabaena* CPB4337 is that low amounts of anions such as phosphate and carbonate increased metal toxicity; this could not be related to significant changes in metal speciation (Table 1) but could be due to a modulating effect of these anions, both nutrients for cyanobacteria (Rippka 1988), on metal uptake/toxicity. Heijerick et al. (2003) also found that low hardness levels (as $CaCO_3$) increased Zn toxicity to *Daphnia magna*; Herrero et al. (2005) reported a slight increase in Hg uptake with background salt concentrations as nitrate but not as chloride salts. Deryabin and Aleshina (2008) recently reported that carbonates and hydrocarbonates had a pronounced inhibitory effect on the bioluminescence of *Photobacterium phosphoreum* and a recombinant luminescent *E. coli* strain. As these low anion concentrations can be found in natural waters and they could affect the performance of other bioreporters in a similar way, laboratory tests should be done before assaying environmental samples in order to fully understand ecotoxicity data.

Finally, the point of ecological relevance/significance is important when assessing the performance of bacterial bioreporters. The assays based on marine luminescent bacteria such as *Vibrio fischeri* might not be very appropriate for soil and freshwater ecotoxicity testing because sample filtration is required and they work only in saline solution (2% NaCl) (Villaescusa et al. 1996). Because of the salinity, the insolubility of some organic substances that might be present in the environmental sample is enhanced, producing turbid solution. As discussed earlier and similar to other authors (Deheyn et al. 2004; Newman and McCloskey 1996), we have found that 2% NaCl substantially changed metal speciation (Table 3); this high salt concentration significantly increased the proportion of chloro-complexes while significantly decreasing the proportion of free ions, which, as discussed earlier, are usually the most toxic forms of a metal. For this reason, the bioassays based on marine luminescent bacteria might underestimate metal toxicity in freshwater simples, as already suggested (Deheyn et al. 2004). In this report, we present an application of a recombinant self-luminescent cyanobacterial bioreporter to assess heavy metal toxicity; this organism is a derivative of a freshwater cyanobacterium, and due to its ecological relevance as a primary producer, it could be used as a potential tool for toxicity assessment in freshwater environments (rivers, effluents, lakes, groundwater, etc.).

## Conclusion

We report an application of an ecologically relevant self-luminescent cyanobacterial bioreporter for the assessment of metal toxicity and its modulation in the presence of a range of potential modifying factors. Chemical modeling and correlation analyses proved very useful for linking toxicity and bioavailability. In general, there was a good correlation between the observed toxic effects and free-ion metal concentration. Low concentrations of phosphate and carbonate increased heavy metal toxicity toward the cyanobacterium. This approach of combining toxicity studies with chemical modeling to predict changes in metal speciation might help to interpret complex toxicity data when testing real environmental samples.

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# CHAPTER III

## Eco-toxicity of emerging pollutants I. Individual and mixture toxicity of lipid regulators.

# ECOTOXICITY ASSESSMENT OF LIPID REGULATORS IN WATER AND BIOLOGICALLY TREATED WASTEWATER USING THREE AQUATIC ORGANISMS

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AREA 6.2 • BIO-ASSAYS • RESEARCH ARTICLE

# Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic organisms

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**Abstract**
*Background, aim, and scope* The high consumption of blood lipid regulators is leading to frequent reports of the occurrence of fibrates in natural streams and wastewater effluents. This paper describes a study undertaken to evaluate the acute toxicity of bezafibrate, clofibric acid, gemfibrozil, and fenofibric acid, a metabolite of fenofibrate whose ecotoxicity has not been previously reported.
*Materials and methods* The bioassays used were based on *Vibrio fischeri*, *Daphnia magna*, and *Anabaena* CPB4337 tests. *Anabaena* CPB4337 is a novel bioassay based on *Anabaena* sp. PCC 7120 strain CPB4337 bearing in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens*.
*Results* The higher toxicity corresponded to fenofibric acid, with $EC_{50}$ as low as 1.72 mg/l for *V. fischeri*. Gemfibrozil was also toxic for *Anabaena* sp. with $EC_{50}$ of 4.42 mg/l. The study reports the results from toxicity tests using fortified real wastewater samples taken from the effluent of a wastewater treatment plant. The wastewater itself was found to be very toxic to *Anabaena* CPB4337 (84% of bioluminescence inhibition) whereas it did not have any negative effect on *D. magna* or *V. fischeri*. On the contrary, *V. fischeri* luminescence exhibited a stimulatory effect in wastewater.
*Discussion* Except for fenofibric acid, the *Anabaena* bioassay was more sensitive than the *D. magna* and *V. fischeri* bioassays to bezafibrate, clofibric acid, and gemfibrozil. For the three toxicity tests, fortification resulted in lower measured toxicity for the four compounds, probably indicating a reduced bioavailability due to the interaction with other chemicals in the wastewater or with particulate matter. The observed decrease in toxicity associated to the use of a wastewater matrix was higher for the more hydrophobic compounds reaching one order of magnitude for bezafibrate and gemfibrozil.
*Conclusions* The *Anabaena* CPB4337 bioassay revealed a certain risk associated with the three less toxic compounds tested. Based on *V. fischeri* and *D. magna* bioassays, bezafibrate and gemfibrozil would have been considered non-toxic and harmful, respectively. The use of $EC_{50}$ data measured in wastewater increases the risk estimation.
*Recommendations and perspectives* Cyanobacteria, as primary producers with a key role in the carbon and nitrogen cycles, are a substantial component of the microbial food webs. Any detrimental effect on this group may have a negative impact in nutrient availability to organisms of higher trophic levels and should be considered in ecotoxicity assessment tests.

**Keywords** *Anabaena* sp. · *Daphnia magna* · Ecotoxicity · Lipid regulators · *Vibrio fischeri* · Wastewater

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## 1 Background, aim, and scope

The occurrence and fate of pharmaceutically active compounds in the aquatic environment have become a major cause for concern due to their effects on humans and aquatic *ecosystems* (Daughton and Ternes 1999; Kümmerer 2001; Gagné et al. 2006). Pharmaceuticals are continuously released into the environment and lead to a widespread distribution (Jørgensen and Halling-Sørensen 2000), with the effluents of sewage treatment plants (STP) being the prevailing path that these compounds follow to enter surface water streams (Gagné et al. 2006). The risks associated with the discharge of pharmaceuticals into the environment are due not only to their acute toxicity but also their genotoxicity, development of pathogen resistance, and endocrine disruption (Halling-Sørensen et al. 1998). The constant presence of these biologically active xenobiotics exposes aquatic organisms to accumulative and multigenerational exposure with a risk of changes that may remain undetected while causing irreversible damage (Daughton and Ternes 1999). Two effects contribute to enhance this risk: first, many pharmaceuticals are not completely destroyed in conventional STP (Carballa et al. 2004); second, the metabolic and environmental degradation of these compounds produce a huge variety of metabolites and degradation products that increase the complexity of wastewater mixtures. The existence of matrix effects and non-additive interactions suggests the necessity of considering combination effects even for less toxic substances (Cleuvers 2003).

Fibrates are a class of drugs derived from fibric acid widely used to reduce plasma triglycerides and raise the level of high-density lipoprotein cholesterol. Their active forms, fibric acids, exert their biological effects by binding peroxisome proliferator-activated receptor α, a member of the nuclear receptor superfamily of ligand-activated transcription factors. It has been stated that most fibrates are excreted unmodified (Isidori et al. 2007). Clofibric acid is a biological metabolite of the active substance clofibrate, ethyl 2-(4-chlorophenoxy)-2-methylpropanoate, a persistent drug used as lipid regulator that has been detected in surface waters even years after it fell out of use (Buser et al. 1998). The presence of clofibric acid in STP has been repeatedly reported after its detection in the effluent of a German treatment plant (Ternes 1998; Andreozzi et al. 2003; Tauxe-Wuersch et al. 2005). Heberer and Stan (1997) reported the presence of clofibric acid in drinking water from the Berlin area at concentrations up to 270 ng/l, probably due to artificial groundwater enrichment. Weigel et al. (2002) detected over 1 ng/l of clofibric acid in samples taken in the North Sea, whereas Boyd et al. (2003) reported 103 ng/l of clofibric acid at the inlet of a drinking treatment plant fed by Detroit River water. Bezafibrate, *p*-[4-[chlorobenzoylamino-ethyl]-phenoxy]-b-methylpropionic acid, is a drug extensively used as a lipid regulator whose consumption in developed countries has greatly increased during the last years (Lambropoulou et al. 2008). Due to its large use and its persistence, bezafibrate has been detected in surface and drinking waters as well as in effluents of STP in the range of nanogram per liter (Metcalfe et al. 2003a, b; Fent et al. 2006). Gemfibrozil, 5-(2,5- dimethylphenoxy)-2,2-dimethylpentanoic acid, is also a growingly used lipid-regulating agent generically classified as a fibric acid derivative. Metcalfe et al. (2003a, b) found levels around 1 μg/l in effluents of Canadian sewage treatment plants. Sanderson et al. (2003) detected similar values (0.75–1.50 μg/l) for the highest concentrations of gemfibrozil in surface waters in North America and Europe. Fenofibric acid, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, is the active metabolite of fenofibrate and has been reported in concentrations of up to 0.5 μg/l in the influent of several Brazilian STP (Stumpf et al. 1999). The same authors reported a low (45%) efficiency of removal of fenofibric acid by activated sludge conventional treatments and also reported an average of 40 ng/l in several natural streams in the state of Rio de Janeiro. In Europe, Andreozzi et al. (2003) reported the occurrence of lipid regulators in the effluent of several STP in Italy, France, and Greece at concentrations of up to 0.68 μg/l (clofibric acid), 4.76 μg/l (gemfibrozil), 1.07 μg/l (bezafibrate), and 0.16 μg/l (fenofibrate). In a former work (Rosal et al. 2008), bezafibrate and gemfibrozil were detected in amounts of 139 and 608 ng/l, respectively, in the same STP from which the samples used in this work were taken. In previous analyses for the same STP, Rodríguez et al. (2008) reported 165 ng/l of fenofibric acid, 61 ng/l of bezafibrate, and 143 ng/l of gemfibrozil. In the same sampling program, clofibric acid was detected with an average concentration of 24 ng/l.

The luminescence inhibition bioassay with marine *Vibrio fischeri* photobacteria has proven to be a useful tool in estimating the acute toxicity of many chemicals. Other standard ecotoxicity methods using algae, bacteria, invertebrates, and higher organisms many of which have been defined by Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals (OECD 2008) are also routinely used. This work used a novel toxicity test based on the strain denominated *Anabaena* sp. PCC 7120 strain CPB4337 that bears in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) (Szittner and Meighen 1990). This strain shows a high constitutive self-luminescence with no need to add exogenous aldehyde; cell viability is not significantly affected by the Tn5 insertion and the endogenous generation of aldehyde (Fernandez-

Pinas and Wolk 1994). Luminescence was shown to be high in a wide range of temperatures and pH values (Fernandez-Pinas and Wolk 1994; Fernandez-Pinas et al. 2000). Cyanobacteria were the first organisms to carry out the oxygenic photosynthesis and are thought to be the ancestors of the chloroplasts; thus, they are usually models to study photosynthetic processes whose results can be extrapolated to higher plants. Cyanobacteria are found virtually in all regions of the world. Originated during the Precambrian Era, they have been found to be the most abundant photosynthetic organisms on the planet today; they are at the base of the aquatic food webs, making air, light, and water into food for other forms of life. Due to its ecological relevance as a prokaryotic primary producer, this novel cyanobacterial bioreporter may be particularly useful to test toxicity in aquatic environments.

The objective of this study was to evaluate the toxicity of several fibric acid derivatives commonly used to treat hypercholesterolemia and universally found in urban and domestic wastewaters even after biological conventional treatments. The ecotoxicity tests used combined the well-established tests based on OECD and Deutsches Institut für Normung standards for *Daphnia magna* and bioluminescent bacteria *V. fischeri* with the novel bioassay that used a self-luminescent cyanobacterium denominated *Anabaena* sp. PCC 7120 strain CPB4337 (hereinafter, *Anabaena* CPB4337). Toxicity bioassays have also been performed in a real wastewater from the effluent of a secondary clarifier in an STP. Special attention has been paid to fenofibric acid, a compound whose presence has been recently reported in wastewaters but whose toxicity has not been experimentally assessed before.

## 2 Material and methods

### 2.1 Materials

Wastewater samples were collected from the secondary clarifier of an STP located in Alcalá de Henares (Madrid) that receives domestic wastewater with a minor contribution of industrial effluents from facilities located near the city, notably chemical and pharmaceutical small plants. The main wastewater characterization parameters are shown in Table 1. This STP used conventional activated sludge treatment and has been designed for a total capacity of 375,000 equivalent inhabitants with a maximum flow rate of 3,000 $m^3$/h. More detailed information has been given elsewhere and revealed that the individual pollutants detected in higher amounts are those commonly encountered in biologically treated wastewater, with a high contribution of stimulants, anti-inflammatories, antibiotics, β-blockers, and lipid regulators (Rosal et al. 2008).

**Table 1** Main wastewater parameters

| | Anions and cations (mg/L) | | |
|---|---|---|---|
| pH | 7.73 | $NO_3^-$ | 36.03 |
| Turbidity (NTU) | 2.68 | $PO_4^{3-}$ | 3.18 |
| Conductivity (μS/cm) | 702 | $SO_4^{2-}$ | 81.66 |
| TOC (mg/l)[a] | 8.1 | $Cl^-$ | 89.17 |
| COD (mg/l) | 62 | $Na^+$ | 83.12 |
| $PO_4$-P (mg/l) | 1.1 | $K^+$ | 15.10 |
| $NH_4^+$-N (mg/l) | 1.5 | $Mg^{2+}$ | 19.45 |
| $NO_3$-N (mg/l) | 7.0 | $Ca^{2+}$ | 38.02 |

[a] Filtered at 0.45 μm

Gemfibrozil (+99%), clofibric acid (97%), and bezafibrate (+98%) were purchased from Sigma Aldrich. Fenofibric acid was produced from fenofibrate (Sigma Aldrich, +99% purity) by way of hydrolysis. A suspension of fenofibrate in isopropanol (30 wt.%, 400 ml) was refluxed during 4 h with an aqueous sodium hydroxide solution (2.0 M, 200 ml). After cooling to less than 70°C, a solution of hydrochloric acid (1.0 M, 325 ml) was slowly added while keeping the temperature over 60°C. The product crystallized after cooling and keeping room temperature during the course of four more hours. The product was filtered and rinsed with water and dried overnight at 60°C under nitrogen. The purity of the product was over 97% and thereafter checked by high-performance liquid chromatography (HPLC) as described. Table 2 shows the main physicochemical properties of the four compounds studied. Solubility, being an important property, has not been included as the solubility of acidic drugs in water is strongly pH dependent with few data considering this variable. Comerton et al. (2007) reported a solubility of 10.9 mg/l of gemfibrozil in water, but we could solve over 125 mg/l in 2 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 6 and higher quantities for the pH at which *V. fischeri* and *D. magna* bioassays were performed. In all cases, we avoided the use of solvents and the upper limit for the concentrations of the studied compounds was their solubility in pure water or wastewater at the pH of the bioassay.

### 2.2 Standard toxicity tests

Bioassays with the photo-luminescent bacteria *V. fischeri* were carried out according to ISO 11348-3 standard protocol (International Organization for Standardization 2007). During the prescribed incubation period, this bioassay measures the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The bacterial assay used the commercially available Biofix Lumi test (Macherey-Nagel, Germany).

The bacterial reagent is supplied freeze-dried (*V. fischeri* NRRL-B 11177) and was reconstituted and incubated at 3° C for 5 min before use. The analysis media was 0.34 M NaCl (2% *w*/*v*) adjusted to the desired pH with NaOH or HCl. The measurements were performed at 15°C using an Optocomp I luminometer. The effect of toxics was measured as percent inhibition with respect to the light emitted under test conditions in the absence of any toxic influence. Toxicity values are routinely obtained after 15- or 30-min exposure and are usually expressed as the median effective concentration values ($EC_{50}$) at which a 50% loss of luminescence is obtained. Phenol and $ZnSO_4$•$7H_2O$ have been used as toxicity standards and all tests have been replicated to ensure reproducibility and in order to obtain acceptable confidence intervals. In analyses performed using wastewater as a matrix, the reference was always the same wastewater after adjusting osmotic pressure.

Acute immobilization tests with *D. magna* were conducted following the standard protocol described in the European Guideline (Commission of the European Communities 1992). The *D. magna* bioassay used a commercially available text kit (Daphtoxkit F™ magna, Creasel, Belgium). The dormant eggs were incubated in standard culture medium imitating natural freshwater at 20±1°C under continuous illumination of 6,000 lx in order to induce hatching. Between hatching and test steps, the daphnids were fed with the microalgae *Spirulina* to avoid mortality during tests. The pH of samples was adjusted to be in the tolerance interval of the test organisms (Seco et al. 2003). Test plates with *D. magna* neonates were incubated for 24–48 h in the dark at 20°C. Acute toxicity was assessed by observing the effects of the test compounds on the mobility of *D. magna*. The neonates are considered immobilized if they lie on the bottom of the test plate and do not resume swimming within a period of 15 s. Acute toxicity is expressed in this test as the median effective concentration ($EC_{50}$) leading to the immobilization of 50% of the daphnids after 48 h.

## 2.3 Cyanobacterial test methods

### *2.3.1 Strain and culture conditions*

*Anabaena* CPB4337 was routinely grown at 28°C in the light, approximately 65 µmol photons per square meter per second on a rotary shaker in 50 ml AA/8 (Allen and Arnon 1955) supplemented with nitrate (5 mM) in 125-ml Erlenmeyer flasks and 10 µg/ml of neomycin sulfate (Nm).

### *2.3.2 Determination of acute toxicity*

The toxicity bioassays using *Anabaena* CPB4337 are based on the inhibition of constitutive luminescence caused by the presence of a toxic substance. Acute (1 and 24 h of exposure) luminescence inhibition-based toxicity assays were performed as follows: 160 µl from five to seven serial dilutions of each tested toxicant plus a control ($ddH_2O$ buffered with MOPS at pH 5.8) were disposed in an opaque white 96-well microtiter plates. Cells, grown as described, were washed twice and resuspended in $ddH_2O$ buffered with MOPS at pH 5.8 and were added to the microtiter plate wells to reach a final cell density at $OD_{750\ nm}$ of 0.5. For 1-h toxicity testing, the luminescence of each sample was recorded every 5 min for up to 1 h in the Centro LB 960 luminometer. For 24-h toxicity testing, the 96-well microtiter plates were kept at room temperature (28°C) at low light (approximately 30 µmol photons per square meter per second) during 24 h and luminescence was recorded for 5 min. Three independent experiments with quadruplicate samples were carried out for all *Anabaena* toxicity assays.

### *2.3.3 Reference toxicant and inter-assay variability calibration*

Copper sulfate ($CuSO_4$) was selected as reference toxicant for calibration in all assays. The calibration allows one to calculate the mean $EC_{50\text{-}1\,h}$ and $EC_{50\text{-}24\,h}$ of copper in order to refuse or accept the experiment if those $EC_{50}$ values fall in or out of the 95% of confidence limits previously fixed for this reference toxicant (USEPA 1994, 2002). To achieve

**Table 2** Physicochemical properties of bezafibrate, clofibric acid, fenofibrate, and gemfibrozil

| | Bezafibrate | Clofibric acid | Fenofibrate | Gemfibrozil |
|---|---|---|---|---|
| CAS no. | 41859-67-0 | 882-09-7 | 49562-28-9 | 25812-30-0 |
| Molecular formula | | | | |
| Molecular weight (g/mol) | 361.819 | 214.645 | 360.831 | 250.333 |
| $pK_a$ | 3.6 (1) | 3.18 (2) | 2.86 (3) [a] | 4.8 (4) |
| log $K_{ow}$ | 4.25 (5) | 2.88 (6) | 5.19 (7) | 4.77 (5) |
| Percent removal in STP (8) | 27–50 | 15–34 | 6–45 [a] | 16–46 |

(*1*) Huber et al. 2003, (*2*) Packer et al. 2003, (*3*) Lewis and Lakeb 1998, (*4*) Lin et al. 2006, (*5*) Han et al. 2006, (*6*) Scheytt et al. 2005, (*7*) Hernando et al. 2007, (*8*) Stumpf et al. 1999

[a] Fenofibric acid

this, five copper dilutions were tested by quadruplicate in control wells of each assay.

### 2.4 Data analysis

The quantitative response to toxic exposure in *V. fischeri* and *D. magna* tests was estimated by fitting the experimental luminescence inhibition or immobilization data to a logistic function:

$$E[Y|x] = \frac{\alpha}{1 + \left(\frac{x}{EC_{50}}\right)^{\beta}} \quad (1)$$

Where E[$Y \mid x$] represents the average response at dosage $x$, and $\alpha$ and $\beta$ are the upper asymptote of response and parameters related to the rate of change at the inflection point of the curve, respectively. The elaboration of experimental data was performed by non-linear regression analysis using conventional statistical inference tools through which $EC_{50}$ results were expressed with their corresponding intervals at a given $p$ level of confidence (Schabenberger et al. 1999).

Toxicity response of the cyanobacterium was estimated also as $EC_{50}$ values, the median effective concentration of a toxicant that causes 50% of bioluminescence inhibition with respect to a non-treated control. $EC_{50}$ and its related statistical parameters, standard deviation, coefficient of variation, and confidence intervals (CI) were estimated using the linear interpolation method (Norberg-King 1993; USEPA 1994, 2002).

### 2.5 Fortification of wastewater

In order to assess the matrix effect associated with real wastewater samples, bezafibrate, clofibric acid, fenofibric acid, and gemfibrozil were dissolved in wastewater at the pH required by each bioassay. The concentration range was adapted as required to obtain acceptable standard deviation in the computing of $EC_{50}$ values.

### 2.6 Stability analyses

The stability of target compounds under bioassay conditions was assessed according to OECD guidance (OECD 2008). In this work, analyses have been performed at the start and at the end of tests lasting 24 h (*Anabaena* CPB4337) and 48 h (*D. magna*). The test has been carried out for the lower and higher concentrations as well as for a concentration near $EC_{50}$ in pure water for each compound. Analyses were conducted at room temperature using an HPLC–diode array liquid chromatograph (Varian) equipped with automated injection of 50-µl sample volumes. The column used was a C18 of 250×4.6 mm, 5 µm (Phenomenex). Isocratic elution of gemfibrozil, clofibric, and fenofibric acids was performed using a mixture of acrylonitrile and deionized water (with 4 ml/l of phosphoric acid and 50 ml/l of methanol) at 1 ml/min and 60:40 (fenofibric acid and gemfibrozil) or 70:30 (clofibric acid). Bezafibrate was analyzed using isocratic elution of methanol and deionized water (70:30) with 0.09 M of acetic acid at 1 ml/min. Detection was performed at 280 nm for gemfibrozil and fenofibric acid, at 230 nm for clofibric, and at 228 nm for bezafibrate.

## 3 Results and discussion

The toxicity can be assessed from $EC_{50}$ values according to the categories established in the technical guidance on risk assessment of substances in the European Union (Commission of the European Communities 1996). In it, wastewater samples or standard compounds are considered "harmful to aquatic organisms" if $EC_{50}$ falls in the 10–100-mg/l range, "toxic" if $1 < EC_{50} < 10$ mg/l and "very toxic" if $EC_{50} <$ 1 mg/l. The results of toxicity tests for fibrates in pure water are reported in Table 3 together with their 95% confidence intervals. The data showed that fenofibric acid could be considered toxic based on *V. fischeri* and *D. magna* tests. Gemfibrozil and bezafibrate exhibited $EC_{50} < 10$ mg/l using

**Table 3** Toxicity of lipid regulators in pure water as $EC_{50}$ values (mg/l) with confidence limits (95% probability)

| Compounds | *V. fischeri*[a] | *V. fischeri*[b] | *Anabaena* sp. 4337[c] | *Anabaena* sp. 4337[d] | *D. magna* |
|---|---|---|---|---|---|
| Bezafibrate | 178.73 (162.06–197.12) | 172.73 (155.52–191.85) | 37.28 (32.60–41.79) | 7.62 (7.01–8.41) | 240.40 (230.12–250.68) |
| Clofibric acid | 290.04 (269.24–310.84) | 240.65 (202.57–278.73) | 48.08 (45.82–55.92) | 30.80 (26.50–42.39) | 83.52 (70.41–96.63) |
| Fenofibric acid | 1.86 (1.64–2.08) | 1.72 (1.48–1.96) | 10.82 (8.46–13.35) | 10.85 (6.16–13.16) | 4.90 (3.74–6.06) |
| Gemfibrozil | 35.34 (33.22–37.66) | 29.07 (26.77–31.37) | 8.44 (7.81–9.24) | 4.42 (4.06–4.57) | 22.85 (17.01–28.69) |

[a] Fifteen minutes of exposure

[b] Thirty minutes of exposure

[c] One hour of exposure

[d] Twenty-four hours of exposure

the new ecotoxicity test based on *Anabaena* CPB4337 and both could also be considered toxic to the cyanobacterium, whereas, according to *V. fischeri* and *D. magna* tests, they would have been classified only as harmful and non-toxic, respectively. Exposure of *Anabaena* to bezafibrate, clofibric acid, and gemfibrozil for 24 h resulted in a significant increase of toxicity (Student's $t$ test, $P<0.05$). It is interesting to point out that the four fibrates can be considered at least harmful to *Anabaena* CPB4337 even in the case of substances that do not evidence toxicity such as bezafibrate or the less toxic clofibric acid when using classic tests. The stability assessment tests showed that the concentrations of all tested compounds were essentially constant during the analyses, always inside the boundary established by OECD (2008).

Table 4 shows toxicity data reported by other authors that used ecotoxicity tests based on *V. fischeri* with 30 min of exposure and *D. magna* at 48 h. The estimations of Sanderson et al. (2003) that used a model based on structure activity relationship (SAR) have been included for comparison. Our results show that gemfibrozil proved more toxic to *V. fischeri* and *D. magna* than most data reported in the literature. The cyanobacterial bioassay that we have developed showed the greatest sensitivity towards bezafibrate, clofibric acid, and gemfibrozil, thus indicating the suitability of this new test to check toxicity of these types of pollutants. Table 4 also evidences an apparent variability of data both within the same and between different tests that is usually found in ecotoxicity data reported from different sources. For example, bezafibrate toxicity to *D. magna* ($EC_{50}$, 48 h) varies between 30.3 and over 200 mg/l (168.3 mg/l in this work). This low reproducibility due to the complexity of biological tests has been attributed to different actual exposure concentrations, changing sensitivities of the organisms, and diverse laboratory performances (Fent et al. 2006).

The toxicity of fenofibric acid has been assessed for the first time in this work. Previously, only an estimation based on SAR models whose precision was determined to be low not only for fenofibric acid but also for other fibrates, as indicated in Table 4 (Sanderson et al. 2003), was reported. Among the four fibrates, the toxicity of fenofibric acid was highest for *V. fischeri* and lowest for *Anabaena* CPB4337, a result opposite to that obtained with bezafibrate, clofibric acid, and gemfibrozil, for which the new test exhibited a higher sensitivity. These results stress the need for gathering information from different organisms before assessing the environmental risks associated with drugs spread into the environment. A restriction to conventional well-established tests could yield a risk underestimation.

Table 5 shows the toxicity of wastewater to the three test organisms used in this work. The wastewater, whose main

**Table 4** Literature toxicity data for bezafibrate, clofibric acid, and gemfibrozil

| Compound | Bioassay | $EC_{50}$ (mg/l) and 95% CI | Reference |
|---|---|---|---|
| Bezafibrate | *V. fischeri* (30 min) | >100 | Isidori et al. 2007 |
| | *D. magna* (24 h) | 100.08 (80.02–120.54) | Isidori et al. 2007 |
| | *D. magna* (48 h) | >200 | Hernando et al. 2004 |
| | *D. magna* (48 h) | 30.3 | Han et al. 2006 |
| | *D. magna* (48 h) | 25.00[a] | Sanderson et al. 2003 |
| Clofibric acid | *V. fischeri* (30 min) | 100 | Henschel et al. 1997 |
| | *V. fischeri* (30 min) | 91.8 | Ferrari et al. 2003 |
| | *D. magna* (48 h) | >200 | Ferrari et al. 2003 |
| | *D. magna* (48 h) | 106 | Henschel et al. 1997 |
| | *D. magna* (48 h) | 150 | Hernando et al. 2004 |
| | *D. magna* (48 h) | 72 | Cleuvers 2003 |
| | *D. magna* (48 h) | 141.2 | Han et al. 2006 |
| | *D. magna* (48 h) | 293.00[a] | Sanderson et al. 2003 |
| Fenofibric acid | *D. magna* (48 h) | 38.00[a] | Sanderson et al. 2003 |
| Gemfibrozil | *V. fischeri* (15 min) | 180 (174–186) | Zurita et al. 2007 |
| | *V. fischeri* (30 min) | 85.74 (77.22–91.74) | Isidori et al. 2007 |
| | *D. magna* (24 h) | 74.30 (66.15–83.45) | Isidori et al. 2007 |
| | *D. magna* (24 h) | 228 (214–242) | Zurita et al. 2007 |
| | *D. magna* (48 h) | 100 | Hernando et al. 2004 |
| | *D. magna* (48 h) | 170 (156–184) | Zurita et al. 2007 |
| | *D. magna* (48 h) | 10.4 | Han et al. 2006 |
| | *D. magna* (48 h) | 6.00[a] | Sanderson et al. 2003 |

[a] Predicted by a structure activity relationship model

**Table 5** Toxicity values obtained for an STP effluent

| toxicity test | $EC_{50}$[a] | SD | CV (%) | $CI_L$ 95% | $CI_U$ 95% |
|---|---|---|---|---|---|
| *V. fischeri* | ls | | | | |
| *Anabaena* sp. 4337[b] | 0.11 | 0.01 | 9.09 | 0.09 | 0.16 |
| *Anabaena* sp. 4337[c] | 0.66 | 0.08 | 12.12 | 0.47 | 0.42 |
| *D. magna* | nt | | | | |

*Ls* luminescence stimulation (effect described in the text), *nt* non-toxic with immobilization <5%

[a] STP effluent dilution that causes 50% inhibition of luminescence in *V. fischeri* and *Anabaena* 4337 tests and 50% immobilization of daphnids in *D. magna* test. $CI_L$ 95% and $CI_U$ 95% are lower and upper 95% confidence limits, respectively

[b] One hour of exposure

[b] Twenty-four hours of exposure

characteristics are indicated in Table 1, was non-toxic to *D. magna*, with immobilization of <5% in three replicates. *V. fischeri* tests exhibited a luminescence increase of near 50% during the first 5 min to decay thereafter that resulted in negative toxicity values when compared to the blank. Although rarely reported in *V. fischeri* bioassays, this effect has been repeatedly observed in our laboratory for wastewaters of a different origin. Early works have shown that subinhibitory concentrations of a toxic substance may yield stimulatory effects on an organism (Southman and Ehrlich 1943). This behavior, called hormetic, has been attributed to the salinity correction of the sample in the *V. fischeri* bioassay that may alter the pollutant bioavailability, thereby developing a false-negative toxicity response (Abbondanzi et al. 2003). Recently, Deryabin and Aleshina (2008) found a stimulatory effect of some salts such as chlorides and sulfates on the luminescence response of *Photobacterium phosphoreum* and recombinant *Escherichia coli* with cloned *lux*CDABE genes of *Photobacterium leiognathi* 54D10. Opposite the result found with *D. magna* and *V. fischeri*, the wastewater was very toxic to *Anabaena* CPB4337, causing a luminescence inhibition of 84.24% (76.14–91.42). As shown in the table, a wastewater dilution as low as 0.11 caused 50% inhibition of luminescence in the acute toxicity test. The observed toxicity could be due to the combined toxicities of the chemicals, pharmaceuticals, herbicides, or metals present in this wastewater. As a reference, an analysis of over 30 micropollutants in wastewater from the same STP has been given elsewhere (Rosal et al. 2008). The fact that the wastewater itself was already toxic to *Anabaena* but non-toxic to *V. fischeri* or *D. magna* highlights the need to develop and use new toxicity tests based on organisms of different trophic levels or origins in order to achieve a more complete assessment of the ecotoxicological risk of pollutants.

In order to assess the risk of fibrates in a real matrix, ecotoxicity tests have been applied to wastewater fortified with different concentrations of the compounds studied in this work. The effect of fibrates in wastewater, measured as $EC_{50}$ and corresponding to wastewater fortified with different amounts of the target compounds, is shown in Table 6. When using the *V. fischeri* and *D. magna* tests, the $EC_{50}$ values found in wastewater were considerably higher than those obtained in pure water by a factor in the range of 2–13×. The lower measured toxicity that resulted from fortification of real wastewater is probably reflecting a reduced bioavailability due to interactions with other chemicals or with particulate matter. The observed decrease

**Table 6** Toxicity effects of lipid regulators in fortified STP effluent expressed as $EC_{50}$ values (mg/l) with confidence limits (95% probability)

| Compounds | *V. fischeri*[a] | *V. fischeri*[b] | *Anabaena* sp. 4337[c] | *Anabaena* sp. 4337[d] | *D. magna* |
|---|---|---|---|---|---|
| Bezafibrate | >250 | >250 | >100 | >100 | >500[e] |
| Clofibric acid | >500 | >500 | >400 | 62.70 (56.30–66.03) | 366.69 (337.39–395.99) |
| Fenofibric acid | 4.31 (3.97–4.65) | 4.11 (3.75–4.47) | >20 | 13.80 (13.38–14.50) | 16.88 (14.13–19.63) |
| Gemfibrozil | 134.15 (123.15–145.15) | 153.29 (146.91–212.59) | 4.81 (2.90–5.70) | 59.16 (57.00–66.00) | 121.12 (113.89–128.36) |

[a] Fifteen minutes of exposure

[b] Thirty minutes of exposure

[c] One hour of exposure

[d] Twenty-four hours of exposure

[e] Immobilization <10% at 500 mg/l

in toxicity associated with the use of a wastewater matrix was higher for the more hydrophobic compounds, reaching one order of magnitude for bezafibrate and gemfibrozil, and with a more pronounced effect on *Anabaena* bioassay. In this regard, it should be noted that the wastewater itself was already very toxic to *Anabaena*. One would expect that the addition of fibrates to the wastewater, also toxic to *Anabaena*, would completely inhibit luminescence. However, except for fenofibric acid, for which there was no significant difference, the $EC_{50}$ values were also higher than those reported in pure water. These results favor the already mentioned hypothesis of antagonistic effects of fibrates with some of the chemicals that are in the wastewater (Rosal et al. 2008). A wide diversity of pharmaceuticals, personal care products, or pesticides that are not completely eliminated in conventional wastewater treatments may reach freshwater sources, leading to a long-term exposure of aquatic organisms in a mixture of these compounds; a systematic analysis of the mixture toxicities of these chemicals should be carried out in order to better understand their biological effects.

As indicated before, the reported concentrations of individual pollutants typically exceed the 10 ng/l cutoff value established in the European Medicines Evaluation Agency (EMEA) tiered procedure that corresponds to 100 ng/l in STP effluents which was once considered the conventional dilution factor, as indicated before (European Medicines Evaluation Agency 2006). Predicted no-effect concentrations (PNEC) can be estimated from their corresponding $EC_{50}$ values obtained in acute toxicity tests by application of an assessment factor as indicated in the EMEA procedure. The guideline prescribes an assessment factor of 1,000 when using the information of short-term $EC_{50}$ from each of three trophic levels of the base set (fish, *Daphnia*, and algae). The results show a clear evidence of adverse effects in water as result of measured environmental concentration of fenofibric acid for which *V. fischeri* was particularly sensitive. For the other tested compounds, the cyanobacterium *Anabaena* CPB4337 led to the lowest estimation of PNEC. If ecotoxicity is measured in wastewater, the observed increase in $EC_{50}$ values roughly corresponds with the dilution factor leading to similar risk estimations for the four compounds. The results show the risk of extrapolation of PNEC based on $EC_{50}$ values obtained from the conventional set of toxicity tests on freshwater, as the introduction of a new species or a change in the matrix may considerably alter the risk level for a certain substance. An additional cause for concern is the potential bioaccumulation of fibrates, as indicated by log $K_{ow}>3$ with the lowest value (clofibric acid) very close to the boundary and with the rest clearly above, as shown in Table 2.

## 4 Conclusions

A novel bioassay that used a self-luminescent cyanobacterium denominated *Anabaena* sp. PCC 7120 strain CPB4337 has been compared to standard ecotoxicity tests based on *V. fischeri* and *D. magna* for the ecotoxicity assessment of four lipid regulators. The toxicity of fenofibric acid, a compound whose ecotoxicity had not been previously assessed, was particularly high with $EC_{50}$ values as low as 1.72 mg/l for *V. fischeri*. The new test of *Anabaena* CPB4337 showed a greater sensitivity for bezafibrate, clofibric acid, and gemfibrozil. The introduction of *Anabaena* bioassay would allow one to reclassify bezafibrate and clofibric acid from "non-toxic" ($EC_{50}>$ 100 mg/l) to "harmful to aquatic organisms" (10 mg/l< $EC_{50}<100$ mg/l). The assessment of toxicity using a matrix of real wastewater leads to higher $EC_{50}$ values that correspond with a lower evidence for toxicity. The more hydrophobic the compound, the higher was the difference, thus suggesting that the effect is probably linked to a reduced bioavailability of the less polar compounds. The results encourage the development of new and more sensitive toxicity tests for the detection of unwanted toxic effects that might become overlooked using conventional bioassays.

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# APPLICATION OF THE COMBINATION INDEX (CI)-ISOBOLOGRAM EQUATION TO STUDY THE TOXICOLOGICAL INTERACTIONS OF LIPID REGULATORS IN TWO AQUATIC BIOLUMINESCENT ORGANISMS.

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# Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms

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ABSTRACT

Pharmaceuticals in the aquatic environment do not appear singly and usually occur as complex mixtures, whose combined effect may exhibit toxicity to the aquatic biota. We report an environmental application of the combination index (CI)-isobologram equation, a method widely used in pharmacology to study drug interactions, to determine the nature of toxicological interactions of three fibrates toward two aquatic bioluminescent organisms, *Vibrio fischeri* and the self-luminescent cyanobacterial recombinant strain *Anabaena* CPB4337. The combination index-isobologram equation method allows computerized quantitation of synergism, additive effect and antagonism. In the *Vibrio* test, the fibrate combinations showed antagonism at low effect levels that turned into an additive effect or synergism at higher effect levels; by contrast, in the *Anabaena* test, the fibrate combinations showed a strong synergism at the lowest effect levels and a very strong antagonism at high effect levels. We also evaluated the nature of the interactions of the three fibrates with a real wastewater sample in the cyanobacterial test. We propose that the combination index-isobologram equation method can serve as a useful tool in ecotoxicological assessment.


## 1. Introduction

Fibrates and statins (HMG-CoA reductase inhibitors) are the main lipid-lowering drugs prescribed either alone or in combination therapy in order to decrease plasma cholesterol levels and reduce the incidence of coronary heart disease. Although partially displaced by statins, the total number of fibrate prescriptions is in constant increase in the United States (Holoshitz et al., 2008). Fibric acids are the active forms of fibrates and belong to the nuclear receptor superfamily of ligand-activated transcription factors. Gemfibrozil and fenofibrate are the fibrates currently marketed in the US, whereas bezafibrate is also available in Europe and other developed countries (Lambropoulou et al., 2008). Fenofibric acid, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, is the active metabolite of fenofibrate, the inactive prodrug marketed and dispensed. Gemfibrozil, 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid and bezafibrate, *p*-[4-[chlorobenzoylamino-

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ethyl]-phenoxy]-b-methylpropionic acid, are also fibric acid derivatives with similar pharmacokinetic behaviour (Miller and Spence, 1998).

The occurrence of lipid regulators in the discharge of treated urban and municipal wastewater has been relatively well documented. Bezafibrate has been detected in effluents of two British STP with averages up to 230 ng/L (Kasprzyk-Hordern et al., 2009). Metcalfe et al. (2003) found around 1 μg/L of gemfibrozil in effluents of Canadian STP, whereas fenofibrate has been reported in concentrations up to 0.5 μg/L in the influent of several Brazilian STP (Stumpf et al., 1999). Andreozzi et al. (2003) found lipid regulators in the effluent of several European STP at concentrations up to 4.76 μg/L (gemfibrozil), 1.07 μg/L (bezafibrate) and 0.16 μg/L (fenofibrate). Rosal et al. (2008), reported the occurrence of bezafibrate and gemfibrozil at levels of 139 and 608 ng/L respectively in the effluent of a Spanish STP. In the same plant Rodríguez et al. (2008) found 165 ng/L of fenofibric acid, 61 ng/L of bezafibrate and 143 ng/L of gemfibrozil.

It is also significant that removal efficiencies observed in current STP are not always high. Fent et al. (2006) reported maximum removal rates of 50–75% for fenofibric acid and gemfibrozil and somewhat higher for bezafibrate, although for the later, efficiencies below 15% have also been reported. Stumpf et al. (1999) reported a 45% removal of fenofibric acid by an activated sludge conventional treatment. Kasprzyk-Hordern et al. (2009) encountered an average degradation of bezafibrate not higher than 67%. On the other hand, Castiglioni et al. (2006) reported that the removal efficiency of bezafibrate during an activated sludge treatment greatly varied from 15% in winter to 87% in summer.

At measured environmental concentrations as those reported above (mostly in the ng/L and μg/L range), many studies have shown that the risk of acute toxicity is unlikely (Fent et al., 2006; Han et al., 2006; Borgmann et al., 2007); however, there is a lack of data on chronic toxicity effects. Moreover, pharmaceuticals in the aquatic environments occur as complex mixtures from different classes, not as single contaminants (Gros et al., 2007); thus, although the concentration of individual pharmaceuticals is low, their mixture could prove ecotoxicologically significant (Brain et al., 2004). Current methods of risk assessment usually focus on the assessment of single chemicals, which may underestimate the risk associated with toxic action of mixtures; probably for this reason, in the last years there is an increasing number of studies dealing with complex mixtures of pharmaceuticals (Cleuvers, 2003, 2004; Crane et al., 2006; Han et al., 2006; Borgmann et al., 2007; Christensen et al., 2007; Pomati et al., 2008; Quinn et al., 2009). However, assessment of combined toxicities is not an easy issue. Basically, two different models are in use for the prediction of mixture toxicity, i.e., concentration addition, when pharmaceuticals have a similar mode of toxic action, and response addition or independent action, when pharmaceuticals have different modes of toxic action (Cleuvers, 2003; Teuschler, 2007). However, toxicological interactions, synergisms or antagonisms, between the pharmaceuticals and their effects can occur independently of mode of action; moreover, in most cases, the pharmacological mechanisms of action is known but the toxic mode of action may remain unknown (Cleuvers, 2003; Chou, 2006). In an effort to overcome this limitation, we report an environmental application of a method widely used in pharmacology to interpret drug interactions; this method, termed as the median-effect/combination index (CI)-isobologram equation (Chou, 2006) allows quantitative determinations of chemical interactions where CI $<1$, $=1$ and $>1$ indicate synergism, additive effect and antagonism, respectively. One important property of the method is that previous knowledge of the mechanisms of action of each chemical is not required. Besides, the method takes into account both the potency and the shapes of the dose-effect curve of each chemical. The method has been computerized allowing an automated simulation of synergism and antagonism at different concentrations and at different effect levels of the chemicals in a mixture.

The aim of our study was to assess the nature of the toxicological interactions of three fibrates, gemfibrozil, bezafibrate and fenofibric acid, by the method of combination index (CI)-isobologram equation. The three pharmaceuticals were used singly or in two- and three-drug combinations. As toxicity endpoint we have chosen the bioluminescent response of two prokaryotes, the naturally luminescent *Vibrio fischeri* and the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC 7120 CPB4337 (hereinafter, *Anabaena* CPB4337), both bioluminescent organisms have proved very useful in evaluating the toxicity of individual fibrates in a previous study (Rosal et al., 2009). For *Anabaena* CPB4337, we also evaluated the nature of the interactions of the three fibrates with a wastewater sample from a local STP, which already proved very toxic to the cyanobacterium (Rosal et al., 2009).

## 2. Materials and methods

### 2.1. Materials

Gemfibrozil (+99%) and bezafibrate (+98%) were purchased from Sigma–Aldrich. Fenofibric acid was produced from fenofibrate (Sigma–Aldrich, +99% purity) by hydrolysis. A suspension of fenofibrate in isopropanol (30 wt.%, 400 mL) was refluxed during 4 h with an aqueous sodium hydroxide solution (2.0 M, 200 mL). After cooling to less than 70 °C, a solution of hydrochloric acid (1.0 M, 325 mL) was slowly added while keeping the temperature over 60 °C. The product crystallized after cooling and keeping at room temperature during 4 or more h. The product was filtered and rinsed with water and dried overnight at 60 °C under nitrogen. The purity of the product was over 97% checked by HPLC. Solubility of acidic drugs in water is strongly pH dependent with few data considering this variable. Comerton et al. (2007) reported a solubility of 10.9 mg/L of gemfibrozil in water, but we could solve over 125 mg/L in 2 mM MOPS (3-[N-morpholino] propanesulfonic acid) at pH 6 and higher quantities for the pH at which *V. fischeri* bioassays were performed. In all cases, we avoided the use of solvents and the upper limit for the concentrations of the studied compounds was their solubility in pure water or wastewater at the pH of the bioassay.

Wastewater samples were collected from the secondary clarifier of a STP located in Alcalá de Henares (Madrid) that

receives domestic wastewater with a minor contribution of industrial effluents from facilities located near the city. This STP used a conventional activated sludge treatment and has been designed for a total capacity of 375,000 equivalent inhabitants with a maximum flow rate of 3000 $m^3$/h. In a recent previous study (Rosal et al., 2009), we found that this wastewater was very toxic to *Anabaena* cells with a wastewater dilution as low as 0.11 causing 50% luminescence inhibition (wastewater $EC_{50}$).

### 2.2. Toxicity tests

Bioassays with the photo-luminescent bacteria *Vibrio fischeri* were carried out according to ISO 11348-3 standard protocol (ISO, 2007). This bioassay measures, during the prescribed incubation period, the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The bacterial assay used the commercially available Biofix Lumi test (Macherey-Nagel, Germany). The bacterial reagent is supplied freeze-dried (*Vibrio fischeri* NRRL-B 11177) and was reconstituted and incubated at 3 °C for 5 min before use. The desired pH was set by using NaOH or HCl. The analysis media was 0.34 M NaCl (2% w/v) and tests were performed at 15 °C and the measurements of light were made using a luminometer (Optocomp I). The effect of toxicants or toxicant mixtures (i.e., fibrates or fibrate combinations) was measured as percent inhibition with respect to the light emitted under test conditions in the absence of any toxic influence. Toxicity values were routinely obtained after 30 min exposure. Phenol and $ZnSO_4 \bullet 7\ H_2O$ have been used as toxicity standards and all tests have been replicated to ensure reproducibility.

The bioassays using the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 were based on the inhibition of constitutive luminescence caused by the presence of any toxic substance (Rodea-Palomares et al., 2009; Rosal et al., 2009). *Anabaena* CPB4337 was routinely grown at 28 °C in the light, ca. 65 μmol photons $m^2\ s^{-1}$ on a rotary shaker in 50 mL AA/8 (Allen and Arnon, 1955) supplemented with nitrate (5 mM) in 125 ml Erlenmeyer flasks and 10 μg/mL of neomycin sulphate (Nm). Luminescence inhibition-based toxicity assays were performed as follows: 160 μL from five to seven serial dilutions of each tested toxicant or toxicant mixture (i.e.; fibrates or fibrate combinations) plus a control (dd$H_2O$ buffered with MOPS at pH 5.8) were disposed in an opaque white 96-well microtiter plates. 40 μL cells, grown as described, were washed twice and resuspended in dd$H_2O$ buffered with MOPS at pH 5.8 and were added to the microtiter plate wells to reach a final cell density at $OD_{750\ nm}$ of 0.5. The luminescence of each sample was recorded every 5 min for up to 1 h in the Centro LB 960 luminometer. Three independent experiments with duplicate samples were carried out for all *Anabaena* toxicity assays. $CuSO_4$ has been used as toxicity standard and all tests have been replicated to ensure reproducibility.

### 2.3. Experimental design of fibrate combinations

Solutions of gemfibrozil (Gm), bezafibrate (Bz) and fenofibric acid (Fn) prepared as described above were used singly and in two (Bz + Gm; Fn + Gm; Fn + Bz) and three (Fn + Gm + Bz) combinations. *Anabaena* and *Vibrio fischeri* cells were treated with serial dilutions of each fibrate individually and with a fixed constant ratio (1:1), based on the individual $EC_{50}$ values, in their binary and ternary combinations. Five dilutions (serial dilution factor = 2) of each fibrate and combination plus a control were tested in three independent experiments with replicate samples.

For evaluating the nature of the interaction of fibrates with wastewater, binary combinations of each fibrate plus wastewater (Fn + WW; Gm + WW; Bz + WW) and a quaternary combination of the three fibrates plus wastewater (Fn + Gm + Bz + WW) were also prepared and tested for *Anabaena* CPB4337. *Anabaena* cells were treated with serial dilutions of each fibrate and wastewater individually and with a fixed constant ratio (1:1), based on the individual $EC_{50}$ values, in their binary and quaternary combinations. Five dilutions (serial dilution factor = 2) of each fibrate and wastewater and their combinations plus a control were tested in three independent experiments with replicate samples. The experimental design is shown in Table 1.

All individual fibrate, wastewater and their combination assays were carried out at the same time as recommended by Chou (2006) to maximize computational analysis of data.

### 2.4. Median-effect and combination index (CI)-isobologram equations for determining combined fibrate interactions

The results were analyzed using the median-effect/combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984) which is based on the median-effect principle (mass-action law) (Chou, 1976) that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition. This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations by using the median-effect equation:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \quad (1)$$

Where $D$ is the dose, $Dm$ is the dose for 50% effect (e.g., 50% inhibition of bioluminescence or $EC_{50}$), $fa$ is the fraction affected by dose $D$ (e.g., 0.75 if cell bioluminescence is inhibited by 75%), $fu$ is the unaffected fraction (therefore, $fa = 1 - fu$), and $m$ is the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively. Therefore, the method takes into account both the potency ($Dm$) and shape ($m$) parameters. If Eq. (1) is rearranged, then:

$$D = Dm[fa/(1 - fa)]^{1/m} \quad (2)$$

The $Dm$ and $m$ values for each fibrate are easily determined by the median-effect plot: $x = \log(D)$ versus $y = \log(fa/fu)$ which is based on the logarithmic form of Eq. (1). In the median-effect plot, $m$ is the slope and $\log(Dm)$ is the $x$-intercept. The conformity of the data to the median-effect principle can be ready manifested by the linear correlation coefficient ($r$) of the data to the logarithmic form of Eq. (1) (Chou, 2006).

**Table 1 – Experimental design for determining toxicological interactions of fenofibric acid [Fn ($D$)$_1$], gemfibrozil [Gm ($D$)$_2$], bezafibrate [Bz ($D$)$_3$] and their binary and ternary combinations for *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests.**

| Pure fibrate experiments | | | | | | | Fibrates plus wastewater experiments | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Vibrio fischeri* | | | *Anabaena* CPB4337 | | | *Anabaena* CPB4337 | | | | |
| Dilutions | Single toxicant | | | Single toxicant | | | | Single toxicant | | | |
| | Fn | Gm | Bz | Fn | Gm | Bz | | Fn | Gm | Bz | WW** |
| | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | | ($D$)$_1$ | ($D$)$_2$ | ($D$)$_3$ | ($D$)$_4$ |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | 37.5 | 2.5 | 2.5 | 12.5 | ¼ ($EC_{50}$) | 2.5 | 2.5 | 12.5 | 0.025 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | 75 | 5 | 5 | 25 | ½ ($EC_{50}$) | 5 | 5 | 25 | 0.05 |
| 1 ($EC_{50}$) | 1.6 | 35 | 150 | 10 | 10 | 50 | 1 ($EC_{50}$) | 10 | 10 | 50 | 0.1 |
| 2 ($EC_{50}$) | 3.2 | 70 | 300 | 20 | 20 | 100 | 2 ($EC_{50}$) | 20 | 20 | 100 | 0.2 |
| 4 ($EC_{50}$) | 6.4 | 140 | 600 | 40 | 40 | 200 | 4 ($EC_{50}$) | 40 | 40 | 200 | 0.4 |
| | Two toxicant combo | | | Two toxicant combo | | | | Two toxicant combo | | | |
| | ($D$)$_1$ + ($D$)$_2$ (1.6:35) | | | ($D$)$_1$ + ($D$)$_2$ (1:1) | | | | ($D$)$_1$ + ($D$)$_4$ (1:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | | 2.5 | 2.5 | | ¼ ($EC_{50}$) | 2.5 | | | 0.025 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | | 5 | 5 | | ½ ($EC_{50}$) | 5 | | | 0.05 |
| 1 ($EC_{50}$) | 1.6 | 35 | | 10 | 10 | | 1 ($EC_{50}$) | 10 | | | 0.1 |
| 2 ($EC_{50}$) | 3.2 | 70 | | 20 | 20 | | 2 ($EC_{50}$) | 20 | | | 0.2 |
| 4 ($EC_{50}$) | 6.4 | 140 | | 25* | 25* | | 4 ($EC_{50}$) | 40 | | | 0.4 |
| | ($D$)$_1$ + ($D$)$_3$ (1.6:150) | | | ($D$)$_1$ + ($D$)$_3$ (1:5) | | | | ($D$)$_2$ + ($D$)$_4$ (1:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | | 37.5 | 2.5 | | 12.5 | ¼ ($EC_{50}$) | | 2.5 | | 0.025 |
| ½ ($EC_{50}$) | 0.8 | | 75 | 5 | | 25 | ½ ($EC_{50}$) | | 5 | | 0.05 |
| 1 ($EC_{50}$) | 1.6 | | 150 | 10 | | 50 | 1 ($EC_{50}$) | | 10 | | 0.1 |
| 2 ($EC_{50}$) | 3.2 | | 300 | 20 | | 100 | 2 ($EC_{50}$) | | 20 | | 0.2 |
| 4 ($EC_{50}$) | 6.4 | | 600 | 30* | | 150* | 4 ($EC_{50}$) | | 40 | | 0.4 |
| | ($D$)$_2$ + ($D$)$_3$ (35:150) | | | ($D$)$_2$ + ($D$)$_3$ (1:5) | | | | ($D$)$_3$ + ($D$)$_4$ (1:0.002) | | | |
| ¼ ($EC_{50}$) | | 8.75 | 37.5 | | 2.5 | 12.5 | ¼ ($EC_{50}$) | | | 12.5 | 0.025 |
| ½ ($EC_{50}$) | | 17.5 | 75 | | 5 | 25 | ½ ($EC_{50}$) | | | 25 | 0.05 |
| 1 ($EC_{50}$) | | 35 | 150 | | 10 | 50 | 1 ($EC_{50}$) | | | 50 | 0.1 |
| 2 ($EC_{50}$) | | 70 | 300 | | 20 | 100 | 2 ($EC_{50}$) | | | 100 | 0.2 |
| 4 ($EC_{50}$) | | 140 | 600 | | 40 | 200 | 4 ($EC_{50}$) | | | 200 | 0.4 |
| | Three toxicant combo | | | Three toxicant combo | | | | Four toxicant combo | | | |
| | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ (1.6:35:150) | | | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ (1:1:5) | | | | ($D$)$_1$ + ($D$)$_2$ + ($D$)$_3$ + ($D$)$_4$ (1:1:5:0.01) | | | |
| ¼ ($EC_{50}$) | 0.4 | 8.75 | 37.5 | 2.5 | 2.5 | 12.5 | 1/8 ($EC_{50}$) | 1.25 | 1.25 | 6.25 | 0.0125 |
| ½ ($EC_{50}$) | 0.8 | 17.5 | 75 | 5 | 5 | 25 | ¼ ($EC_{50}$) | 2.5 | 2.5 | 12.5 | 0.025 |
| 1 ($EC_{50}$) | 1.6 | 35 | 150 | 10 | 10 | 50 | ½ ($EC_{50}$) | 5 | 5 | 25 | 0.05 |
| 2 ($EC_{50}$) | 3.2 | 70 | 300 | 20 | 20 | 100 | 1 ($EC_{50}$) | 10 | 10 | 50 | 0.1 |
| 4 ($EC_{50}$) | 6.4 | 140 | 600 | 40 | 40 | 200 | 2 ($EC_{50}$) | 20 | 20 | 100 | 0.2 |

For the *Anabaena* test, the design for the experiment with the wastewater [WW ($D_4$)] sample is also included. The experimental design is based on $EC_{50}$ ratios as proposed by Chou and Talalay (1984). $EC_{50}$ is the effective concentration of a toxicant which caused a 50% bioluminescence inhibition. The combination ratio was approximately equal to the $EC_{50}$ ratio of the combination components (i.e., close to their equipotency ratio). *Upper maximal possible dose due to the solubility limit of fibrates in pure water. **$EC_{50}$ for wastewater is the dilution which caused 50% luminescence inhibition. ($D$)$_1$, ($D$)$_2$ and ($D$)$_3$ in mg/L, ($D$)$_4$ is the dilution of wastewater in dd$H_2O$.

These parameters were then used to calculate doses of the fibrates and their combinations required to produce various effect levels according to Eq. (1); for each effect level, combination index (CI) values were then calculated according to the general combination index equation for *n* chemical combination at *x*% inhibition (Chou, 2006):

$$^{n}(\mathrm{CI})_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{[D]_j / \sum_1^n [D]\right\}}{(D_m)_j \left\{(f_{ax})_j / \left[1-(f_{ax})_j\right]\right\}^{1/mj}} \quad (3)$$

where $^{n}(\mathrm{CI})_x$ is the combination index for *n* chemicals (e.g., fibrates) at *x*% inhibition (e.g., bioluminescence inhibition); $(D_x)_{1-n}$ is the sum of the dose of *n* chemicals that exerts *x*% inhibition in combination, $\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the dose of each of *n* chemicals that exerts *x*% inhibition in combination; and $(D_m)_j \{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts *x*% inhibition. From Eq. (3), CI <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively.

### 2.5. Analysis of results

Computer program CompuSyn (Chou and Martin, 2005, Compusyn Inc, USA) was used for calculation of dose-effect curve parameters, CI values, *fa*-CI plot (plot representing CI versus *fa*, the fraction affected by a particular dose; see Eq. (1)) and polygonograms (a polygonal graphic representation depicting synergism, additive effect and antagonism for three or more drug combinations). Linear regression analyses were computed using MINITAB Release 14 for Windows (Minitab Inc; USA).

## 3. Results

### 3.1. Toxicological interactions of fibrate combinations in Vibrio fischeri and Anabaena CPB4337 bioluminescence tests

Applying the combination index-isobologram method, we evaluated the nature of gemfibrozil (Gm), fenofibric acid (Fn) and bezafibrate (Bz) interactions both in *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests. Table 2 shows the dose-effect curve parameters (*Dm*, *m* and *r*) of the three fibrates singly and their binary and ternary combinations, as well as mean combination index (CI) values of fibrate combinations. *Dm* was the dose required to produce the median-effect (analogous to the $EC_{50}$); *Dm* values for Fn were the lowest both, in *Vibrio* and *Anabaena* tests, *Dm* values for Gm were in the same range for both *Vibrio* and *Anabaena* while Bz

**Table 2 – Dose-effect relationship parameters and mean combination index (CI) values (as a function of fractional inhibition of luminescence) of gemfibrozil (Gm), fenofibric acid (Fn), and bezafibrate (Bz) individually and of their binary and ternary combinations on *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests.**

| Drug combo | *Vibrio fischeri* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Dose-effect parameters | | | | CI values | | | | | |
| | *Dm* | | *m* | *r* | $EC_{10}$ | | $EC_{50}$ | | $EC_{90}$ | |
| | mg/L | (μM) | | | | | | | | |
| Fn | 1.45 | (4.01) | 0.78 | 0.989 | – | | – | | – | |
| Gm | 20.58 | (82.11) | 1.53 | 0.966 | – | | – | | – | |
| Bz | 252.07 | (696.46) | 1.15 | 0.975 | – | | – | | – | |
| Gm + Bz | 78.20 | (234.20) | 1.54 | 0.991 | 1.13 ± 0.13 | Add | 0.97 ± 0.04 | Add | 0.86 ± 0.05 | Syn |
| Fn + Bz | 153.79 | (424.93) | 1.09 | 0.981 | 2.98 ± 0.15 | Ant | 1.71 ± 0.03 | Ant | 1.17 ± 0.06 | Ant |
| Fn + Gm | 9.84 | (38.74) | 1.15 | 0.973 | 0.99 ± 0.17 | Add | 0.75 ± 0.05 | Syn | 0.86 ± 0.08 | Syn |
| Fn + Gm + Bz | 55.69 | (166.69) | 1.23 | 0.993 | 1.46 ± 0.06 | Ant | 1.01 ± 0.02 | Add | 0.99 ± 0.03 | Add |
| | *Anabaena* CPB4337 | | | | | | | | | |
| | Dose-effect parameters | | | | CI values | | | | | |
| | *Dm* | | *m* | *r* | $EC_{10}$ | | $EC_{50}$ | | $EC_{90}$ | |
| | mg/L | (μM) | | | | | | | | |
| Fn | 8.53 | (23.62) | 0.96 | 0.971 | – | | – | | – | |
| Gm | 10.69 | (42.67) | 0.81 | 0.959 | – | | – | | – | |
| Bz | 12.56 | (34.70) | 1.08 | 0.990 | – | | – | | – | |
| Gm + Bz | 19.17 | (56.88) | 0.84 | 0.972 | 1.06 ± 0.15 | Add | 1.57 ± 0.06 | Ant | 2.5 ± 0.22 | Ant |
| Fn + Bz | 13.92 | (38.49) | 0.76 | 0.965 | 0.55 ± 0.06 | Syn | 1.19 ± 0.04 | Ant | 2.59 ± 0.14 | Ant |
| Fn + Gm | 12.26 | (41.45) | 0.46 | 0.955 | 0.13 ± 0.02 | Syn | 1.29 ± 0.05 | Ant | 12.9 ± 2.33 | Ant |
| Fn + Gm + Bz | 6.62 | (19.45) | 0.53 | 0.960 | 0.09 ± 0.01 | Syn | 0.57 ± 0.02 | Syn | 3.92 ± 0.19 | Ant |

The parameters *m*, *Dm* and *r* are the antilog of *x*-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency ($EC_{50}$), and conformity of the data to the mass-action law, respectively (Chou, 1976; Chou and Talalay, 1984; Chou, 2006). *Dm* and *m* values are used for calculating the CI values (Eq. (3)); CI <1, =1, and >1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. $EC_{10}$, $EC_{50}$ and $EC_{90}$, are the doses required to inhibit bioluminescence 10, 50 and 90%, respectively. Computer software CompuSyn was used for automated calculation and simulation.

*Dm* values were an order of magnitude higher in the *Vibrio* test (Rosal et al., 2009); *m* was the Hill coefficient used to determine the shape of the dose-response curve, hyperbolic ($m = 1$), sigmoidal ($m > 1$) or negative sigmoidal ($m < 1$); also shown in the table, linear regression correlation coefficients (*r*-values) of the median-effect plots were >0.95 in all cases, indicating the conformity of the data to the median-effect principle which qualifies for further studies using this method.

The *Dm* and *m* values for single fibrates and for their combination mixtures were used for calculating synergism or antagonism based on the CI Eq. (3) (Chou, 2006). Fig. 1 shows the *fa*-CI plot of fibrate interactions both for *Vibrio* (Fig. 1a) and *Anabaena* tests (Fig. 1b); the *fa*-CI plot depicts the CI value versus *fa* (effect level or fraction of luminescence inhibited by a fibrate singly or in combination with respect to the control) for two (Fn + Bz; Fn + Gm and Bz + Gm) and three fibrate (Fn + Gm + Bz) combinations. The *fa*-CI plot is an effect-oriented plot that shows the evolution of the kind of interaction (synergism, antagonism, additive effect) as a function of the level of the effect (*fa*) of a particular toxicant on the reference organism (*fa*, where $EC_a = fa \times 100$; i.e., $EC_{10} = f10 \times 100$). In the *Vibrio* test (Fig. 1a), the Bz + Gm and Fn + Gm binary combination showed a slight antagonism at very low *fa* values and slight synergism (Fn + Gm) or nearly additive effects (Bz + Gm) at the highest *fa* values, the Fn + Bz combination showed a strong antagonism at low effect levels but the antagonism decreased and approached an additive kind of interaction at the highest *fa* levels; the ternary combination (Fn + Gm + Bz) showed a moderate antagonism at low *fa* values that also turned into a nearly additive effect at *fa* values above 0.4. Correlation analyses were made between CI values of the fibrate ternary combination and CI values of each of the fibrate binary combinations to determine which binary combination interaction was predominant in the ternary mixture (Table 3); the highest correlation coefficient was found for the Fn + Bz combination ($r = 0.91$), suggesting that this combination interaction predominated in the three fibrate mixture. The *fa*-CI plot of the *Anabaena* test (Fig. 1b) showed the opposite pattern of interactions as the three binary and the ternary combinations showed from slight to strong synergism at the lowest *fa* values that turned into a very strong antagonism at *fa* values over 0.5; the ternary combination (Fn + Gm + Bz) closely followed the interaction pattern of the binary Fn + Gm combination, this is confirmed by the highest correlation coefficient found between the CI values of the ternary combination and the CI values of the Fn + Gm combination ($r = 0.996$) which suggests that in the *Anabaena* test, this particular combination seemed to be the predominant in the ternary toxicological interaction. Selected average CI values for both *Vibrio fischeri* and *Anabaena* CPB4337 tests at three representative dose levels ($EC_{10}$, $EC_{50}$ and $EC_{90}$) and the combined effects are summarized in Table 2.

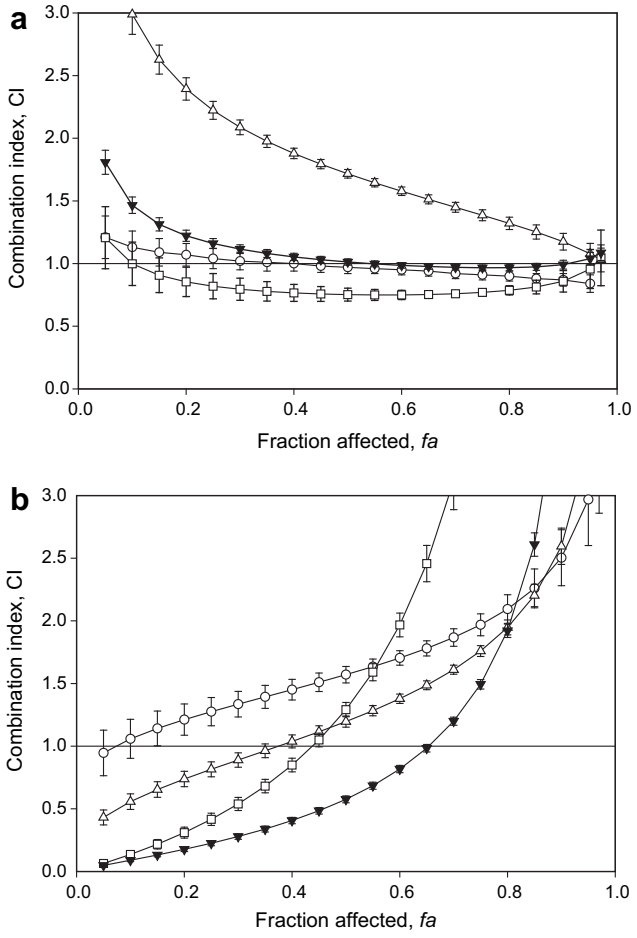


**Fig. 1 – Combination index plot (*fa*-CI plot) for a set of three fibrate combinations: Fn + Bz (–△–), Bz + Gm (–○–), Fn + Gm (–□–) and Fn + Gm + Bz (–▼–) for *Vibrio fischeri* test (a) and *Anabaena* CPB4337 test (b). CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa* = 0.10 to 0.95. CI <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively. At least three independent experiments with two replicates were used. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (SDA) (Chou and Martin, 2005). Fn = fenofibric acid, Bz = bezafibrate and Gm = gemfibrozil.**

### 3.2. *Toxicological interactions of wastewater and fibrate combinations in the* Anabaena *CPB4337 bioluminescence test*

In a recent previous study (Rosal et al., 2009), we found that a wastewater sample collected from a local STP was very toxic to *Anabaena* cells with a wastewater dilution of 0.11 causing 50% luminescence inhibition (wastewater $EC_{50}$). The observed toxicity was attributed to the combined toxicities of over thirty micropollutants, which included fibrates as well as other pharmaceuticals (Rosal et al., 2008). We sought to investigate the nature of the interaction between the wastewater (WW) and the three fibrates in binary (Fn + WW; Bz + WW and Gm + WW) and quaternary (Fn + Gm + Bz + WW) combinations; for these experiments, the wastewater itself was regarded as a toxicant; the experimental design was analogous to the one for the three fibrate interactions and is also shown in Table 1. The *r*-values of the median-effect plots were >0.95 in all cases, indicating that the data conformed to the median-effect principle (not shown). Fig. 2 shows the *fa*-CI plot for each

**Table 3 – Correlation analyses between CI values of fibrate ternary and fibrate + wastewater quaternary combinations (*y*) and their binary combinations (*x*) for *Vibrio fischeri* and *Anabaena* CPB4337 tests.**

| Test organism | Combinations | | | Regression parameters | | |
|---|---|---|---|---|---|---|
| | | | | $x_o$ | *m* | *r* |
| *V. fischeri* | Fn + Gm + Bz | versus | Gm + Bz | −0.614 | 1.77 | 0.83 |
| | | | Fn + Bz | 0.594 | 0.281 | 0.91 |
| | | | Fn + Gm | −0.067 | 1.40 | 0.81 |
| *Anabaena* CPB4337 | Fn + Gm + Bz | versus | Gm + Bz | −5.876 | 4.39 | 0.91 |
| | | | Fn + Bz | −2.716 | −3.00 | −0.941 |
| | | | Fn + Gm | 0.282 | 0.247 | 0.996 |
| | WW + Fn + Gm + Bz | versus | Gm + Bz | −0.079 | 0.372 | 0.999 |
| | | | Fn + Bz | 0.199 | 0.246 | 0.998 |
| | | | Fn + Gm | 0.464 | 0.017 | 0.897 |
| | | | Fn + WW | −0.253 | 1.31 | 0.999 |
| | | | Gm + WW | 2.131 | −2.41 | −0.89 |
| | | | Bz + WW | 0.003 | 0.865 | 0.999 |

Fn = fenofibric acid, Bz = bezafibrate, Gm = gemfibrozil, WW = wastewater. The parameters of linear regression equations: $x_0$ (value of *y* when *x* = 0); *m* (slope) and *r* (correlation coefficient) with all *p*-values of 0.001. Analyses were computed using MINITAB Release 14 for Windows.

of the binary fibrate-wastewater combination and the quaternary combination; as can be observed, in a broad range of *fa* values, the binary combinations showed a strong synergism; however, at *fa* values above 0.8, the binary Fn + WW and Bz + WW combinations approached an additive effect and at *fa* values above 0.95, these two combinations yielded antagonism; by contrast, the Gm + WW combination became even more synergistic. The quaternary combination interaction showed a strong synergism through a broad range of *fa* values but also turned into slight antagonism at *fa* values above 0.95,

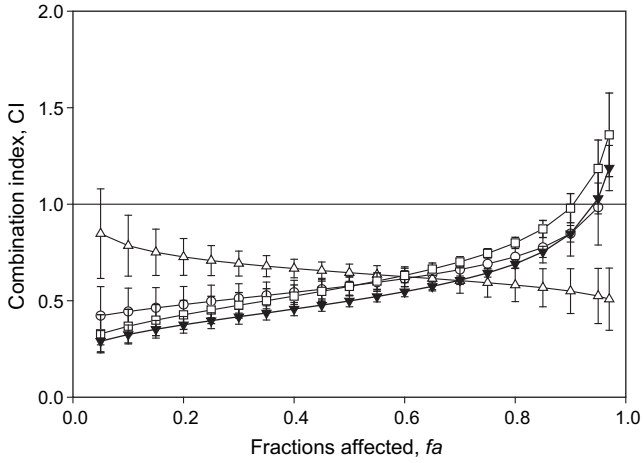


**Fig. 2 – Combination index plot (*fa*-CI plot) for a set of three fibrates and toxic wastewater sample in their binary and quaternary combinations: Gn + WW (–△–), Fn + WW (–○–), Bz + WW (–□–) and Fn + Gm + Bz + WW (–▼–) for the *Anabaena* CPB4337 test. CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa* = 0.10 to 0.95. CI < 1, =1 and > 1 indicates synergism, additive effect and antagonism, respectively. At least three independent experiments with two replicates were used. The vertical bars indicate 95% confidence intervals for CI values based on sequential deletion analysis (SDA) (Chou and Martin, 2005). Fn = fenofibric acid, Bz = bezafibrate, Gm = gemfibrozil, WW = wastewater.**

closely resembling the pattern of the Fn + WW and Bz + WW interactions which is confirmed by the highest *r* value (*r* = 0.999) in the correlation analyses (Table 3), which suggests a predominant effect of Fn and Bz in the quaternary interaction.

The computer software CompuSyn (Chou and Martin, 2005) displays a type of graphic termed polygonogram, which is a semiquantitative method of representing interactions between three or more compounds at a determined *fa* value. This graphic allows a simplified visual presentation of the overall results. Fig. 3 shows the polygonogram for the three fibrates and the wastewater at four *fa* values; synergism is indicated by solid lines and antagonism by broken ones; the thickness of the lines indicates the strength of the interaction. The polygonogram clearly shows the synergistic interaction of wastewater in combination with each of the three fibrates at low *fa* values and the antagonistic interaction that appeared at the highest *fa* value, 0.99, for the Fn + WW and the Bz + WW combinations.

The same wastewater sample collected from a local STP was proved as responsible of stimulation of the bioluminescence activity of *Vibrio fischeri* to 110–120% of that of the control. Moreover, the $EC_{50}$ values for the fibrates in the wastewater were higher than those for fibrates in pure water (Rosal et al., 2009). The same trend was observed comparing the dose-effect curve parameters (*Dm*, *m* and *r*) for the ternary combination (Fn + Gm + Bz) of fibrates in $ddH_2O$ and wastewater. The dose required to produce the median-effect (*Dm*) in *Vibrio fischeri* test when (Fn + Gm + Bz) were solved in wastewater was 131.936 compared to 55.6951 mg/L required when $ddH_2O$ was employed. CI values could not be calculated for *Vibrio fischeri* due to the fact that the wastewater itself was not toxic to this bacterium; synergism or antagonism could not be properly estimated (Chou, 2006).

## 4. Discussion

The three fibrates that we have used in our study are lipid modifying agents that are effective in lowering elevated

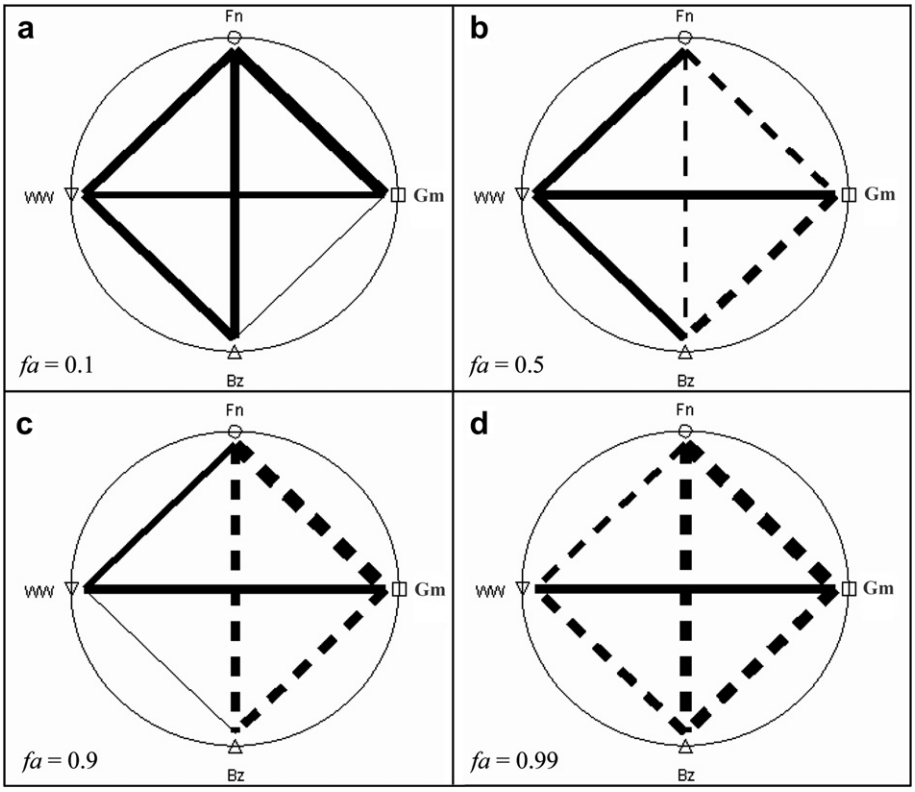


**Fig. 3 – Polygonograms showing the toxicological interactions of three fibrates and a toxic wastewater sample in their binary combinations (Fn + Bz, Bz + Gm, Fn + Gm, Gm + WW, Fn + WW, Bz + WW) as calculated by CompuSyn (Chou and Martin, 2005) for *Anabaena* CPB4337 test at four effect levels: *fa* = 0.1 (a), *fa* = 0.5 (b), *fa* = 0.9 (c) and *fa* = 0.99 (d). Solid lines indicate synergism, broken lines indicate antagonism. The thickness of the line represents the strength of synergism or antagonism. Figure generated by CompuSyn (Chou and Martin, 2005).**

plasma triglycerides and cholesterol in humans (Staels et al., 1998). These pharmaceuticals are highly used, ubiquitous and persistent (Daughton and Ternes, 1999), they are found at ng/L to μg/L levels in many STP effluents, surface waters, estuaries of rivers and even in sea water (for a review, see Hernando et al., 2007). Although non-target organisms; the continuous release of these substances into the environment may cause acute or chronic toxicity to the aquatic biota. Regarding fibrates, in the recent literature there are many reports dealing with individual toxicity of different fibrates in a range of aquatic organisms from primary producers to consumers; a great variability has been found in the sensitivity of the different test organisms toward these pharmaceuticals (Hernando et al., 2007). However, pharmaceuticals such as fibrates do not occur singly in a polluted environment and are usually found as mixtures, therefore, for risk assessment strategies it is important to know the combined effects of pharmaceuticals in non-target organisms (Teuschler, 2007).

There are two concepts widely used for the prediction of mixture toxicity: concentration addition (CA) and independent action (IA) (Backhaus et al., 2003; Vighi et al., 2003; Backhaus et al., 2004; Junghans et al., 2006). CA is used for mixtures whose components act in a similar mode of action while IA is based on the idea of dissimilar action, meaning that the compounds have different mechanisms of action; however, as discussed by Cleuvers (2003) the terms similar/dissimilar action may be misleading. Pharmaceuticals such as fibrates may have the same pharmacological mechanism of action [i.e., interaction with the binding peroxisome proliferator-activated receptor α (PPARα)] in their target organism, humans; however, if fibrates released in the aquatic environments prove toxic to different non-target organisms, the exact mechanism of toxicity (probably different to the pharmacological mode of action) should be investigated in depth before choosing which approach, CA or IA, to use. In fact, only if toxicity is regarded as non-specific at all, the concept of CA may be used although it may also have limitations. Cleuvers (2003) found that two totally different pharmaceuticals, a fibrate and an anti-epileptic drug, followed the concept of CA in the *Daphnia* toxicity test and the concept of IA in an algal test; both pharmaceuticals apparently shared the same non-specific toxic mode of action for both organisms; so it appeared that the concept of CA or IA did not depend on a similar/dissimilar mode of action but on the tested organism. The author also discussed that by definition, when using CA, substances applied below their individual non-effect concentration (NOEC) will contribute to the total effect of the mixture while when using IA, substances applied below their NOEC will not contribute to the total effect of the mixture, meaning that any combination effect will probably be higher if the substances follow the concept of CA and this may be misleading when considering the terms synergism or

antagonism because as also discussed by Chou (2006), synergism or antagonism may occur independently of a similar or dissimilar mode of action. In this context, Fent et al. (2006) tested mixtures of different kinds of pharmaceuticals (including fibrates) that might have estrogenic activity in a yeast reporter system; they applied the CA model and found that it had severe limitations when the dose-response curves of the individual pharmaceuticals were not identical or at low effect concentrations. As pharmaceuticals released in the environment may have such diverse dose-effect relationships, the lack of appropriate prediction suggests limitation of the CA mixtures concept.

To study the nature of the combined fibrate interactions (synergism, additive effect, antagonism) for the *Vibrio fischeri* and *Anabaena* CPB4337 bioluminescence tests, we have followed the combination index (CI)-isobologram equation method of Chou (2006) and Chou and Talalay (1984); a method widely used to study drug interactions in pharmacology. This method may be considered a fractional analysis technique for drug interactions (Berenbaum, 1981; Bovill, 1998) that is independent of the mode of action and considers both the potency ($EC_{50}$, *Dm*) and the shape (*m*) of the dose-effect curve for each drug. The method allows prediction of synergism/antagonism at all effect levels (*fa*) for a combination of *n* drugs; in contrast with the classical graphical isobologram method (Berenbaum, 1981; Bovill, 1998) that cannot be used for more than three compounds and have also graphical limitations to show all effect levels. By using this method, we have been able to determine the nature of interactions for a wide range of effect levels of three fibrates in binary and ternary combinations in two different bioluminescent organisms. However, the nature of these interactions was not uniform along the *fa* levels range in any of the two organisms. In *Vibrio fischeri*, antagonism predominated at low and intermediate *fa* levels but at the highest effect levels, interactions became additive or slightly synergistic. In *Anabaena*, a dual synergistic/antagonistic behaviour was observed with synergism predominating at *fa* levels below 0.4–0.5 and strong antagonism above these *fa* values. It is difficult to give an explanation to this phenomenon because the combination index method only allows quantitative determination of synergism or antagonism and the elucidation of the mechanism by which synergism or antagonism occurs is a separate issue that needs a different kind of approach. However, tentatively, antagonism, which could be considered the predominant interaction in *Vibrio fischeri* and *Anabaena*, might be explained by the structural similarity of fibrates which are related pharmaceuticals that share a common structural motif, a cyclic head and a hydrophobic tail (Rosal et al., 2009); at the *fa* levels where antagonism is found in both organisms, fibrates may compete with one another for the same target/receptor sites. The slight synergism found at very high levels in *Vibrio fischeri* could perhaps be explained by the fact that at very high concentrations, fibrates may somehow combine to increase toxicity by an unspecific way of action that is probably not related to their pharmacological mechanism. Perhaps, the most puzzling interaction is the observed high synergism at very low *fa* levels in *Anabaena*; the mechanism of such synergistic interaction is not readily apparent. One could speculate that these fibrates at very low concentrations could involve what Jia et al. (2009) in their extensive review of mechanisms of drug combinations call ''facilitating actions'' that means that secondary actions of one drug enhances the activity or level of another drug in the mixture or alternatively ''complementary actions'' when drugs act at the same target at different sites, at overlapping sites or at different targets of the same pathway. However, in the literature there are very few reports on possible targets of fibrates on the prokaryotic cell; English et al. (1994) reported that peroxisome proliferators such as fibrates have been shown to induce cytochrome $P450_{BM\text{-}3}$ which catalyzes the hydroxylation of fatty acids, in *Bacillus megaterium*. Garbe (2004) reported that fibrates induced methyltransferase Rv0560c with a function in the biosynthesis of isoprenoid compounds in *Mycobacterium tuberculosis*; Garbe (2004) suggested that both effects may act on the plasma membrane, modulating its properties. In mitochondria, which have significant features that resemble those of prokaryotes, fibrates have been found to inhibit respiratory complex I (NDH-1 complex) and to interfere with mitochondrial fatty acid oxidation (Scatena et al., 2007). Whether fibrates may exert similar effects in *Vibrio fischeri* and *Anabaena* to those observed in *Bacillus* or mitochondria needs further research. In this context, we have found that, as the *fa*-CI plots show, fibrate interactions do not follow the same pattern in both bacteria, this may be due to the different origin and position in the food web of *Vibrio fischeri*, a heterotrophic marine prokaryote and *Anabaena* CPB4337, a recombinant strain of an obligate phototrophic freshwater prokaryote; in fact, *Anabaena* presents intracellular photosynthetic membranes called thylakoids where several functionally distinct NDH-1 complexes have been found with roles both in respiration and photosynthesis (Battchikova and Aro, 2007). If fibrates are also affecting NDH-1 complexes in *Anabaena*, their effects might be very different to those in *Vibrio fischeri*; so, although we have measured the same toxicity endpoint in both bacteria, i.e., luminescence inhibition, the combined effects of fibrates seem to depend on the test organism.

Ince et al. (1999) assessed toxic interactions of heavy metals in binary mixtures on *Vibrio fischeri* and the freshwater aquatic plant *Lemna minor* and found that most binary metal mixtures exhibited only antagonistic interactions in the plant opposed to fewer antagonistic and some synergistic interactions in the heterotrophic bacterium. These authors also found that in the bacterium, the nature of the interaction (synergism or antagonism) also changed with the effect level of the binary metal combinations, although the authors did not provide a mechanistic explanation for this variability. Cheng and Lu (2002) made a comparison of joint interactions of organic toxicants in binary mixtures in *Escherichia coli* and *Vibrio fischeri* and found that toxicants with the same mechanisms of toxicity displayed mostly additive or antagonistic interactions in *E. coli* and *Vibrio fischeri*; however a synergistic interaction was found between glutardialdehyde and butyraldehyde in *Vibrio*. Synergistic effects in both bacteria were mostly associated with toxicants with different mechanisms of toxicity, although antagonism clearly predominated. They also found that for a total of 44 organic binary mixtures, only six mixtures resulted in identical type of interaction in both bacteria. From our results and those of other authors' (Ince et al., 1999; Cheng and Lu, 2002; Cleuvers, 2003) one may

conclude that previous knowledge of the mechanism of toxic action of a compound is not useful enough to predict which kind of interactions it will display when combined with other toxicants with the same or different toxic mechanism; also, as we have shown, the nature of the interaction may depend on the effect level of the mixture. In addition, different types of organisms will show completely different responses to mixtures of potential toxicants.

We previously found that a local wastewater was very toxic for the *Anabaena* CPB4337 test but non-toxic at all for the *Vibrio fischeri* or *Daphnia magna* tests. This wastewater is a mixture of over thirty micropollutants, mostly pharmaceuticals of different therapeutics groups that, besides the fibrates used in this study, included antibiotics, analgesics/anti-inflammatories, β-blockers, antidepressants, anti-epileptics/psychiatrics, ulcer healing compounds, diuretics and bronchodilators; personal care products and some priority organic pollutants are also present (Rosal et al., 2008). The method of Chou allows to combine one drug mixture with another drug mixture and determine their interactions; therefore, we studied the nature of the interaction of fibrates and wastewater in the *Anabaena* bioluminescence test; interestingly, we found that in a wide range of effect levels, the interaction of wastewater and the three fibrate combination was synergistic; particularly, at very low *fa* values which means that fibrates are at low concentrations and the wastewater is diluted several-fold, the method predicted a strong synergism; this may be due, as discussed above, to the observed synergistic interactions of fibrates with one another as well as interactions with some of the detected micropollutants when present at very low concentrations. This observed synergism may be environmentally relevant since most pharmaceuticals such as fibrates do not usually show acute toxicity on non-target organisms when tested at real environmental concentrations (Hernando et al., 2007) but in a mixture, if they act synergistically, they could prove toxic for a test organism even at low concentrations; these results agree with those found by Hernando et al. (2004) who reported synergistic toxic effects for *Daphnia magna* test when wastewater was spiked with environmental concentrations of several pharmaceuticals including fibrates. By contrast, our results show that at high *fa* values ($fa > 0.8$), the combined interaction of the quaternary fibrates + wastewater combination, the binary Fn + WW and Bz + WW combinations approached an additive effect and eventually became antagonistic; in our previous study, the wastewater itself decreased *Anabaena* bioluminescence by 84% with a lower confidence limit of 76% and an upper confidence limit of 91%; when the wastewater was spiked with increasing concentrations of each fibrate we found that, with the exception of gemfibrozil, the $EC_{50}$ values for the fibrates in the wastewater were higher than those for fibrates in pure water; this was attributed either to reduced bioavailability or to antagonistic effects of fibrates with other chemicals present in the wastewater; although we did not use the method of Chou, we obtained similar results to the ones we report in this study; that is, at high effect levels (>84% luminescence inhibition) the interaction of fibrates with wastewater, except the Gm + WW combination, showed antagonism.

Based on our results, we propose that the combination index (CI)-isobologram equation, a method widely used in pharmacology both for *in vitro* and *in vivo* bioassays, may also be applied in environmental toxicology as a general method to define interactions of potential toxicants in mixtures in any test organism and/or toxicological endpoint of interest and could be especially useful for risk assessment strategies that take into account the toxicological interactions of substances in a mixture.

## 5. Conclusions

We report an environmental application of the combination index (CI)-isobologram equation to study the nature of the interactions of fibrate combinations in two bioluminescent aquatic organisms. The method allowed calculating synergism or antagonism of binary and ternary fibrate combinations at all effect levels simultaneously; we could also test the method with a real wastewater sample in binary and quaternary combination with the fibrates, finding that at very low effect levels, the fibrates acted synergistically with the wastewater in the *Anabaena* test. The proposed method may be used with other test organisms and/or toxicological endpoints and could be particularly useful for risk assessment approaches to toxicity of complex mixtures.

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# CHAPTER IV

## Eco-toxicity of emerging pollutants II. Interaction of perfluorinated surfactants with priority and emerging pollutants.

# ECOTOXICOLOGICAL ASSESSMENT OF SURFACTANTS IN THE AQUATIC ENVIRONMENT: COMBINED TOXICITY OF DOCUSATE SODIUM WITH CHLORINATED POLLUTANTS

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# Ecotoxicological assessment of surfactants in the aquatic environment: Combined toxicity of docusate sodium with chlorinated pollutants

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ABSTRACT

The toxicity of perfluorinated surfactants perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorobutane sulfonate (PFBS) and PF-656 as well as the sulfosuccinate surfactant docusate sodium has been examined using two bioluminescence inhibition assays based on the marine bacterium *Vibrio fischeri* and the self-luminescent cyanobacterial recombinant strain *Anabaena* CPB4337. We also determined multigenerational toxicity towards the growth of the algae *Pseudokirchneriella subcapitata*. With $EC_{50}$ values in the 43–75 mg/L range, docusate sodium exhibited a higher toxicity towards the three organisms than PFOS, PFOA, PF-656 and PFBS. We investigated the toxicological interactions of the most toxic surfactant, docusate sodium, with two chlorinated compounds, triclosan and 2,4,6-trichlorophenol (TCP), in their binary and ternary mixtures using the method of the combination index based on the median-effect equation. In general, the binary mixture of the chlorinated compounds triclosan and TCP exhibited antagonism, which was stronger for the growth test using *P. subcapitata*. Except for the green alga, the binary mixtures of docusate sodium with TCP or triclosan showed synergism at medium to high effect levels; the synergistic behaviour predominating in the ternary mixture and in the three tested species. This result highlights the potential toxicological risk associated with the co-occurrence of this surfactant with other pollutants.



## 1. Introduction

The dissemination of anthropogenic pollutants in the aquatic environment takes place either by point-sources associated to the local discharges or from a large variety of activities, the main point-source being the effluents of sewage treatment plants. Xenobiotics are a source of concern not only due to their specific physical and chemical properties, but because they are released in large and increasing quantities and in complex mixtures whose properties are largely unknown. Surfactants are synthetic chemicals used in large amounts in varieties of industrial cleansing processes as well as in consumer products. Spent surfactants, either from domestic or industrial use, reach biological treatment units and, eventually, are discharged to the environment.

Perfluorinated surfactants such as perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and their salts find use in formulating paints or cleaning agents as well as in the production of water impermeable products. The environmental concern about these compounds is due to the fact that they are persistent and bioaccumulative. PFOS has been banned in Europe by the Directive 2006/122/EC and has recently been added to Annex B of the Stockholm Convention on Persistent Organic Pollutants. It has been suggested that PFOA can be generated from certain precursors during biological wastewater treatment (Murakami et al., 2009). The potential substitutes to replace PFOS and PFOA are still mainly perfluoroalkyl based surfactants due to the polarity properties given by the carbon–fluorine bond. 3 M Company introduced in 2003 the shorter-chain compound perfluorobutane sulfonate (PFBS) under the trade name 3 M's Novec™. PolyFox PF-656 is a fluorinated and hydroxylated polyether produced by Omnova Solutions Inc. Several companies market products based on sulfosuccinate derivatives which can be an alternative to fluorinated surfactants. Docusate sodium, bis(2-ethylhexyl) sodium sulfosuccinate, is an anionic surfactant, potentially bioaccumulative and widely used in pharmaceutical formulations. Perfluorinated surfactants have been detected in the effluent of wastewater treatment plants at levels of hundreds of nanograms per liter (Loganathan et al., 2007; Guo et al., 2010). In surface water they appear in highly populated and industrialized areas such as Yangtze River for which Jin et al. (2009) reported a median concentration of 4.2 ng/L for PFOS and 5.4 ng/L for PFOA with peaks as high as 298 ng/L (PFOA). In drinking water they have also been frequently reported. Ericson

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et al. (2009) found up to 58.1 ng/L (PFOS), 57.4 ng/L (PFOA) and 69.4 ng/L (PFBS) in municipal drinking water from Catalonia (Spain). The toxicity of PFBS, PF-656 and docusate sodium to aquatic organisms has been seldom reported with no data for aquatic microorganisms prior to this work except a value of 36 mg/L of docusate sodium for a 48 h *Daphnia magna* test attributed to CYTEC Industries and included in the IUCLID Dataset (Carlsson et al., 2006) and a report from NICNAS (2005) indicating for PFBS a $EC_{50}$ value of 5733 mg/L for 96 h algal growth inhibition.

Perfluorinated/sulfosuccinate surfactants may interact with other xenobiotics with an additional cause for concern due to their ability to solubilize non-polar compounds (Haigh, 1996). There are very few studies of the toxicological interactions of these surfactants with other organic compounds and those reported deal mainly with PFOS (Liu et al., 2008). Chlorinated organic pollutants have been a subject of extensive research, many of them having been banned in different regulatory schemes. Of particular concern are chlorophenols such as 2,4,6-trichlorophenol (TCP) which can originate in the disinfection of water with chlorine or chlorinated compounds (Correa et al., 2003). Triclosan, 5-chloro-2-(2,4-dichlorophenoxy)-phenol, is an emergent pollutant widely used in consumer and professional health care products as disinfecting agent. It has been repeatedly reported in natural water and wastewater from the early detection of 50–150 ng/L of Okumura and Nishikawa (1996) to the recent work of Rosal et al. (2010a) who measured an average concentration of 219 ng/L in the effluent of an activated sludge sewage treatment plant.

The objective of this study was to evaluate the individual toxicity of the perfluorinated surfactants PFOS, PFOA, PFBS, PF-656 and docusate sodium towards three aquatic organisms. In addition, we aimed to assess the toxicological interaction of the most toxic of the surfactants, docusate sodium, with two environmentally relevant chlorinated pollutants, TCP and triclosan. For it, we used the method of the combination index (CI)-isobologram equation; a method that we have previously used to assess the nature of interactions of lipid regulators in non-target organisms (Rodea-Palomares et al., 2010).

## 2. Material and methods

### 2.1. Materials

Perfluorooctane sulfonate (PFOS) potassium salt (98%) was purchased from Fluka. PFOA (96%), docusate sodium (98%) triclosan (>97%) and TCP (98%), were obtained from Sigma–Aldrich. PFBS (98.2%) and Polyfox 656 (PF-656) were kindly provided by the 3 M Company and Omnova respectively. We avoided the use of solvents and for the cases in which we reached the solubility limit at the pH of the bioassay this value has been stated as lower boundary.

### 2.2. Toxicity bioassays

The chronic toxicity was determined following the algal growth inhibition test following OECD TG 201 *Pseudokirchneriella subcapitata* open system using 96-well microplates in which the algae was cultured in a total volume of 200 μL. The results showed that nominal and measured exposure concentrations did not show significant deviations Bioassays with the photo-luminescent bacteria *Vibrio fischeri* were performed according to ISO 11348-3 standard protocol (International Organization for Standardization, 2007). This bioassay measures the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The incubation period used in this work was 15 min in all cases. The bacterial assay used the commercially available Biofix Lumi test (Macherey–Nagel, Germany) in which the bacterial reagent is supplied freeze-dried (*V. fischeri* NRRL-B 11177), reconstituted and incubated at 3 °C for 5 min before use. The analysis media was 0.34 M NaCl (2% w/v) and tests were performed at 18 °C and the measurements of light were made using a microplate luminometer. The bioassays using the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 were based on the inhibition of constitutive luminescence caused by the presence of any toxic substance (Rodea-Palomares et al., 2009). *Anabaena* CPB4337 was routinely grown at 28 °C in the light, ca. 65 mmol photons $m^2$ $s^{-1}$ on a rotary shaker in 50 mL AA/8 supplemented with nitrate (5 mM) in 125 ml Erlenmeyer flasks and 10 mg/mL of neomycin sulphate (Nm). Details are given elsewhere (Rodea-Palomares et al., 2010). The stability of target compounds under chronic bioassay conditions was assessed according to OCDE Guidance (OECD, 2008). In this work, analyses have been performed at the start and at the end of tests lasting 72 h (*P. subcapitata*) for the compounds studied in mixtures. The test has been carried out for the higher concentration and for a concentration near $EC_{50}$ for each compound using an HPLC-Diode Array Liquid Chromatograph as indicated elsewhere. The stability of chemicals in short acute assays was not examined in view of results published elsewhere (Rosal et al., 2010b).

### 2.3. Median effect and combination index (CI) equations for determining individual and combined toxicities

The response to toxic exposure in the three microorganisms was estimated using the median-effect equation based on the mass-action law as derived by Chou and Talalay (1984):

$$\frac{f_a}{1-f_a} = \left(\frac{D}{EC_{50}}\right)^m \quad (1)$$

where $f_a$ represents the fraction of the population/system affected by a certain dose, $D$, expressed as concentration of toxicant. $EC_{50}$ is the median effect–dose or the concentration required to inhibit or affect a system by 50% (e.g., 50% inhibition of bioluminescence or growth). The power, $m$, identifies the shape of the dose–effect relationship curve, that is hyperbolic, sigmoidal and negative sigmoidal if $m = 1$, $m > 1$, and $m < 1$ respectively (Chou, 2006).

The quantification of synergism or antagonism for a combination of a set of $n$ substances (i.e., sodium docusate, triclosan and TCP) is given by a combination index, CI:

$$^n(\mathrm{CI})_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\frac{D_j}{\sum_1^n [D]}}{(D_m)_j\left[\frac{(f_{ax})_j}{1-(f_{ax})_j}\right]^{1/mj}} \quad (2)$$

where $^n(\mathrm{CI})_x$ is the combination index for $n$ chemicals at $x$% inhibition (e.g., bioluminescence/growth inhibition); $(D_x)_{1-n}$ is the sum of the dose of $n$ chemicals that exerts $x$% inhibition in combination, $D_j/\sum_1^n [D]$ is the proportionality of the dose of each of $n$ chemicals that exerts $x$% inhibition in combination; and $(D_m)_j$ $\{(f_{ax})_j/[1-(f_{ax})_j]^{1/mj}$ is the dose of each drug alone that exerts $x$% inhibition. From Eq. (2), CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, respectively.

Combination index for different $f_a$ values can be determined from the preceding equations together with the experimental data from toxicant mixtures (Chou, 2006). The experimental design for sodium docusate-chlorinated compounds combinations was carried out at a fixed constant ratio (1:1) based on the individual $EC_{50}$ values with five levels using a serial dilution factor of 2. Individual compounds and all combinations plus a control were tested in at least three independent experiments with replicate samples performed simultaneously.

## 3. Results and discussion

### 3.1. Toxicity of individual compounds

Table 1 lists the results of toxicity tests for the surfactants and chlorinated compounds tested in this work together with their 95% confidence intervals. Confidence intervals were determined using a linear interpolation method that did not assume any particular dose–effect model (USEPA, 2002). PFOS could be classified as harmful to *P. subcapitata* (10 mg/L < $EC_{50}$ < 100 mg/L), $EC_{50}$ values were considerably larger for *Anabaena* CPB4337 and, particularly, for *V. fischeri*. Concerning literature data for PFOS, Boudreau et al. (2003) obtained $EC_{50}$ values for the 96 h growth inhibition test on *P. subcapitata* of 78.2 mg/L (cell density) and 59.2 mg/L (chlorophyll A), not very different to those reported here although obtained for a different growth time. The toxicity of PFOA was also low, with the lower $EC_{50}$ for *Anabaena* CPB4337. Mulkiewicz et al. (2007) measured a $EC_{50}$ of 571.6 ± 57.5 mg/L for *V. fischeri*, in agreement with our value 524 (505–538) mg/L. No previous data on PFOS and PFOA toxicity is available for cyanobacteria. Literature data for technical surfactants are scarce. A report from NICNAS (2005) indicated for PFBS $EC_{50}$ values of 5733 mg/L (96 h growth inhibition) and 5733 mg/L (96 h biomass), both for *P. subcapitata*. In our work, we found a much lower toxicity, with only a 37% growth inhibition at 20250 mg/L. For the other microorganisms, the toxicity of PFBS was also very low. For the case of PF-656, luminescence inhibition of *V. fischeri* and *Anabaena* CPB4337 was very low for concentrations <100 mg/L, but the inhibition of algal growth reached 50% at 43.4 mg/L; so, PF-656 could also be classified as harmful to the green alga. The data showed that the sulfosuccinate docusate sodium exhibited a considerable acute toxicity for all three organisms and could also be classified as "harmful to aquatic organisms"; this is the first report of docusate sodium toxicity to aquatic organisms as no toxicity values have been reported in the scientific literature except for the value of 36 mg/L for *D. magna* (Carlsson et al., 2006).

The data for TCP and triclosan are in agreement with previously reported values for *V. fischeri* (Hoffmann et al., 2003), being highly toxic towards *P. subcapitata* and *Anabaena* CPB4337. Concerning triclosan we also obtained high toxicity in line with other values reported for *P. subcapitata* and *V. fischeri* (Orvos et al., 2002; Tatarazako et al., 2004; de Lorenzo et al., 2008). All toxicants fitted well to the median-effect equation except docusate sodium in *V. fischeri* that clearly deviated from the common sigmoidal or hyperbolic monotonic shape exhibiting a biphasic dose–response curve (Fig. 1). The logarithmic form of Eq. (1) shows a broken line with a turning point at a dose of about 55 mg/L. For the computation of CI (Eq. (2)), we used as reference for docusate sodium acting individually the biphasic response as shown in the inset of Fig. 1. We also determined that the inflection is not related to critical micelle concentration, that appears somewhat below 200 mg/L in the presence of 2% NaCl in solution (*V. fischeri* medium).

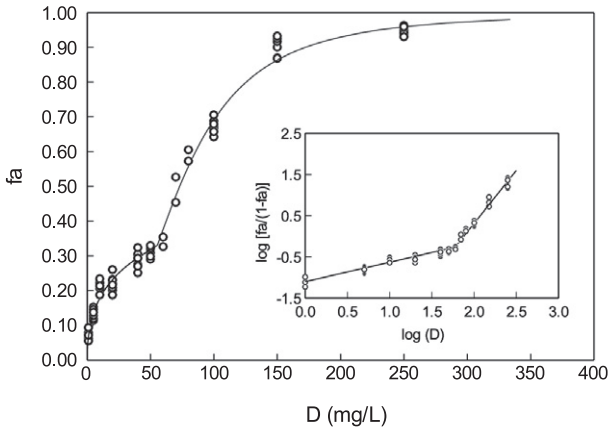


**Fig. 1.** Dose–effect curve and logarithmic median-effect plot for docusate sodium in *Vibrio fischeri* ecotoxicity test (15 min).

### 3.2. Toxicity of binary and ternary mixtures

From the tested surfactants, the sulfosuccinate surfactant docusate sodium proved to be the most toxic to the three organisms; we used the combination index method to determine the nature of its interaction with TCP and triclosan that were also toxic or very toxic for the three microorganisms tested in this work. Table 2 shows the dose–effect curve parameters from Eq. (1), namely, $EC_{50}$, $m$ and the linear correlation coefficient corresponding to the data in logarithmic form, $r$ (Chou, 2006). These values are listed together with the combination index (CI) for all combinations and three degrees of effect levels ($EC_{10}$, $EC_{50}$ and $EC_{90}$). The linear correlation coefficient was >0.97 in all cases indicating the conformity of the data to the median-effect principle. The data from single and combined toxicants was used to quantify synergism or antagonism by applying sequential deletion analysis as indicated elsewhere (Chou and Martin, 2005). The response for the three microorganisms are represented in Figs. 2–4 as a function of the fraction affected from $f_a$ = 0.05 to 0.95 with explicit indication of their 95% confidence intervals as error bars. Some representative results for every mixture are also shown in Table 2. In the *P. subcapitata* test, the binary mixtures containing docusate sodium exhibited a strong antagonism, being their toxicity apparently marked by

**Table 1**
Toxicity of surfactants and chlorinated compounds expressed as $EC_{50}$ values (mg/L) with confidence limits (95% probability) towards *Pseudokirchneriella subcapitata*, *Vibrio fischeri*, and *Anabaena* CPB4337.

| | *Pseudokirchneriella subcapitata* | | *Vibrio fischeri* (15 min) | | Anabaena CPB4337 | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (mg/L) | C.I. 95% | $EC_{50}$ (mg/L) | CI 95% | $EC_{50}$ (mg/L) | C.I. 95% |
| Docusate sodium | 39.5 | 38.1–40.8 | 74.5 | 70.6–77.1 | 43.0 | 36.6–50.1 |
| PFOA | 96.2 | 88.6–113.7 | 524 | 505–538 | 72.3 | 57.96–82.9 |
| PFOS | 35.0 | 34.2–35.5 | >500[a] | – | 143.27 | 120.3–155.9 |
| PF-656 | 43.0 | 41.1–44.9 | >250[b] | – | >250[c] | – |
| PFBS | >20 250[d] | – | 17520 | 16 850–18 200 | 8386 | 7752–8693 |
| Triclosan | 0.037 | 0.036–0.038 | 0.95 | 0.91–0.99 | 1.15 | 0.86–1.46 |
| TCP | 0.061 | 0.058–0.062 | 18.4 | 17.5–19.2 | 0.37 | 0.32–0.64 |

[a] 12% luminescence inhibition at 500 mg/L.
[b] 15% luminescence inhibition at 250 mg/L.
[c] Luminescence between 100% and 250% that of the control.
[d] 37% growth inhibition at 20 250 mg/L.

**Table 2**
Dose–effect relationship parameters and mean combination index (CI) values of docusate sodium, triclosan and TCP in their binary and ternary combinations for *Pseudokirchneriella subacpitata*, *Vibrio fischeri* and *Anabaena* CPB4337 tests. (Synergism emphasized in bold.)

| | Dose effect parameters | | | CI values | | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (mg/L) | *m* | *r* | $EC_{10}$ | $EC_{50}$ | $EC_{90}$ |
| *Anabaena CPB4337* | | | | | | |
| Docusate + triclosan | 12.7 | 4.85 | 0.987 | **0.73 ± 0.12** | **0.43 ± 0.11** | **0.29 ± 0.24** |
| Docusate + TCP | 8.54 | 3.44 | 0.971 | 1.53 ± 0.59 | **0.47 ± 0.10** | **0.17 ± 0.01** |
| Triclosan + TCP | 1.65 | 0.72 | 0.975 | **0.34 ± 0.04** | 2.11 ± 0.18 | 12.8 ± 3.76 |
| Docusate + triclosan + TCP | 7.87 | 2.09 | 0.974 | 0.89 ± 0.30 | **0.66 ± 0.19** | **0.61 ± 0.07** |
| *Pseudokirchneriella subacpitata* | | | | | | |
| Docusate + triclosan | 39.9 | 1.32 | 0.980 | 30.3 ± 6.9 | 14.7 ± 1.4 | 7.6 ± 1.2 |
| Docusate + TCP | 46.2 | 0.86 | 0.997 | 523 ± 209 | 933 ± 114 | 1724 ± 768 |
| Triclosan + TCP | 0.23 | 2.01 | 0.975 | 11.1 ± 1.9 | 4.60 ± 0.36 | 1.92 ± 0.43 |
| Docusate + triclosan + TCP | 0.54 | 3.00 | 0.972 | 4.3 ± 0.7 | 1.17 ± 0.09 | **0.33 ± 0.05** |
| *Vibrio fischeri* | | | | | | |
| Docusate + triclosan | 6.85 | 3.15 | 0.978 | 2.16 ± 0.16 | 1.01 ± 0.05 | **0.69 ± 0.03** |
| Docusate + TCP | 22.5 | 1.13 | 0.990 | 0.96 ± 0.06 | 1.01 ± 0.03 | **0.64 ± 0.06** |
| Triclosan + TCP | 8.56 | 2.41 | 0.991 | 1.77 ± 0.05 | 1.26 ± 0.02 | 1.01 ± 0.02 |
| Docusate + triclosan + TCP | 12.8 | 4.46 | 0.987 | 3.00 ± 0.13 | 1.05 ± 0.03 | **0.52 ± 0.02** |

The parameters *m*, $EC_{50}$ and *r* are the antilog of *x*-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency ($EC_{50}$), and conformity of the data to the mass-action law, respectively (Chou and Talalay, 1984; Chou, 2006). $EC_{50}$ and *m* are used for calculating the CI values (Eq. (3)); CI < 1, CI = 1, and CI > 1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. $EC_{10}$, $EC_{50}$ and $EC_{90}$, are the doses required to reach a response inhibition of 10%, 50% and 90%, respectively.

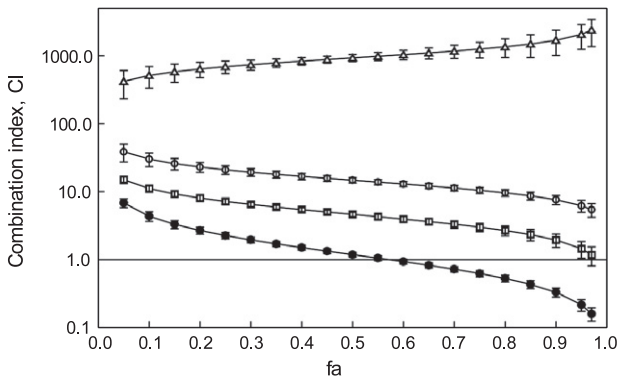


**Fig. 2.** Combination index plot for binary and ternary combinations of docusate sodium, triclosan and TCP for the *Pseudokirchneriella subcapitata* growth test: TCP + triclosan (-□-), docusate + TCP (-Δ-), docusate + triclosan (-○-) and the ternary mixture (-●-). The line at CI = 1 represents additivity.

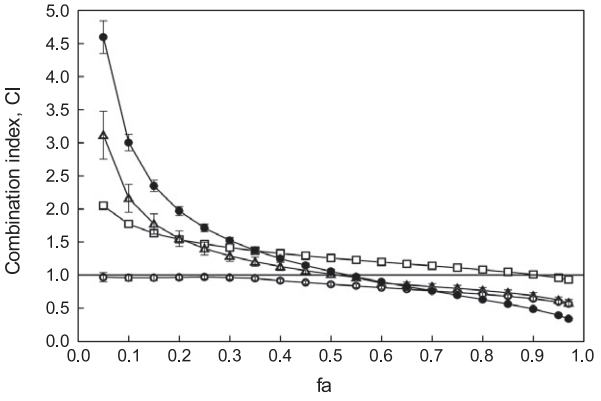


**Fig. 3.** Combination index plot for binary and ternary combinations of docusate sodium, triclosan and TCP for the *Vibrio fischeri* test: docusate + TCP (-Δ-), docusate + triclosan (-○-), TCP + triclosan (-□-) and the ternary mixture (-●-). The line at CI = 1 represents additivity.

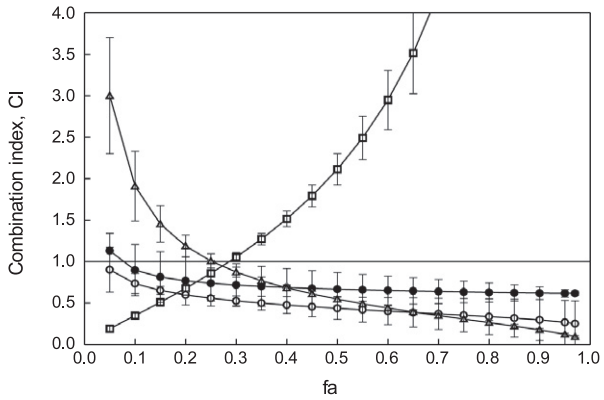


**Fig. 4.** Combination index plot for binary and ternary combinations of docusate sodium, triclosan and TCP for the *Anabaena* CPB4337 test: docusate + TCP (-Δ-), docusate + triclosan (-○-), TCP + triclosan (-□-) and the ternary mixture (-●-). The line at CI = 1 represents additivity.

docusate sodium with low or negligible contribution of TCP and triclosan, compounds individually much more toxic to the alga (Fig. 2). This effect was not observed either for *Anabaena* CPB4337 or for *V. fischeri* for which the difference in individual toxicity of the chlorinated compounds with respect to sodium docusate was less intense. The binary mixture of triclosan and TCP were also antagonistic at all *fa* levels in the green alga.

The results for *V. fischeri* and *Anabaena* CPB4337 tests exhibited certain similarities. The binary combination of docusate sodium and TCP showed a relatively strong antagonism at low effect levels to approach an additive or synergistic effect at the highest $f_a$ levels. The combination of docusate sodium and triclosan also showed a certain synergism at high $f_a$ values, the same behaviour was observed for the ternary mixture. Except for the strong antagonism exhibited by the binary mixtures of triclosan and TCP at $f_a > 0.3$, the toxicity pattern obtained for *V. fischeri* and *Anabaena* CPB4337 were similar, with a tendency to synergism at higher levels of effect. The presence of sodium docusate in the ternary mixture resulted in a synergistic behaviour for the three organisms. In the case of the green alga, synergism predominated only at high *fa* levels ($f_a > 0.6$); in *V. fischeri*, synergism was found at $f_a$ levels greater than 0.5 (Fig. 3) whilst in *Anabaena* CPB4337, synergism of the ternary mixture was evident almost throughout the whole $f_a$ range

as shown in Fig. 4. A correlation analysis between CI values suggests that the binary interactions of mixtures containing docusate sodium predominated in the three component mixture both for *Anabaena* CPB4337 and *V. fischeri*. For the case of *P. subcapitata* the result was opposite, with a higher correlation with the mixture of triclosan and TCP.

Risk assessment tools are usually based on single component data, a procedure which may lead to a misestimation of the actual risk associated with complex mixtures. The classic models for the prediction of mixture toxicity, concentration addition and independent action, are based on simple assumptions on the mode of toxic action (Teuschler, 2007). The idea is misleading as the mode of action have proved irrelevant after being demonstrated that toxicological interactions, namely synergism or antagonism, can occur irrespective of the primary mode of action (Chou, 2006). Moreover, the mode of action of a substance as toxic in environmentally relevant conditions is largely unknown and different for different organisms and trophic levels. Cleuvers (2003) noted that, even for substances such as drugs, whose biochemical mechanism is known, the action in a non-target organism, once released in the environments is essentially unknown. Liu et al. (2008) have reported that PFOS may increase the membrane fluidity and permeability to hydrophobic substances. If this was the case, a synergistic effect would be expected as general rule in binary mixtures PFOS-organic compounds; however, Liu et al. (2009) reported a synergistic effect PFOS-PCP but antagonism in the binary mixtures PFOS-diuron and PFOS-atrazine in the green alga *Scenedesmus obliquus*; the authors indicated that PFOS may differentially affect the toxicity of structurally different compounds stressing the effect of hydrophobicity between the compounds they tested. In this work we present data on the interactive effects of one surfactant, docusate sodium whose toxicity to aquatic organisms has been tested for the first time in this work, with two chlorinated compounds; we have made binary and, ternary mixtures, finding that particularly in the ternary mixture, docusate sodium increased the toxicity of both chlorinated compounds in the three tested organisms with the difference that synergism was present in a wider range of effect levels in prokaryotes (particularly *Anabaena* CPB4337) than in the eukaryotic green alga; the observed synergism indicated a potential toxicological risk associated with the co-existence of docusate sodium and other organic pollutants in aquatic environments. It has been stated that the interaction of surfactants and chemicals, including other surfactants, affects different functions and multiple cellular response targets. Such interaction generates a complex cascade of events in biological systems that cannot be summarized in a simple pattern (Wei et al., 2009). As a consequence, synergism or antagonism may occur independently of a similar or dissimilar mode of action and, with the current knowledge of toxicity mechanisms cannot be predicted. On the other hand, ecotoxicity studies conducted on several species and trophic levels may show a completely different response to the same toxicant mixture. Moreover, the nature of the interaction may depend on the effect level, but what we found is a general tendency of mixtures to exhibit synergistic responses as they become more complex. In a preceding work we proved that mixtures of several fibrates in wastewater showed a synergistic behaviour practically all over the fa range, while the same compounds in pure water were antagonistic in binary mixtures (Rodea-Palomares et al., 2010).

## 4. Conclusions

We derived the median effect–dose of the perfluorinated surfactants PFOS and PFOA as well as those of several alternative surfactants that included docusate sodium and the fluorinated substances 3 M's PF-656 and PFBS. Docusate sodium showed the highest toxicity to *Anabaena* CPB4337 and *V. fischeri*, and exhibited a similar toxicity than PFOS and PF-656 towards algal growth. Both PFOS and PF-656 were, however, not toxic to *Anabaena* CPB4337 and *V. fischeri.* PFBS exhibited significant toxicity only towards the algae *P. subcapitata*. The data indicated that docusate sodium can be classified as "harmful to aquatic organisms" for the three species tested in this work. The chlorinated compounds triclosan and TCP were highly toxic to *P. subcapitata*.

The application of the combination index (CI) isobologram method to mixtures of docusate, triclosan and TCP, showed that they behaved antagonistically for most binary mixtures that turned into synergistic in ternary mixtures for the three organisms and at least for a certain range of effect. For *Anabaena* CPB4337, also the mixtures of docusate sodium and triclosan were synergistic all over the range of effect levels. The toxicity pattern obtained for *V. fischeri* and *Anabaena* tests were similar due most probably to the prokaryotic nature of both. The increased toxicity of the ternary mixtures containing sodium docusate suggested a potential risk associated to the co-occurrence of this surfactant with other xenobiotics in the same environments that needs further research. This displacement from antagonistic to synergistic behaviour in complex mixtures may led to an important underestimation of mixture toxicity when assuming additive behaviour from single component data.

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# TOXICOLOGICAL INTERACTIONS OF PERFLUOROOCTANE SULFONIC ACID (PFOS) AND PERFLUOROOCTANOIC ACID (PFOA) WITH SELECTED POLLUTANTS.

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**SUBMITTED**

# Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants.

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**Abstract**

**The combined toxicity of the perfluorinated surfactants perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and several pollutants ($Hg^{2+}$, $Cd^{2+}$, 2,4-D, propylparaben, mitomycin C and furazolidone) has been examined with a bioluminescent cyanobacterial toxicity test. $Hg^{2+}$, $Cd^{2+}$, mitomycin C and furazolidone could be included in the "Acute aquatic hazard" category established in the Regulation (EC) No 1272/2008 being "very toxic to aquatic life". Toxicological interactions of PFOA, PFOS with these pollutants in binary, ternary and multicomponent mixtures were studied using the Combination- Index method. PFOA and PFOS showed an antagonistic interaction at the whole range of effect levels, this may explain in part the finding that PFOA and PFOS interacted in an inverse way with the organic pollutants; the relative hydrophobicity of the tested compounds would also explain this interaction pattern. The interaction of both PFOS and PFOA with heavy metals was mostly antagonistic, decreasing metal toxicity. With increasing complexity of the mixtures, the CI method predicted synergism at low to very low levels of effect; pollutant combinations at their mixture NOECs were tested and confirmed the predicted synergism.**

*Keywords: Antagonism; Cyanobacterium; Combination index; PFOA & PFOS; Synergism.*

## 1. Introduction

Surfactants are synthetic chemicals used in large amounts in a variety of industrial cleansing processes as well as in consumer products. Perfluorinated chemicals (PFCs) are synthetic fluorinated surfactants composed of a carbon backbone and a charged functional group. The eight-carbon backbone perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are two of the most widely used PFCs. The strong covalent bond between the fluor and carbon ions makes PFCs thermally and chemically stable; they are also oil and water repellent; these unique properties make these chemicals highly resistant to both chemical and biological degradation under normal environmental conditions and have been found to be highly persistent in the environment [1, 2]. Their global occurrence, persistence in the environment and bioaccumulation in biota has increased the concerns about possible toxic effect of PFCs. in 2000, the US-EPA declared PFOS and PFOA withdrawal to avoid environmental pollution and potential health risks; the OECD in 2002 declared these substances as biopersistent, bioaccumulative and toxic to mammalians; PFOS was finally banned in Europe by the directive 2006/122/EC and recently added to the Annex B of the Stockholm Convention on Persistent Organic Pollutants.

Due to the bioaccumulation of PFCs in humans and associated potential toxicity, most toxicological studies have been made in rodents and/or human cell lines; however, there is comparatively less information on the ecotoxicity of these chemicals in

the aquatic environment. In addition, in the aquatic environment, various PFCs co-exist and co-occur with a variety of other xenobiotics[3-5]; thus, to obtain a full picture of the true impact of PFCs, studies on aquatic toxicity of representative PFCs such as PFOS and PFOA applied singly and in combination as well as combined with other xenobiotics are needed. Chemicals in a complex mixture may either not interact or interact synergistically or antagonistically [6-9]; interactions which should be taken into account when considering risk assessment strategies. There are very few reports on the interaction between PFOA and PFOS themselves or on the interactions of PFOA and PFOS with other xenobiotics which is of special concern considering the ability of PFCs to solubilise non-polar compounds[10]. Most of these interactions studies have been performed with PFOS [11-14]; to our knowledge, no previous studies about toxicological interactions of PFOA with other xenobiotics have been reported.

The aim of this study was to assess the nature of the interactions between PFOS and PFOA as well as PFOS and/or PFOA combined with selected priority and emerging pollutants. As toxicity endpoint we have chosen the bioluminescent response of the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 [7, 15, 16]. To identify and quantify the nature of the interactions between the fluorinated surfactants and the pollutants, we made binary, ternary and complex mixtures of these pollutants with PFOS and PFOA which were analyzed by the method of the combination index (CI)-isobologram equation which we have previously used to study the combined effects of pollutant mixtures [7, 8].

## 2. Material and Methods

### *2.1. Materials*

PFOS (98%) was obtained from Fluka, PFOA (96%), mitomycin C (MMC) (97%), $Hg^{2+}$ (as $HgCl_2$) (99%) and $Cd^{2+}$ (as $CdCl_2$) (97.5%) were purchased from Sigma-Aldrich. Propyl 4-hydroxybenzoate (propylparaben; PPB), 2-(2,4-dichlorophenoxi) acetic acid (2,4-D) and 3-{[(5-nitro-2-furyl)methylene]amino}-1,3-oxazolidin-2-one (furazolidone; FURA) (98%) were obtained from Alfa Aesar. CAS No, Molecular formula and main physicochemical properties of these compounds are summarized in Table 1, log $K_{ow}$ and log $D_{ow}$ are included as descriptors of the hydrophobicity of the tested chemicals. Polar Surface Area (PSA) is included as a descriptor of passive molecular transport through membranes [17].

We avoided the use of solvents when possible, with the only exceptions of the stock solutions of mitomycin C which was prepared in methanol, and 2,4-D and furazolidone, which were prepared in DMSO. Final concentrations of methanol and DMSO in the assay medium were always below 0.005% (V/V). No significant effect on bioluminescence of *Anabaena* CPB 4337 was found for these concentrations of solvents (not shown). Stock solutions and dilutions used in the bioassays were stored in the dark at -20 ºC.

### *2.2. Toxicity bioassays*

The bioassays using the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 were based on the inhibition of constitutive luminescence caused by the presence of any toxic substance and were performed as previously described [15, 16, 18]. The stability of target compounds under the bioassay conditions was examined according to OECD Guidance [19]. Analyses have been performed at the start and at the end of the 24h-exposure test for the highest concentration and for a concentration near the $EC_{50}$ (*Dm*) using an HPLC-Diode Array Liquid Chromatograph or ICP-MS, except for those for which stability was previously assessed [8]. HPLC analyses were performed using a Hewlett Packard 1200 Series device (Agilent Technologies, Palo Alto, USA) equipped with a reversed phase Kromasil 5u 100A C18 analytical column. The mobile phase was a mixture of acrylonitrile (50%) and acidified water (50%). UV detection was carried out at 230 nm (MMC), 360 nm (FURA), 254nm (PPB) and 360 nm (2,4-D). Inductively Coupled Plasma-Mass Spectrometry analyses were used to determine the exposure concentration of mercury and cadmium. The equipment used was a quadrupole mass spectrometer Agilent 7700X operating at 3 MHz in

helium cell gas mode. No significant differences were found between the nominal and measured exposure concentrations for $Hg^{2+}$, $Cd^{2+}$, 2,4-D, PPB, PFOA and PFOS; thus, throughout the present study, their nominal concentrations were used for data analyses. In the case of MMC and FURA, the final concentration/initial concentration ratios (in abiotic conditions) were 0.038 for MMC and 0.73 for FURA. In both cases, for data analyses, exposure concentrations were used instead of nominal concentrations according to OECD Guidance (OECD, 2008).

### 2.3. *Experimental design of PFOS/PFOA/selected chemicals combinations*

Solutions of PFOS, PFOA, $HgCl_2$, $CdCl_2$, propylparaben, 2,4-D, furazolidone and mitomycin C were used singly and in the binary, ternary and multicomponent mixtures shown in Table 2. *Anabaena* cells were treated with serial dilutions of each chemical individually and with a fixed constant ratio (1:1), based on the individual $EC_{50}$ values, in their combinations. Five to seven dilutions (serial dilution factor = 2) of each chemical and combination plus a control were tested in three independent experiments with replicate samples as described elsewhere [7].

### *2.4. Median-Effect and combination index (CI)-isobologram equations for determining individual and combined toxicities.*

The response to toxic exposure in *Anabaena* CPB4337 test was estimated using the median-effect equation based on the mass-action law [21]:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \qquad (1)$$

Where $D$ is the dose, $Dm$ is the dose for 50% effect ($EC_{50}$), $fa$ is the fraction affected by dose $D$ (e.g., 0.75 if cell bioluminescence is inhibited by 75%), $fu$ is the unaffected fraction (therefore, $fa = 1\text{-}fu$), and $m$ is the coefficient of the sigmoidicity of the dose-effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curve, respectively. Therefore, the method takes into account both the potency ($Dm$) and shape ($m$) parameters. If equation 1 is rearranged, then:

$$D = Dm[fa/(1\text{-}fa)]^{1/m} \qquad (2)$$

The $Dm$ and $m$ values for each individual compound or mixture were determined by the median- effect plot: x = log ($D$) versus y = log ($fa/fu$) which is based on the logarithmic form of equation 1. In the median-effect plot, $m$ is the slope and $Dm = 10^{-(\text{y-intercept})/\text{m}}$. The conformity of the data to the median-effect principle can be ready manifested by the linear correlation coefficient ($r$) of the data to the logarithmic form of equation 1 [22].

These parameters were then used to calculate doses of individual compound and their mixtures required to produce various effect levels according to equation 1; for each effect level, combination index (CI) values were then calculated according to the general combination index equation for $n$-chemical combination at $x$% inhibition [22]:

$${}^n(\mathrm{CI})_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{[D]_j \Big/ \sum_1^n [D]\right\}}{(D_m)_j \left\{(f_{ax})_j / \left[1-(f_{ax})_j\right]\right\}^{1/mj}} \qquad (3)$$

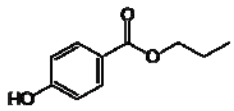
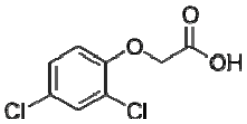
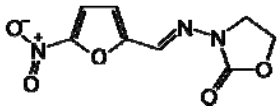
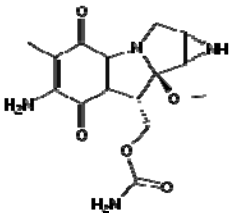
where ${}^n(\mathrm{CI})_x$ is the combination index for $n$ chemicals at $x$% inhibition; $(D_x)_{1\text{-}n}$ is the sum of the dose of $n$ chemicals that exerts $x$% inhibition in combination, $\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the dose of each of $n$ chemicals that exerts $x$% inhibition in combination; and $(D_m)_j \{(f_{ax})_j/[1\text{-}(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts $x$% inhibition. From equation 3, CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, respectively.

### *2.5. Analysis of results*

Computer program CompuSyn [23] was used for calculation of the individual and combined dose-effect curve parameters; CI values of the different mixtures; *fa*-CI plots and Polygonograms. Linear regression analyses were computed using MINITAB Release 14 for Windows (Minitab Inc; USA). The

mixture NOECs (no observed effect concentrations) were determined by Dunnett`s multiple comparison procedure [24, 25] ($p \leq 0.05$) using also Minitab.

**Table 1. Physicochemical properties of $Hg^{2+}$ (Hg), $Cd^{2+}$ (Cd), propyl 4-hydroxybenzoate (Propylparaben, PPB), (2,4-Dichlorophenoxy) acetic acid (2,4-D), furazolidone (FURA), mitomycin C (MMC), perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS).**

| Compound | CAS No. | Molecular structure | Molecular weight (g/mol) | Water Solubility (g/L) | pKa | Log $K_{ow}$[1] | Log $D_{ow}$[2] | PSA[3] ($A^2$) |
|---|---|---|---|---|---|---|---|---|
| $Hg^{2+}$ (as $HgCl_2$) | 7487-94-7 | - | 271.52 | 74 | - | 3.2 | 3.2 | - |
| $Cd^{2+}$ (as $CdCl_2$) | | - | 183.32 | 1350 | - | -0.07 | -0.07 | - |
| propyl 4-hydroxybenzoate (Propylparaben, PPB) | 94-13-3 |  | 180.2 | 1.1 | 8.23 | 2.901 | 2.90 | 46.5 |
| (2,4-Dichlorophenoxy) acetic acid (2,4-D) | 94-75-7 |  | 221.04 | 367 | 2.98 | 2.426 | -0.59 | 46.5 |
| 3-{[(5-nitro-2-furyl)methylene]amino}-1,3-oxazolidin-2-one (Furazolidone, FURA) | 67-45-8 |  | 225.158 | 0.12 | -1.98 | -0.050 | -0.05 | 101 |
| Mitomycin C | 50-07-7 |  | 334.33 | 0.57 | 13.27 | -0.298 | -0.30 | 147 |
| Perfluorooctanoic acid (PFOA) | 335-67-1 |  | 414.07 | 13.6 | 0.50 | 6.444 | 0.94 | 37.3 |
| Perfluorooctane sulfonic acid (PFOS) | 1763-23-1 |  | 500.13 | 7.5 | -3.27 | 4.512 | -4.76 | 62.8 |

**[1]:Log $K_{ow}$ (log P)= log of octanol water partition coefficient.**

**[2]:Log $D_{ow}$ = The octanol-water partition coefficient, $K_{ow}$, is a measure of the hydrophobicity of a given neutral compound. For compounds that dissociate in aqueous solution, the corresponding acid-base equilibrium has to be considered originating an apparent octanol-water partition coefficient, usually represented $D_{ow}$. For the computation of $D_{ow}$, both pH and the dissociation constant of acidic of basic compounds, $pK_a$ are required. For acidic compounds, the Herderson-Hasselbalch equations yield:**

$$D_{ow} = \frac{K_{ow}}{1 + 10^{pH - pK_a}} \quad \textbf{(1)}$$

**For basic drugs, the apparent partition coefficient can be expressed by means of the $pK_a$ for their conjugate acids:**

$$D_{ow} = \frac{K_{ow}}{1 + 10^{pK_a - pH}} \quad \textbf{(2)}$$

**For neutral substances, $D_{ow} = K_{ow}$[20]**

**[3]: PSA = Polar surface area: The polar surface area (PSA) is defined as the surface overall sume of polar atoms, (usually oxygen and nitrogen), including also attached hydrogens.**
**PSA is a commonly used medicinal chemistry metric for the optimization of cell permeability. Molecules with a polar surface area of greater than 140 angstroms squared are usually believed to be poor at permeating cell membranes. [17].**

## 3. Results

### *3.1. Toxicity of individual compounds*

Table 2 shows the dose-effect curve parameters (*Dm*, *m* and *r*) of the eight compounds tested in this study using the *Anabaena* CPB4337 24-h toxicity test singly and in their binary, ternary and multi-component mixtures (6 to 8 components). For single components, *Dm* ($EC_{50}$) in mg/l were as follow: MMC (0.014), Hg (0.070), Cd (0.091), FURA (0.974), 2,4-D (3.74), PPB (11.91), PFOS (16.29) and PFOA (19.81). *Dm* values of MMC, $Hg^{2+}$ and $Cd^{2+}$ were the lowest, and could be included together with FURA in the "Acute aquatic hazard" category established in the Regulation (EC) No 1272/2008 ($EC_{50}$ < 1 mg/l) and classified as "very toxic to aquatic life" (H400).

### *3.2. Toxicological interactions of PFOA and PFOS with selected pollutants in binary and ternary combinations in the Anabaena CPB4337 bioluminescence test*

Figure 1 shows the *fa*-CI plots of binary and ternary mixtures for the *Anabaena* tests. The *fa*-CI plot depicts the CI value versus *fa* (the effect level or fraction of luminescence inhibited with respect to the control). Average CI values for three representative effect levels ($EC_{10}$, $EC_{50}$ and $EC_{90}$) are also shown in table 2.

Figure 1a and b show the *fa*-CI plots for PFOA/PFOS/ Heavy metals binary and ternary combinations. The $Hg^{2+}$ + $Cd^{2+}$ combination showed a slight antagonism in almost the whole range of effect levels, approaching an additive effect at the highest *fa* values. Regarding PFOA/heavy metals mixtures (Figure 1a), the PFOA + $Hg^{2+}$ combination showed a strong antagonism in the *fa* range; the PFOA + $Cd^{2+}$ combination was also antagonistic but to a lesser degree than the PFOA + $Hg^{2+}$ combination. The ternary mixture PFOA + $Hg^{2+}$ + $Cd^{2+}$ led to dual synergistic/antagonistic behaviour being synergistic at *fa* values below 0.2, additive at *fa* values between 0.2 and 0.4, and turning into antagonism at *fa* values above 0.4. Correlation analyses were made between CI values of the ternary combinations and CI values of each of the binary combinations to determine which binary combination was predominant in the ternary mixture (Table 3). In this correlation analysis, for the ternary mixture PFOA + $Hg^{2+}$ + $Cd^{2+}$, the highest correlation coefficient was found for the PFOA + $Cd^{2+}$ combination ($r$ = 0.988), suggesting that his combination interaction predominated in the three compound mixture.

In the PFOS – Heavy metal mixtures (Figure 1 b), binary combinations of PFOS + $Hg^{2+}$ and PFOS + $Cd^{2+}$ also showed a strong antagonism in the whole range of effect levels (*fa*), but with a tendency of increasing antagonism in the case of PFOS + $Cd^{2+}$ while in the PFOS + Hg combination, there was a tendency towards additive effect. The ternary mixture of PFOS + $Hg^{2+}$ + $Cd^{2+}$ was clearly antagonistic in the whole range of *fa* values meaning that the presence of PFOS significantly increased the observed antagonism of the binary $Hg^{2+}$ + $Cd^{2+}$ mixture. The highest correlation coefficients were those of the PFOS + Hg combination ($r$ = 0.995) and Hg + Cd combination ($r$ = 0.994), suggesting them as predominant in the three component mixture (Table 3).

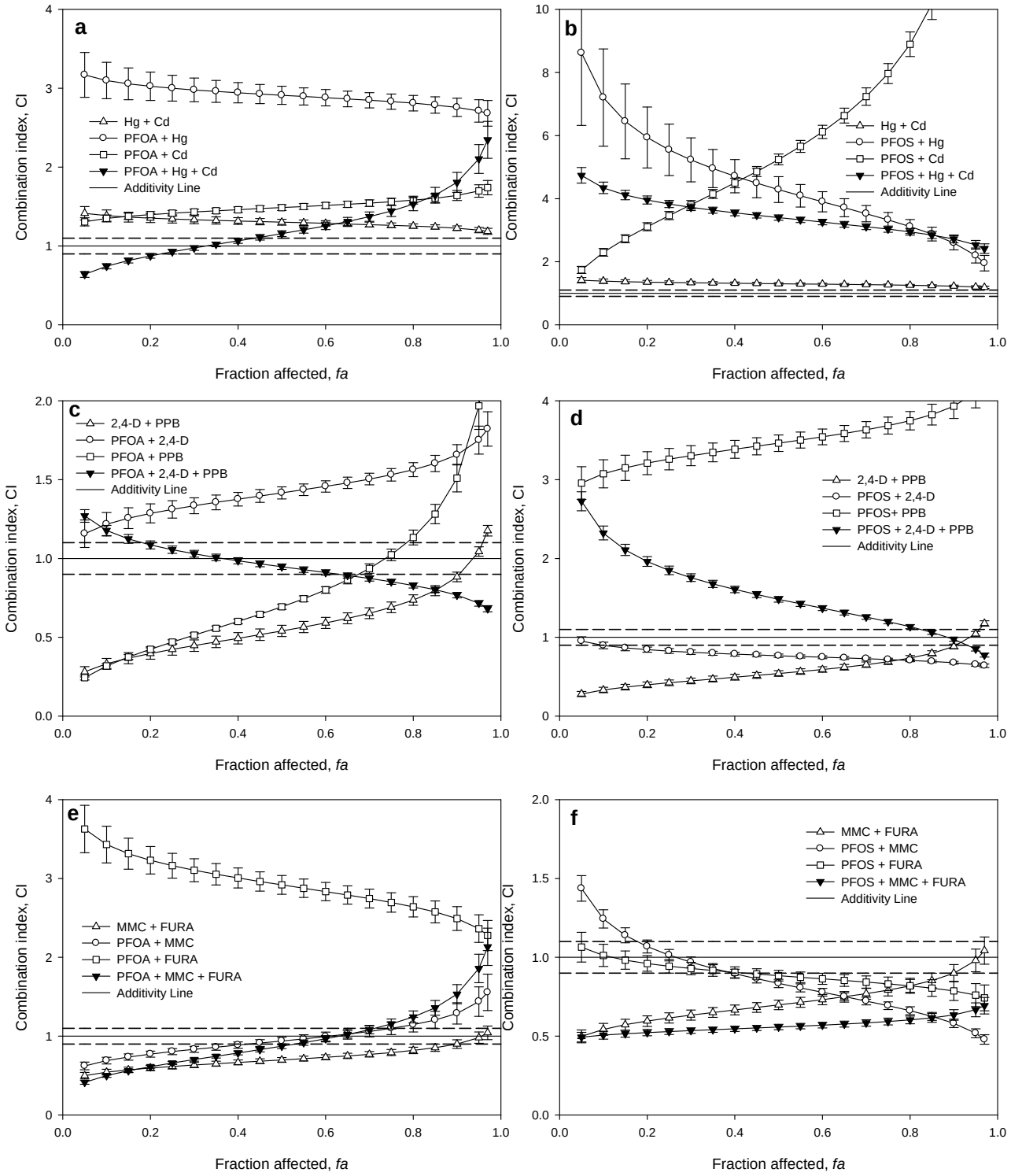
In the PFOA – biocides mixtures (Figure 1c), the binary mixture of 2,4-D + PPB was synergistic at *fa* levels below 0.75 and became additive at *fa* values above this value. The binary combinations of PFOA + 2,4-D was increasingly antagonistic in the whole *fa* range. The binary mixtures of PFOA + PPB led to a dual synergistic/antagonistic behaviour: it was synergistic at *fa* levels below 0.7, additive at *fa* levels between 0.7-0.8 and it turned into an increasing antagonism at *fa* levels below 0.8. The ternary PFOA + 2,4-D + PPB mixture also led to dual antagonistic/synergistic behaviour being antagonistic at *fa* values below 0.1, additive at *fa* values between 0.1 and 0.7 and turning into synergistic at *fa* values above 0.7; just the opposite of the observed behaviour of the binary PFOA + PPB combination. In this case, all the correlation coefficients were negative, indicating an inverse relationship between the pattern of the interaction of the ternary mixture with respect to any of the binary combinations. In the PFOS and biocides mixtures (Figure 1d), the binary

**Table 2. Dose-effect relationship parameters and mean combination index (CI) values of Hg, Cd, PPB, 2,4-D, FURA, MMC, PFOA and PFOS, individually and of their binary, ternary and multicomponent (six or more components) combinations on *Anabaena* CPB4337 bioluminescence test.**

| | Dose-effect parameters | | | CI Values at | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Drug Combo | *Dm* | *m* | *r* | **$EC_{10}$** | | **$EC_{50}$** | | **$EC_{90}$** | |
| **Drug/Combo** | **Dm** | **m** | **r** | | | | | | |
| Hg | 0.070 | 2.618 | 0.960 | - | | - | | - | |
| Cd | 0.091 | 2.321 | 0.935 | - | | - | | - | |
| PPB | 11.91 | 2.198 | 0.916 | - | | - | | - | |
| 2,4-D | 3.740 | 2.606 | 0.933 | - | | - | | - | |
| FURA | 0.974 | 2.399 | 0.949 | - | | - | | - | |
| MMC | 0.014 | 2.654 | 0.912 | - | | - | | - | |
| PFOA | 19.81 | 2.122 | 0.950 | - | | - | | - | |
| PFOS | 16.29 | 1.805 | 0.884 | - | | - | | - | |
| Binary Mixtures | | | | | | | | | |
| Hg + Cd | 0.111 | 2.556 | 0.953 | 1.38 ± 0.07 | Ant | 1.30 ± 0.03 | Ant | 1.22 ± 0.02 | Ant |
| 2,4-D + PPB | 2.720 | 1.618 | 0.925 | 0.33 ± 0.03 | **Syn** | 0.54 ± 0.03 | **Syn** | 0.88 ± 0.03 | **Syn** |
| MMC + FURA | 0.210 | 1.985 | 0.938 | 0.54 ± 0.03 | **Syn** | 0.69 ± 0.03 | **Syn** | 0.89 ± 0.05 | **Syn** |
| PFOA + PFOS | 93.85 | 3.367 | 0.923 | 4.18 ± 0.73 | Ant | 3.77 ± 0.34 | Ant | 3.41 ± 0.25 | Ant |
| PFOA + Hg | 41.15 | 2.386 | 0.900 | 3.09 ± 0.10 | Ant | 2.91 ± 0.11 | Ant | 2.75 ± 0.11 | Ant |
| PFOA + Cd | 13.20 | 2.026 | 0.939 | 1.34 ± 0.04 | Ant | 1.48 ± 0.03 | Ant | 1.64 ± 0.06 | Ant |
| PFOA + PPB | 11.45 | 1.221 | 0.928 | 0.31 ± 0.01 | **Syn** | 0.69 ± 0.01 | **Syn** | 1.50 ± 0.08 | Ant |
| PFOA + 2,4-D | 10.05 | 2.117 | 0.918 | 1.21 ± 0.07 | Ant | 1.41 ± 0.03 | Ant | 1.65 ± 0.06 | Ant |
| PFOA + FURA | 27.04 | 2.717 | 0.878 | 3.42 ± 0.23 | Ant | 2.91 ± 0.11 | Ant | 2.49 ± 0.15 | Ant |
| PFOA + MMC | 4.695 | 1.841 | 0.921 | 0.68 ± 0.04 | **Syn** | 0.93 ± 0.04 | Add | 1.28 ± 0.13 | Ant |
| PFOS + Hg | 60.52 | 3.356 | 0.903 | 7.20 ± 1.54 | Ant | 4.28 ± 0.40 | Ant | 2.59 ± 0.22 | Ant |
| PFOS + Cd | 58.00 | 1.119 | 0.946 | 2.29 ± 0.12 | Ant | 5.24 ± 0.17 | Ant | 12.1 ± 0.66 | Ant |
| PFOS + 2,4-D | 6.787 | 2.568 | 0.949 | 0.89 ± 0.08 | Add | 0.76 ± 0.03 | **Syn** | 0.67 ± 0.02 | **Syn** |
| PFOS + PPB | 53.14 | 1.699 | 0.938 | 3.07 ± 0.17 | Ant | 3.46 ± 0.10 | Ant | 3.93 ± 0.15 | Ant |
| PFOS + FURA | 9.955 | 2.215 | 0.928 | 1.01 ± 0.07 | Add | 0.88 ± 0.03 | Add | 0.78 ± 0.06 | **Syn** |
| PFOS + MMC | 6.360 | 3.490 | 0.946 | 1.24 ± 0.02 | Ant | 0.83 ± 0.01 | **Syn** | 0.58 ± 0.03 | **Syn** |
| % of Synergistic mixtures | | | | | 25% | | 31% | | 31% |
| Ternary mixtures | | | | | | | | | |
| PFOA + Hg + Cd | 8.717 | 1.560 | 0.921 | 0.74 ± 0.03 | **Syn** | 1.15 ± 0.03 | Ant | 1.80 ± 0.12 | Ant |
| PFOA + 2,4-D + PPB | 7.317 | 3.210 | 0.935 | 1.17 ± 0.03 | Ant | 0.94 ± 0.01 | **Syn** | 0.76 ± 0.01 | **Syn** |
| PFOA + MMC + FURA | 3.507 | 1.515 | 0.933 | 0.49 ± 0.02 | **Syn** | 0.87 ± 0.03 | **Syn** | 1.53 ± 0.12 | **Syn** |
| PFOS + Hg + Cd | 34.06 | 2.530 | 0.927 | 4.33 ± 0.18 | Ant | 3.39 ± 0.08 | Ant | 2.72 ± 0.12 | Ant |
| PFOS + 2,4-D + PPB | 13.58 | 3.908 | 0.921 | 2.32 ± 0.04 | Ant | 1.48 ± 0.02 | Ant | 0.97 ± 0.01 | Add |
| PFOS + MMC + FURA | 3.562 | 1.987 | 0.933 | 0.50 ± 0.05 | **Syn** | 0.55 ± 0.02 | **Syn** | 0.63 ± 0.02 | **Syn** |
| % of Synergistic mixtures | | | | | 50% | | 50% | | 50% |
| Multicomponet mixtures | | | | | | | | | |
| Mix 6 | 1.745 | 2.063 | 0.940 | 0.62 ± 0.01 | **Syn** | 0.75 ± 0.01 | **Syn** | 0.91 ± 0.02 | Add |
| PFOA + Mix 6 | 2.859 | 1.585 | 0.935 | 0.45 ± 0.01 | **Syn** | 0.74 ± 0.05 | **Syn** | 1.22 ± 0.27 | Add |
| PFOS + Mix 6 | 3.848 | 1.801 | 0.920 | 0.57 ± 0.01 | **Syn** | 0.75 ± 0.01 | **Syn** | 1.00 ± 0.04 | Add |
| PFOA + PFOS + Mix 6 | 5.263 | 1.829 | 0.940 | 0.66 ± 0.01 | **Syn** | 0.85 ± 0.01 | **Syn** | 1.11 ± 0.03 | Add |
| % of Synergistic mixtures | | | | | 100% | | 100% | | 0% |

**Hg = $Hg^{2+}$, Cd = $Cd^{2+}$, PPB = Propylparaben, 2,4-D = (2,4-Dichlorophenoxy) acetic acid , FURA = furazolidone, MMC = mitomycin C, PFOA = perfluorooctanoic acid, and PFOS = perfluorooctane sulfonic acid. The parameters *m*, *Dm* and *r* are the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency ($EC_{50}$), and conformity of the data to the mass-action law, respectively [20, 21]. CI < 1, CI = 1, and CI > 1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. $EC_{10}$, $EC_{50}$ and $EC_{90}$, are the doses required to inhibit bioluminescence 10%, 50% and 90%, respectively.**

**Figure 1. Combination index Plot (*fa-CI plot*) for binary and ternary mixtures PFOA and PFOS with selected pollutants for the *Anabaena* CPB4337 test. CI values are plotted as a function of the fractional inhibition of bioluminescence (*fa*) by computer simulation (CompuSyn) from *fa*= 0.10 to 0.95. CI< 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively. The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005). Broken lines indicated upper and lower limits of additivity [22]. Hg = $Hg^{2+}$, Cd = $Cd^{2+}$, PPB = Propylparaben, 2,4-D = (2,4-Dichlorophenoxy) acetic acid, FURA = furazolidone, MMC = mitomycin C.**




combination of PFOS + 2,4-D was synergistic in practically the whole range of *fa* values and the binary combination PFOS + PPB was strongly antagonistic (CI > 3) in the whole range of *fa* values. The ternary combination PFOS + 2,4-D + PPB led to a dual antagonistic/synergistic behaviour, similar to

that found for the PFOA + 2,4-D + PPB mixture (Fig. 1c) but being antagonistic in practically the whole range of *fa* values and only becoming synergistic at the highest *fa* values (above 0.97). The highest correlation coefficient was found for the PFOS + 2,4-D combination ($r$ = 0.998), suggesting that this combination interaction predominated in the three component mixture (Table 3).

In the PFOA – pharmaceuticals mixtures (Figure 1e), the binary mixture of FURA + MMC was synergistic in practically the whole range of *fa* values, becoming nearly additive at *fa* values close to 1. The binary combination PFOA + FURA was strongly antagonistic in the whole *fa* range while he binary mixture of PFOA + MMC showed a dual synergistic/ antagonistic behaviour, synergistic at low to mean *fa* values (*fa* < 0.5), additive at *fa* values between 0.45 – 0.7, and slightly antagonistic at *fa* values above 0.7. The ternary mixture PFOA + FURA + MMC also showed a dual synergistic/ antagonistic behaviour, being synergistic at *fa* values below 0.5, additive at *fa* values between 0.5 and 0.7 and turning into antagonism at *fa* values above 0.7. As expected, the highest correlation coefficient was found for the PFOA + MMC combination ($r$ = 0.994), suggesting that this combination interaction predominated in the three component mixture (Table 3). In the PFOS – pharmaceuticals mixtures (Figure 1f), both binary mixtures of PFOS + MMC and PFOS + FURA led to a dual antagonistic/ synergistic behaviour being antagonistic at *fa* values below 0.2, additive at *fa* values between 0.2 and 0.45 and dominated by synergism at *fa* values above 0.45; however, although the interaction pattern for both binary mixtures was similar, the PFOS + MMC mixture showed a higher synergism at low *fa* values and higher antagonism at the higher *fa* levels. The ternary PFOS + MMC + FURA mixture was synergistic in practically the whole range of *fa* values being more synergistic than any of the corresponding binary mixtures. The highest correlation coefficient was found for the MMC + FURA combination ($r$ = 0.988), suggesting that this combination interaction clearly influenced the pattern of the observed interaction in the three component mixture.

Figure 2 shows a polygonogram of eight components which summarizes the evolution of the interactions of PFOA and PFOS with the six selected pollutants in binary mixtures at three representative levels of effect (*fa* = 0.1, 0.5 and 0.9). Quantitative values of CI at this *fa* levels can be seen in Table 2. The polygonogram (Fig. 2) clearly shows that the pattern of the interactions in binary mixtures is globally dominated by antagonistic interactions of the perfluorinated surfactants with the different pollutants particularly at the highest levels of effect; the exception to this were the interactions of PFOS with the herbicide 2,4-D and with the antibacterial furazolidone which were synergistic along all the representative *fa* levels, and the synergistic interactions of PFOA with MMC and PPB at low to mean effect levels.

**Figure 2. Polygonograms showing the toxicological interactions of PFOA and PFOS with selected pollutants in their binary combinations for the *Anabaena* CPB4337 test at three representative effect levels: *fa* = 0.1, *fa* = 0.5, *fa* = 0.9. Green solid lines indicate synergism, red broken lines indicate antagonism. The thickness of the line represents the strength of synergism or antagonism. Hg = $Hg^{2+}$, Cd = $Cd^{2+}$, PPB = Propylparaben, 2,4-D = (2,4-Dichlorophenoxy) acetic acid, FURA = furazolidone, MMC = mitomycin C.**

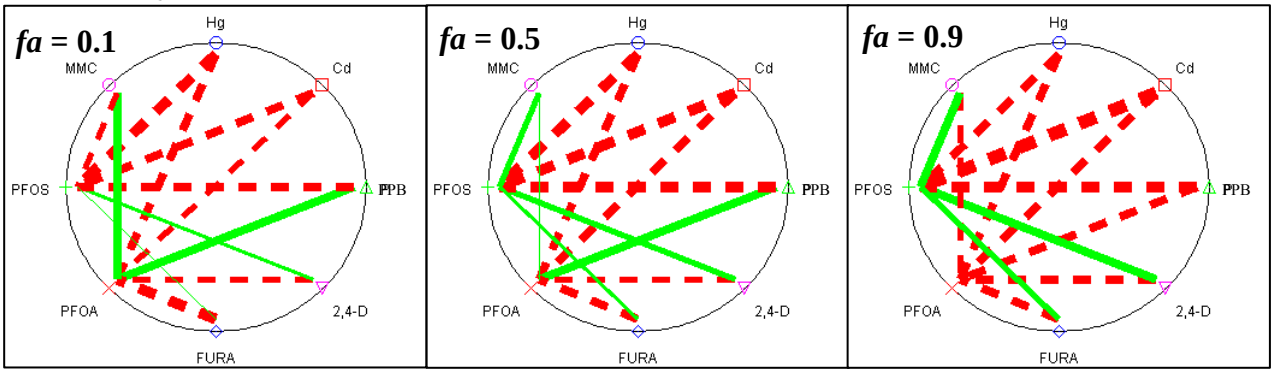


**Table 3. Correlation analyses between CI values of PFOA and PFOS ternary and multicomponent combinations (*y*) and their binary and ternary combinations (*x)* for *Anabaena* CPB4337 test.**

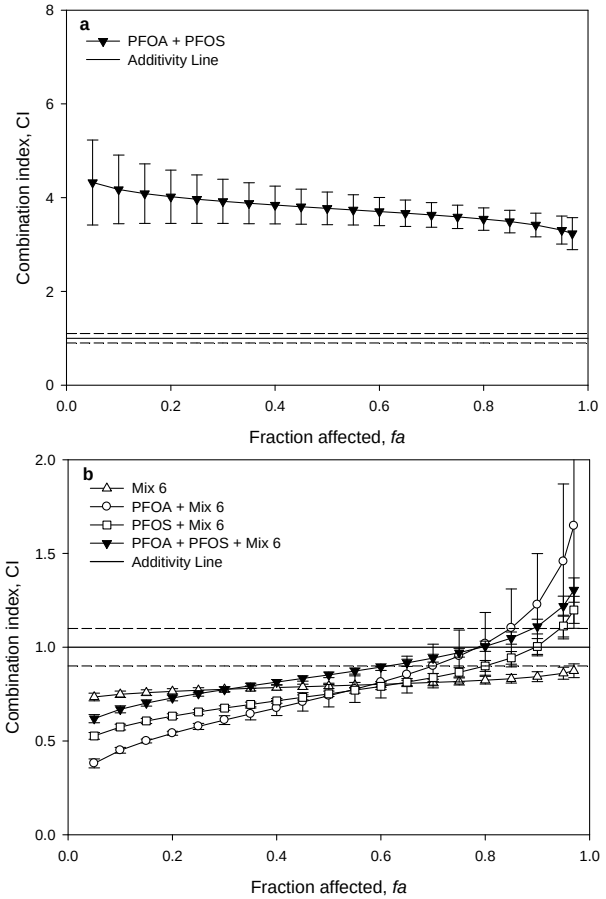
| Combinations | | | *Regression parameters* | | |
|---|---|---|---|---|---|
| Ternary combinatios | | | $x_o$ | *m* | *r* |
| PFOA + Hg +Cd | *vs* | Hg + Cd | 10.655 | -7.240 | - 0.969 |
| | *vs* | PFOA + Hg | 11.059 | -3.369 | - 0.957 |
| | *vs* | PFOA + Cd | -4.583 | 3.893 | 0.988 |
| PFOS + Hg + Cd | *vs* | Hg + Cd | -9.537 | 9.981 | 0.994 |
| | *vs* | PFOS + Hg | 1.855 | 0.349 | 0.995 |
| | *vs* | PFOS + Cd | 4.169 | -0.111 | - 0.878 |
| PFOA + 2,4-D + PPB | *vs* | 2,4-D + PPB | 1.316 | -0.618 | - 0.947 |
| | *vs* | PFOA + 2,4-D | 2.189 | -0.861 | - 0.980 |
| | *vs* | PFOA + PPB | 1.162 | -0.249 | - 0.907 |
| PFOS + 2,4-D + PPB | *vs* | 2,4-D + PPB | 2.683 | -1.936 | - 0.907 |
| | *vs* | PFOS + 2,4-D | -3.202 | 6.142 | 0.998 |
| | *vs* | PFOS + PPB | 6.479 | -1.409 | - 0.950 |
| PFOA + MMC + FURA | *vs* | MMC + FURA | -1.266 | 3.122 | 0.990 |
| | *vs* | PFOA + MMC | -0.816 | 1.829 | 0.994 |
| | *vs* | PFOA + FURA | 4.514 | -1.211 | - 0.946 |
| PFOS + MMC + FURA | *vs* | MMC + FURA | 0.302 | 0.368 | 0.998 |
| | *vs* | PFOS + MMC | 0.739 | -0.199 | - 0.928 |
| | *vs* | PFOS + FURA | 1.101 | -0.601 | - 0.956 |
| Multicomponent combinations | | | | | |
| Mix 6 | *vs* | Hg + Cd | 1.587 | -0.608 | - 0.995 |
| | *vs* | 2,4-D + PPB | 0.705 | 0.153 | 0.985 |
| | *vs* | MMC + FURA | 0.612 | 0.255 | 0.997 |
| PFOA + Mix 6 | *vs* | PFOA + Hg +Cd | 0.211 | 0.423 | 0.998 |
| | *vs* | PFOA + 2,4-D + PPB | 1.879 | -1.198 | - 0.966 |
| | *vs* | PFOA + MMC + FURA | 0.329 | 0.419 | 0.999 |
| PFOS + Mix 6 | *vs* | PFOS + Hg +Cd | 1.638 | -0.268 | - 0.953 |
| | *vs* | PFOS + 2,4-D + PPB | 1.200 | -0.313 | - 0.927 |
| | *vs* | PFOS + MMC + FURA | -1.105 | 3.210 | 0.999 |
| PFOA + PFOS + Mix6 | *vs* | PFOA + Hg +Cd | 0.479 | 0.435 | 0.998 |
| | *vs* | PFOA + 2,4-D + PPB | 2.196 | -1.234 | 0.967 |
| | *vs* | PFOA + MMC + FURA | 0.601 | 0.430 | 0.994 |
| | *vs* | PFOS + Hg +Cd | 2.089 | -0.310 | 0.958 |
| | *vs* | PFOS + 2,4-D + PPB | 1.583 | -0.363 | 0.934 |
| | *vs* | PFOS + MMC + FURA | -1.074 | 3.693 | 0.999 |

**Hg = $Hg^{2+}$, Cd = $Cd^{2+}$, PPB = Propylparaben, 2,4-D = (2,4-Dichlorophenoxy) acetic acid , FURA = furazolidone, MMC = mitomycin C, PFOA = perfluorooctanoic acid, and PFOS = perfluorooctane sulfonic acid. The parameters of linear regression equations: $x_0$ (value of *y* when *x* = 0); *m* (slope) and *r* (correlation coefficient) with all *p*-values <0.05.**

*3.3. Toxicological interactions of PFOA and PFOS in multicomponent mixtures with selected pollutants in the Anabaena CPB4337 bioluminescence test.*

In order to evaluate any antagonistic or synergistic effect between PFOA and/or PFOS and complex mixtures of the selected pollutants, we made 4 different multicomponent mixtures: a mixture of 6 components which includes the selected pollutants previously used ($Hg^{2+}$, $Cd^{2+}$, PPB, 2,4-D, MMC and FURA), named Mix6; 2 mixtures of 7 components in which we analysed the effect of the addition of PFOS or PFOA to the complex mixture Mix6: PFOA + Mix 6 and PFOS + Mix 6, and an 8 component

**Figure 3. Combination index Plot (*fa-CI plot*) for the binary mixture PFOA + PFOS (a), and multicomponent mixtures of PFOA and PFOS with selected pollutants (b) for the *Anabaena* CPB4337 test. CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively. The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) [21]. Broken lines indicated upper and lower limits of additivity [22]. Mix 6 = $Hg^{2+}$, $Cd^{2+}$, PPB, 2,4-D, FURA, MMC.**




mixture including both PFOA and PFOS and the 6 selected pollutants, named PFOA + PFOS + Mix 6; perfluorinated surfactants on the mixture behaviour. Figure 3 shows the *fa*-CI plots for the binary mixture PFOA + PFOS (Figure 3a), as well as those of the different multicomponent mixtures Mix 6, PFOA + Mix 6, PFOS + Mix 6 and PFOA + PFOS + Mix 6 (Figure 3b). As shown in the figure, binary mixture of both perfluorinated surfactants PFOA + PFOS sowed a strong antagonism in the whole range of *fa* values.

Regarding multicomponent mixtures (Figure 3b), the multicomponent mixture Mix6 was synergistic in the whole range of *fa* values, keeping a constant CI value of around 0.7. The addition of PFOA or PFOS to mix 6 resulted in a dual effect on the *fa*-CI behaviour: at low to mean levels, both mixtures, PFOA + Mix6 and PFOS + Mix 6 became more synergistic than Mix 6 with a decrease in the CI values down to 0.47 at very low effect levels (fa < 0.1); however, at high *fa* values (>0.8), both 7-component mixtures approached additivity. The addition of both PFOA and PFOS to the multicomponent mixture Mix6 had a marked effect leading to a dual synergistic/antagonistic behaviour; at the lowest *fa* values, the mixtures was more synergistic than mix 6 but the synergism significantly decreased with increasing *fa* levels until it approached an additive effect at *fa* levels between 0.3 and 0.7 and turned into antagonism at *fa* values > 0.7. Correlation analyses were also made between CI values of the 4 complex mixtures and their binary (for Mix 6) or ternary combinations (for PFOA + Mix 6 and PFOS + Mix 6) to determine which component mixture interactions were predominant in the multicomponent mixtures (Table 3).

For the Mix6 mixture, the highest positive correlation coefficient was found for the binary mixture of the two pharmaceuticals MMC + FURA ($r$ = 0.997) suggesting that this combination interaction predominated in this mixture. In both the PFOA + Mix6 and PFOS + Mix6 combinations, the ternary mixtures including pharmaceuticals (PFOA + MMC + FURA and PFOS + MMC + FURA) showed the highest correlation coefficients ($r$ = 0.999 for both mixtures); besides, for the PFOA + Mi x6 combination, the ternary PFOA + heavy metals mixture also showed appositive high correlation coefficient ($r$ = 0.998). In the most complex mixture which included both perfluorinated surfactants and all the selected pollutants (PFOA + PFOS + Mix 6), the highest correlation coefficient was found for the ternary PFOS + MMC + FURA and PFOA + Hg + Cd mixtures ($r$ = 0.999 and 0.998, respectively), closely followed by PFOA + MMC + FURA ($r$ = 0.994), indicating that the mixtures including these two pharmaceuticals followed by those containing both heavy metals clearly influenced the observed interaction behaviour of the four complex mixtures.

Synergism is predicted by the CI method at low to

**Table 4. Observed toxicities of the multicomponent mixtures Mix 6, PFOA + Mix 6, PFOS + Mix 6 and PFOA + PFOS + Mix 6 and Combination Index (CI) calculations at mixture NOECs concentrations.**

| Mixture | Total Mixture concentration[1] (mg/l) | Observed toxicity (% inhibition) | Experimental CI values |
|---|---|---|---|
| Mix 6 | 0.664 | **17.91 ± 5.02** | 0.66 ± 0.04 |
| PFOA + Mix 6 | 1.210 | **23.16 ± 9.65** | 0.54 ± 0.06 |
| PFOS + Mix 6 | 1.835 | **19.09 ± 5.03** | 0.63 ± 0.02 |
| PFOA + PFOS + Mix 6 | 2.382 | **21.40 ± 8.03** | 0.73 ± 0.04 |

**[1]: Total Mixture Concentrations at mixture NOECs as estimated by Dunnett's test [23, 24]. The individual concentrations (mg/l) of each component at mixture NOECs were as follows: $Hg^{2+}$ = $7.81 \cdot 10^{-4}$, $Cd^{2+}$ = 0.0031, PPB = 0.426, 2,4-D = 0.3905, MMC = 0.0011, FURA = 0.033, PFOA = 0.5464; PFOS = 1.171.**

very low effect levels in the complex mixtures tested (Fig. 3b), this might have implications in risk assessment; thus, in order to find out whether these predicted CIs could be real, we made a set of experiments in which, for the four complex mixtures, chemicals were mixed at their calculated mixture NOECs concentrations. At these concentrations, individual components of complex mixtures did not exert any toxicity (not shown). The results on observed toxicity and calculated Ci values for the four mixtures are shown in Table 4. All these mixtures inhibited luminescence by around 20 % (*fa* = 0.2), the computed CI values for these levels of effect for each mixture clearly indicated synergism (CI in the range from 0.54 to 0.73), confirming the predicted synergism at low/very low effect levels.

## 4. Discussion

In this work, we describe by the first time the toxicological interactions of two of the most environmentally relevant fluorinated surfactants, PFOS and PFOA [3, 4] with several priority and emerging pollutants in a bioluminescent cyanobacterium which has previously proved very useful in ecotoxicity studies [7, 8, 15, 16].

Except for PFOA/PFOS interaction with heavy metals, we found that, in general, PFOA and PFOS interacted in an inverse way with the organic pollutants in their binary and ternary combinations, this fact may be related to their relative hydrophobicity if perfluorinated compounds may increase the accessibility of co-existing chemicals as suggested by Liu et al. [14]. The chemicals we have used have different hydrophobicity as shown by their log $D_{ow}$ values (a better descriptor of hydrophobicity for compounds that dissociate in aqueous solution) (table 1); according to these values, propylparaben and PFOA are the most hydrophobic and PFOS and 2,4D the most polar of the organic compounds; the polar surface area (table 1) gives an idea of the ability of a particular chemical to permeate cell membranes since it is a descriptor that was shown to correlate well with passive molecular transport through membranes [17]. PFOA has a lower polar surface area than PFOS, perhaps indicating a higher capability of permeating membranes; in fact, Nobels et al. [26] reported a higher level of membrane damage by PFOA than that induced by PFOS. Our results show that PFOA interacted synergistically with the most hydrophobic compound propylparaben and antagonistically with the most polar one, 2,4-D, in their binaries while PFOS interacted just in the opposite way with both chemicals. Liu et al. [14] found that PFOS differentially affected the toxicity of compounds which differed in their hydrophobicity; unfortunately, there seems to be no reports on the combined toxicity of PFOA with other substances; our results suggest that, although relative hydrophobicity may be a relevant factor in the interactions of PFOS and PFOA with co-existing organics, both also interacted in opposite ways with compounds with similar hydrophobicity such as mitomycin C and furazolidone; this may imply more complex and different mechanisms of interaction of PFOA and PFOS with these compounds not directly related with their relative hydrophobicity. Several authors have already suggested that the toxicity of PFOS and PFOA should be addressed separately as they seem to behave differently, independently of the toxicity endpoint [26-28].

The interaction of both PFOS and PFOA with heavy metals was totally different to the ones with the

organic chemicals; in the case of Hg and Cd, both PFCs interacted mostly antagonistically; the most plausible explanation could be the stabilization of the metals through either complexation or counter-ion exchange with the negatively charged surfactants at the assay pH as proposed for the reduction of Cd and Pb uptake in a macroalga in the presence of the anionic surfactant SDS [29].

With regards to complex mixtures, the mixture of the six compounds was clearly synergistic at almost all effect levels, addition of PFOA or PFOS increased the synergism, particularly at low effect levels, with the most hydrophobic PFC, PFOA, inducing a higher synergistic interaction; as expected due to their antagonistic behaviour in their binary, the addition of both PFOA and PFOS to mix 6 decreased the observed synergistic interaction in practically the whole *fa* range, indicating that the presence of both PFCs could decrease the toxicity of co-existing chemicals.

The predicted synergism at low to very low effect levels in all the complex mixtures indicated a potential toxicological risk associated with the co-existence of these compounds at low or very low concentrations in the aquatic environment; to demonstrate that the predicted synergism by CI was real of each mixture, we tested the individual toxicity of each compound at the concentration present in these mixtures, and we found no toxic effect. Then we made new mixtures based on these concentrations, finding a luminescence inhibition of around 20% with experimental CIs smaller than 1, indicating that the predicted synergism by the CI method was real and could be of environmental relevance.

As different PFCs may co-exist in the same environment and toxicological interactions among them as the ones showed in this report could occur, studies of the combined toxicities between as many PFCs as possible as well as between them and other substances should be performed, specially directed to find out compounds with might interact non-additively or which may greatly influence the pattern of interactions in complex mixtures. We propose that the CI method which quantifies the interactions, if any, and which is independent of the mechanism of action of the tested compounds may be a useful approach to carry out such studies.

## 4. Conclusions

When individual toxicities were tested, the perfluorinated surfactants PFOS and PFOA, 2,4-D and propylparaben showed lower toxicity than $Hg^{2+}$, $Cd^{2+}$, mitomycin C and furazolidone which could be considered as "very toxic to aquatic life". The antagonistic interaction between PFOA and PFOS at all effect levels as well as the relative hydrophobicity of the tested compounds could explain the opposite interaction pattern of both perfluorinated surfactants with the organic chemicals. Both PFOA and PFOS interacted antagonistically with both heavy metals; this could be explained by stabilization of the cations in the solution by the negatively charged surfactants. The CI method predicted synergism in all the complex mixtures at low effect levels which may have implications in the real environment; pollutant combinations at their mixture NOECs confirmed the predicted synergism.

## 5. Acknowledgements

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# CHAPTER V

## Physicochemical Characterization and Ecotoxicological Assessment of $CeO_2$ Nanoparticles Using Two Aquatic Microorganisms.

# PHYSICOCHEMICAL CHARACTERIZATION AND ECOTOXICOLOGICAL ASSESSMENT OF $CEO_2$ NANOPARTICLES USING TWO AQUATIC MICROORGANISMS.

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**(HIGHLIGTED ARTICLE[1])**

[1]**Editor's Highlight:** This research integrated extensive characterization of cerium oxide ($CeO_2$) nanoparticles with toxicity studies in green algae and cyanobacterium. There was no evidence for uptake of nanoparticles by the cells, but cytotoxicity was observed in a concentration-dependent manner that was associated with nanoparticle aggregates and cell membrane disruption. Characterization of the nanoparticles as a function of concentration and toxicity was exemplary, and the data are highly relevant for ecotoxicity assessment of nanomaterials. **Toxicological Sciences** 119(1):135-45.

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# Physicochemical Characterization and Ecotoxicological Assessment of $CeO_2$ Nanoparticles Using Two Aquatic Microorganisms

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**The physicochemical properties of nanoparticles determine their interaction with living organisms. Four different cerium oxide nanoparticles, including commercial materials, were characterized and compared with a micron-sized ceria. The formation of aggregates as well as ζ-potential, surface area, and chemical composition were determined. The formation of primary particle aggregates was a slow process that led to different particle sizes depending on the composition of the medium. In this paper, we describe the toxicity of cerium oxide for the self-luminescent cyanobacterial recombinant strain *Anabaena* CPB4337 and the green alga *Pseudokirchneriella subcapitata*. The toxicity for *Anabaena* exposed to nanoparticles in pure water for 24 h ranged from 0.27 to 6.3 mg/l; *P. subcapitata* $EC_{50}$ (yielded effective concentration of nanoparticles that inhibits the cellular function of interest by 50%) values in the 2.4–29.6 mg/l range. Images of both organisms showed membrane disruption and highly damaged cells. Free cerium was highly toxic for both organisms, but the negligible amount found dissolved in the nanoparticle suspensions could not explain the observed toxic effect of nanoceria on the aquatic organisms; the dissolution of zinc could contribute to the toxicity of bulk material but could not explain the toxic effect of nanoceria either. We found no evidence of nanoparticle uptake by cells, but our observations suggested that their toxic mode of action required direct contact between nanoparticles and cells; in the case of the cyanobacterium, cells completely coated by layers of ceria nanoparticles were observed. Cell damage most probably took place by cell wall and membrane disruption; further research is needed to find out whether the oxidative activity of ceria could be responsible.**

*Key Words:* **cerium oxide; microalgae; cyanobacteria; nanoparticles; membrane disruption.**

Nanosized particles have always been present in nature, but the accelerating penetration of engineered nanoparticles (ENP) in the market is raising serious concerns over their potential impact on the environment. A key aspect for the ecotoxicity assessment of ENP is the need to address their physicochemical behavior in biologically relevant media. This is because their environmental exposure is strongly dependent on adsorption and aggregation phenomena that may limit exposure but also promote locally high concentrations in sediments or biofilms (Handy *et al.*, 2008). Cerium oxide nanoparticles have a wide range of applications. They are used as an additive in diesel fuel, but the commercial success of this application is limited; the main use of nanoceria being the formulation of slurries for the chemomechanical planarization (CMP) of silicon wafers in the production of integrated circuits.

Recent works have studied certain aspects of the effect of nano-$CeO_2$ in human cells. Xia *et al.* (2008) and Park *et al.*, (2008b) studied their effect on bronchial epithelial cells (BEAS-2B) and found a toxic effect, which was related to the production of harmful reactive oxygen species (ROS). Both works demonstrated the effective uptake of nanoparticles by the cells. However, the toxicity of nanoceria is still not clear and Park *et al.* (2008a) reported, under the same conditions, the absence of any toxic effect in cell lines from the human brain and rat cardiomiocytes.

As far as ecologically relevant aquatic organisms are concerned, Roh *et al.* (2010) encountered a marked size-dependent effect of $CeO_2$ nanoparticles on the fertility and survival of the soil nematode *Caenorhabditis elegans*. Rogers *et al.* (2010) monitored the growth inhibition of *Pseudokirchneriella subcapitata* and assessed damage in cell membranes by measuring its permeability. They reported an effective concentration of nanoparticles that inhibits the cellular function of interest by 50% ($EC_{50}$) value of 10.3 mg/l of a 10- to 20-nm cerium oxide. Van Hoecke *et al.* (2009) found a significant chronic toxicity of three different-sized nanoceria for *P. subcapitata* and *Daphnia magna*, with $EC_{50}$ for algal growth in the 7.6–28.8 mg/l range. Thill *et al.* (2006) and Zeyons *et al.* (2009) studied the toxicity of nano-$CeO_2$ for the bacteria *Synechocystis* PCC6803 and *Escherichia coli*. They reported close contact in the case of *E. coli* and nanoparticles, suggesting that toxicity could be because of an oxidative response associated with the reduction of Ce(IV) to Ce(III).

Fundamental questions regarding metal oxide nanoparticle toxicity remain unsolved. It is not clear whether the internalization of particles is relevant to the induction of intracellular effects or toxicity may be because of the adsorption of nanoparticles on the cellular membrane. This holds particularly for negatively charged particles because cell membranes possess large negatively charged domains, which should repel them. The question about the importance of particle size is still unanswered with very different toxic effects being reported for particles of similar size (Aruoja *et al*., 2009; Hartmann *et al*., 2010). Surface chemistry and the reason for toxic effects observed in non-nanoparticles are other points that require further attention and possibly a change in dose metrics, generally based on mass concentration.

The existing measurements of cerium oxide in the environment lead to exposure levels much lower than the estimated no effect dose for chronic human exposure (Park *et al*., 2008b). It has also been argued that exposure models predict concentrations significantly lower than those for which ecotoxicity investigations have encountered toxic effects. Therefore, most nanoparticles, in particular nanoceria, might not have any environmental impact (Tiede *et al*., 2009). Other data are more worrying. Limbach *et al*. (2008) have indicated that the majority of the nanoparticles are captured by clearing sludge in wastewater treatment plants, but up to 6 weight percentage of $CeO_2$ reaches the exit stream. On the other hand, although Registration, Evaluation, Authorization, and Restriction of Chemical substances regulation fails to consider the new risks posed by nanoparticles, the European Parliament recently published a report advocating extensive safety testing for manufactured nanomaterials.

The reason for choosing a green alga and a cyanobacterium was the ecological position of these organisms at the base of the aquatic food web and their essential role in nutrient cycling and oxygen production. Cyanobacteria constitute a phylum of bacteria that obtain their energy through plant-like photosynthesis. They are the most widespread primary producers at the base of the marine food chain and are also crucial in many other habitats including freshwaters, saline lakes, and biological soil crusts. Using different toxicity endpoints, this paper describes the toxic effect on these organisms of several nanoparticulate and micron-sized ceria, including commercially important samples. The physicochemical characterization of particles was extensive and helped to explain the observed toxicity for both organisms, as well as facilitating the integration of our data with previous or new research.

## MATERIALS AND METHODS

***Materials.*** In this work, we tested five different types of uncoated $CeO_2$ (CAS no. 1306-38-3) particles. The set included four types of nanoparticles with nominal primary particles in the 10–60 nm range and a micron-sized material (Sigma-Aldrich, powder, < 5000 nm, 99.9% trace metals basis) used as bulk reference (B5000). The nanoparticles included (1) Sigma-Aldrich cerium(IV) oxide nano powder, < 25 nm particle size (N25) either as powder or 5 weight percentage of suspension in water and (2) Sigma-Aldrich nano powder, < 50 nm particle size (N50), and two nanoceria provided by the Organization for Economic Cooperation and Development (OECD's) Working Party on Manufactured Nanomaterials. In accordance to their stated primary particle size, we referred to these two materials as N10 and N60. Both are commercial nanomaterials intended for use as an ingredient for diesel fuel and a component of CMP slurries, respectively. Cerium(III) chloride (CAS no. 7790-86-5) > 99.99% was purchased from Sigma-Aldrich. Water suspensions were prepared with high-purity water obtained from a Milipore Mili-Q system with a resistivity of at least 18 MΩ cm at 25°C. pH adjustments were made with analytical grade sodium hydroxide or hydrochloric acid from Merck. The reagents for OECD algal growth medium (AGM) and Allen and Armon modified medium were analytical grade reagents purchased from Sigma-Aldrich.

***Characterization of nanoparticles.*** Concentrated suspensions of $CeO_2$ (100–160 mg/l) were prepared according to the following procedure. The prescribed amount of nanoparticle powder was mixed with a few drops of water in the bottom of a glass in order to create a paste. After a wetting period during which the paste was periodically removed, 50 ml of water or the liquid dispersing media was added. The glass was dispersed using a Sonics "VibraCell" ultrasound disperser (BioBlock Scientific, France) operating at 500 W for 30 s (90% amplitude). The procedure was agreed with other groups from OECD's Working Party on Manufactured Nanomaterials, and its purpose was to ensure a complete wetting and deagglomeration of the particulate solid. Once dispersion was completed, the deagglomerated suspension was taken to the desired volume according to the target final concentration and gently stirred with a magnetic rod for at least 2 h. Stock solutions were kept at 4°C in the dark and gently stirred before use or analysis while the temperature rose to normal room values.

Brunauer-Emmet-Teller surface area measurement and pore size distribution were determined by multipoint nitrogen adsorption at 77 K using a Beckman Coulter SA3100 device. Prior to analysis, samples were vacuum degassed at 200°C for 120 min. Chemical analysis was performed by Total Reflection X-ray Fluorescence (TXRF) using a Caneca 8030C spectrometer adjusted to obtain an excitation energy of 17.4 keV (Mo KR) and equipped with a Si(Li) detector (Oxford Instruments, U.K.). High-resolution transmission electron microscopy (TEM) images of nanoparticles were taken on a JEOL (JEM-2000 FX) microscope operating at 200 kV. TEM samples of nanoceria were prepared from 5 or 100 mg/l suspensions prepared as described previously. The size distribution of nanoparticles (< 6000 nm) was obtained using dynamic light scattering (DLS, Malvern Zetasizer Nano ZS). Zeta potential was measured via electrophoretic light scattering combined with phase analysis light scattering in the same instrument equipped with a Malvern autotitrator MPT-2. The measurements were conducted at 25°C using 10mM in KCl as the dispersing medium. Inductively Coupled Plasma—Mass Spectrometry analyses were performed on ultrafiltrated samples (10 kDa MWCO Vivaspin 6 Centrifugal Concentrator) on an Elan 6000 Perkin-Elmer Sciex device equipped with an autosampler AS 91. Cell counting was performed using a Z2 Coulter Counter Analyzer in line with International Standard (ISO 13319:2000).

***Toxicity bioassays and analytical procedures.*** The toxicity of each suspension was determined by monitoring the growth inhibition of the green alga "*P. subcapitata*" and by determining the constitutive luminescence inhibition of the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337. The final concentrations tested ranged from 0 (control samples) to 100 mg/l. The assays based on algal growth inhibition followed OECD TG 201. They were either performed in 96-well microplates, in which the algae were cultured in a total volume of 200 µl, or in 25-ml cultures conducted in 50-ml flasks mounted on a GFL 3005 orbital shaker. The growth of *P. subcapitata* was monitored daily for 72/96 h and assessed by optical density (OD) at 640 nm using a RAYTO RT-2100 C microplate reader and by direct cell counting using a Coulter Counter device (2.5–8.5 µm). Algae beads and culture media were

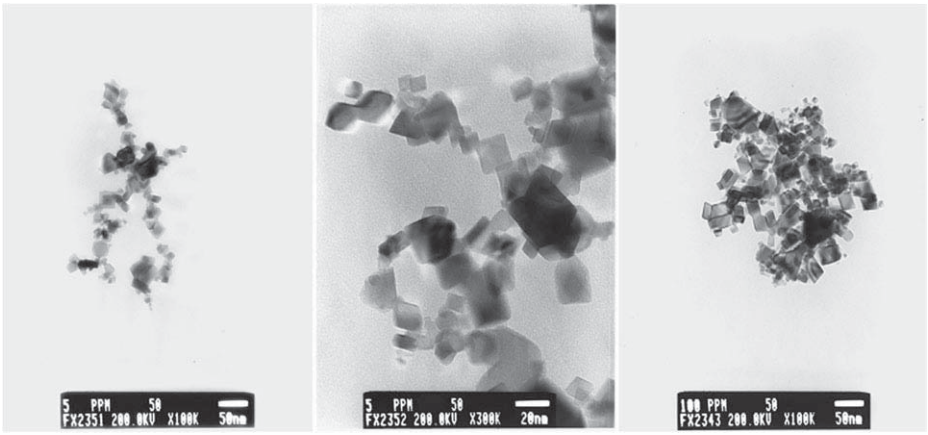


**FIG. 1.** TEM micrographs of suspensions of N50 particles in water at 5 mg/l (a and b) and 100 mg/l (c).

purchased from Microbiotest Inc. Microplates were maintained at 22°C inside a growing chamber with controlled light intensity (~100 μmol photons/$m^2$/s) and humidity levels, with no culture media evaporation and with periodical shaking and aeration. At least four replicates of each toxic concentration or blank were assayed using $ZnSO_4$ as standard for reproducibility control.

The bioassays using the bioluminescent cyanobacterium *Anabaena* CPB4337 were based on the inhibition of constitutive luminescence caused by the presence of toxics (Rodea-Palomares *et al.*, 2009a,b). *Anabaena* CPB4337 was routinely at 28°C in the light, circa 65 μmol photons/$m^2$/s on a rotary shaker in 50 ml AA/8 medium supplemented with nitrate (5mM) in 125-ml Erlenmeyer flasks and 10 mg/ml of neomycin sulfate. The assays were conducted in 96-well microplates in a total volume of 200 μl. Bioassay details are given elsewhere (Rodea-Palomares *et al.*, 2010). The assay media used were as follows: Mili-Q water, referred to as pure water (PW), pH 6; cyanobacterial culture medium AA/8 + N buffered with 2mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 6; and AA/8 + N diluted 1/10 in Mili-Q water buffered with 2mM HEPES, pH 6 (denoted AA/8 + N and AA/8 + N 1/10, respectively). Luminescence was monitored 1, 4, and 24 h after exposure to the nanoparticle suspensions and cerium(III) chloride solutions.

Quantitative measurements of adenosine 5′-triphosphate (ATP) were performed as a direct biochemical indicator of toxicity using a BactTiter-Glo Microbial Cell Viability Assay (PROMEGA). The detection of ATP was based on the production of oxyluciferin whose signal was recorded using a Fluoroskan Ascent FL plate luminometer at spectral response 350–650 nm.

Bright-field micrographs were taken with an Olympus BH-2 microscope equipped with a Leica DC 300F digital camera. For TEM, cyanobacterial and algal cells exposed for different periods to increasing concentrations of the nanoparticle suspensions were collected by centrifugation, washed three times in phosphate buffer (0.1 M Na-phosphate, pH 7.2), and fixed in 3.1% glutaraldehyde made in phosphate buffer for 3 h at 4°C. Samples were washed three times and added to 4% bacteriological agar in phosphate buffer. Postfixation was on 1- to 2-mm agar blocks using osmium tetroxide in phosphate buffer for 2 h at 4°C. The samples were then dehydrated in ethanol and embedded in Durcupan resin (Fluka) sectioned in a Leica Reichert Ultracut S ultramicrotome, stained with uranyl acetate, and observed with a JEOL (JEM 1010) electron microscope at an operating voltage of 100 kV.

***Statistical data treatment.*** The toxicity response of both organisms was estimated as $EC_{50}$ and $EC_{10}$. We used $EC_{10}$ as a surrogate for the no-observed effect concentration (Fox, 2008). For *Anabaena* CPB4337, these values were the effective concentration of toxicant that caused 50 or 10% bioluminescence inhibition with respect to a nontreated control. In *P. subcapitata*, they refer to the growth rate inhibition during the exponential growth phase. $EC_x$ and their related statistical parameters such as standard deviation, coefficient of variation, and confidence intervals were estimated using a model-independent linear interpolation method (Norberg-King, 1993). The data were computed using software (inhibition concentration percentage [ICp]) freely available from Environmental Protection Agency statistical computer programs. ICp approach uses a nonparametric monotonic regression that does not depend on any particular model allowing point estimates and confidence intervals even without the entire dose-response curve.

## RESULTS

### *Particle Characterization*

The characterization of $CeO_2$ nanoparticles was performed using a combination of DLS, ζ-potential, TEM, total-reflection X-ray fluorescence (TXRF), and surface area measurement in order to provide clear insight into chemical composition, surface chemistry, particle size, and morphology. These properties are essential for a better understanding of nanoparticle toxicity for aquatic organisms.

A first essential property governing the behavior of nanoparticle suspension in aqueous media is their tendency to form aggregates or agglomerates. In what follows and in line with Rouquerol *et al.* (1994), the assemblage of primary particles will be denoted as "aggregate" because of their loose and unconsolidated character as shown by TEM. Figure 1 shows microphotographs of two samples of suspensions of N50 at two concentrations (5 and 100 mg/l). The images show the formation of loose structures at a low concentration that tend to become larger and more compact for higher loads. The powder morphology essentially consisted of truncated octahedrons with crystallite sizes considerably lower than the nominal 50-nm cutoff, with some specimens as small as a few nanometers. We never found small independent crystals in the TEM images. Pictures for the rest of the nanoceria samples are shown in supporting information (Supplementary figs. S1–3) and reveal a similar tendency to form aggregates of hundreds of nanometers. Only for the case of N10 was any different with particularly rounded primary particles which formed tighter aggregates, a morphology compatible with its

**TABLE 1**
**Size of Aggregates (nanometer) of Suspensions of 100 mg/l of $CeO_2$ Measured by DLS in Pure Water at pH 6 after 24 h (PW + 24 h), Pure Water at pH 5 after 15 Days (PW + 15 Day), OECD AGM, pH 8, after 24 h (OECD AGM), and Allen and Arnon Modified Medium Diluted 1/10 and Adjusted to pH 6 in 2mM HEPES (AA8 + N 1/10)**

| | PW + 24 h | PW + 15 days | OECD AGM | AA/8 + N /10 | AA/8 + N |
|---|---|---|---|---|---|
| $nCeO_2$-10 | 575 | 658 | 499 | 164 | 1081 |
| $nCeO_2$-25 | 158 | 180 | 2107 | 155 | 2632 |
| $nCeO_2$-50 | 218 | 235 | 1546 | 184 | 2285 |
| $nCeO_2$-60 | 210 | 223 | 1599 | 185 | 2223 |
| $CeO_2$-5000 | 195 | 283 | 417 | 199 | 1224 |

use as diesel fuel additive. The diffraction pattern obtained in TEM showed that all the samples were dominated by cerianite, the cubic form of $CeO_2$.

DLS confirmed that cerium oxide nano powders were always present as aggregates either in water or in algal and cyanobacterial culture media and that they could not be dispersed as primary particles. The aggregate formation was dynamic and took several hours to reach a stable size distribution. Supplementary figure S4 shows the result of monitoring a suspension of N50 for 1 week after preparation. DLS size distribution tends to become broader, and the particles, dispersed as explained in the previous section, still aggregated for some hours before reaching a suspension that was stable for weeks. Higher concentrations led to larger aggregates but always achieved a stable distribution within a few hours except for the case of N10 that aggregated for some days while approaching equilibrium (Supplementary fig. S5). In all cases, the suspension was stirred before analysis as indicated before. Table 1 shows representative results for the aggregate size measured by DLS in the nanometric region both in pure water and in the media used for bioassays. We found larger aggregates for AGM and concentrated Allen and Arnon modified medium. These differences may be because of the interaction of inorganic ions or other particles from the medium with the charged surface of nanoparticles as explained below. In pure water and diluted saline medium, the aggregates were in all cases below micron size. In all cases, the size of aggregates increased with the concentration of nanoparticles and, once prepared, tended to increase over time until achieving stable dispersion. The case of micron-sized ceria (B5000), nominally a bulk material, is particularly noteworthy. Both DLS and TEM pictures show that the dispersions contained a certain amount of primary nanoparticles, with aggregates not unlike from those found with nanoceria. TEM images show small crystallites, many of them below 20 nm, together with other small particles adhering to the larger ones (Supplementary fig. S6).

The tendency to form nanoparticle aggregates depends strongly on the surface charge. The particle charge, determined as ζ-potential by electrophoretic light scattering in a Malvern's ZetaSizer instrument, was negative in all cases (with values ranging from −12 to almost −30 mV) except for pure water, pH 6, where all nanoparticle suspensions gave a positive charge with relatively high positive values for N25 and N50. The isoelectric point, which corresponds to the pH at which the surface is neutral, was in the 6.0–7.5 region so that at the bioassay pH particle charge was low in all cases. We also determined that ζ-potential changed over time during the first few hours or days after preparing a suspension. Supplementary figure S7 in the supporting information shows the case of N50 measured just after preparation and 1 week later. After that time, we observed no further changes in any case. This effect can be explained by the adsorption of ions from solution or by the changes in the hydration state of the surface. ζ-Potential and isoelectric point are listed in Table 2.

The specific surface of all nanoparticle samples was in the 30–60 $m^2/g$ range. The bulk oxide, however, had a much smaller surface (4.7 $m^2/g-$). Surface area characterization using nitrogen adsorption at 77 K also allowed the size of primary particles to be determined. The results are listed in Table 2. All materials exhibited a Type II isotherm (International Union of Pure and Applied Chemistry classification of adsorption isotherms) that corresponded to a macroporous adsorbent with strong adsorbate-adsorbent affinity. Supplementary figure S8 displays the full isotherm corresponding to N50. The pore volume for this material was 0.15 $cm^3/g$ with 50% of pores between 15 and 50 nm (using Barrett-Joyner-Halenda desorption isotherm), the rest of the materials displaying similar characteristics. The chemical composition of samples assessed by TXRF detected calcium and very minor amounts of other metals, probably impurities from production precursors. Vanadium, chromium, manganese, iron, nickel, copper, gallium, arsenic, selenium, and bromine were below the detection limit in all samples. For the rest, the data are listed in Table 2. Worth noting is the fact that, in addition to cerium, we detected minor amount of metals including zinc, whose possible toxic effect is discussed below.

### *Ecotoxicity Results*

The data shown in Table 3 correspond to the luminescence inhibition of *Anabaena* CPB4337 in water and in modified Allen and Armon medium (diluted up to one-tenth). The data include dissolved cerium chloride with doses expressed in milligrams per liter of $Ce^{3+}$. The results show a significant toxic effect for ceria nanoparticles duirng a 24-h assay in water at pH 6. Medium effect values were as low as 0.56 and 0.27 mg/l for N25 and N50, respectively. The toxicity of N25 and N50 in water is probably related to the positive ζ-potential measured in these conditions (Table 2). $EC_{50}$ was also below 1 mg/l for dissolved $Ce^{3+}$ and lower than 10 mg/l for the rest of

**TABLE 2**
**Physicochemical Properties of Nanoparticulate and Bulk Cerium Oxide**

| | N10 | N25 | N50 | N60 | B5000 |
|---|---|---|---|---|---|
| Size of primary particles (BET, nm) | 12 | 13 | 22 | 28 | 176 |
| BET surface area ($m^2$/g) | 67.6 | 63.2 | 37.8 | 29.9 | 4.7 |
| ζ-Potential (mV) | | | | | |
| Pure water, 10mM KCl, pH 8 | −23.0 ± 1.7 | −12.8 ± 0.5 | −14.1 ± 0.4 | −21.5 ± 1.1 | −20.7 ± 2.3 |
| Pure water, 10mM KCl, pH 6 | + 0.4 ± 0.8 | + 22.4 ± 1.3 | + 18.7 ± 0.8 | + 0.7 ± 1.1 | + 8.3 ± 0.9 |
| AGM, pH 8 | −12.5 ± 0.9 | −15.5 ± 1.0 | −16.0 ± 0.9 | −10.9 ± 0.3 | −12.2 ± 0.1 |
| AA/8 + N 1/10, pH 6 | −28.5 ± 1.2 | −27.5 ± 1.1 | −28.6 ± 0.8 | −29.4 ± 1.4 | −17.8 ± 0.7 |
| AA/8 + N, pH 6 | −26.9 ± 1.5 | −19.5 ± 0.9 | −20.3 ± 1.4 | −15.6 ± 1.0 | −16.2 ± 1.1 |
| Isoelectric point (10mM KCl) | 6.12 | 7.49 | 7.38 | 6.07 | 6.20 |
| Chemical composition (TXRF, %) | | | | | |
| Cerium (as $CeO_2$) | 91.2 ± 0.8 | 93.2 ± 1.1 | 97.7 ± 1.3 | 95.8 ± 1.4 | 90.8 ± 1.4 |
| Calcium | 5.20 ± 0.14 | 5.69 ± 0.18 | 1.49 ± 0.12 | 4.18 ± 0.24 | 7.45 ± 0.38 |
| Titanium | 1.11 ± 0.09 | 0.15 ± 0.10 | < 0.083 | nd | < 0.277 |
| Zinc | 0.018 ± 0.004 | 0.010 ± 0.006 | 0.021 ± 0.007 | 0.020 ± 0.010 | 0.014 ± 0.011 |
| Sulfur | 1.60 ± 0.35 | 0.93 ± 0.36 | 0.77 ± 0.39 | < 1.236 | 1.03 ± 0.77 |
| Chlorine | 0.53 ± 0.20 | < 0.333 | < 0.234 | < 0.449 | < 0.856 |
| Dissolved metals (10 kDa Ultrafiltrated, µg/l) | | | | | |
| Zinc | 10.54 ± 0.07 | 9.15 ± 0.08 | 10.72 ± 0.07 | 14.89 ± 0.11 | 24.45 ± 0.12 |
| Cerium | nd | nd | 0.11 ± 0.06 | nd | ns |

*Note*. BET, Brunauer-Emmet-Teller; nd, not detected; ns, not significantly different from zero.

oxides including the bulk control. Table 3 also shows results in AA/8 + N 1/10 buffered at pH 6 with 2mM HEPES. The presence of salts considerably decreased the toxicity of particles but not that of dissolved cerium whose $EC_{50}$ was 1.51 mg/l, the rest being above the highest assayed concentration; essentially, the same results were obtained when the undiluted medium, AA/8 + N, was used (not shown). Even without reaching $EC_{50}$, the toxic effect was apparent at very low concentrations in saline media. These results are given as $EC_{10}$. A certain hormetic effect, which appeared as the luminescence maximum in Table 3, was also observed during the first few hours.

The effect of $CeO_2$ particles and dissolved $Ce^{3+}$ on the growth inhibition of *P. subcapitata* was assessed by measuring

**TABLE 3**
**Dose-Effect Relationship Parameters for the Luminescence Inhibition Assays Using *Anabaena* CPB4337. $EC_x$ in milligrams per liter of Particles or Dissolved Metal. The Boundaries Represent 95% Confidence Intervals. The Maximum Observed in AA/8 + N 1/10 Represents a Luminescence Transitory Enhancement**

| | 1 h | | 4 h | | 24 h | |
|---|---|---|---|---|---|---|
| Pure water pH 6 | $EC_{50}$ | Boundaries | $EC_{50}$ | Boundaries | $EC_{50}$ | Boundaries |
| N10 | 69.4 | 56.9–77.0 | 33.7 | 25.9–39.8 | 6.3 | 4.7–8.5 |
| N25 | 37.8 | 33.3–43.1 | 27.5 | 12.8–34.3 | 0.56 | 0.35–1.67 |
| N50 | 44.9 | 41.4–49.8 | 46.6 | 32.0–71.8 | 0.27 | 0.20–0.58 |
| N60 | 42.4 | 31.3–54.7 | 60.1 | 38.7–5.2 | 7.5 | 4.0–12.4 |
| B5000 | > 100 | — | 34.8 | 1.45–39.3 | 8.9 | 4.8–34.4 |
| $Ce^{3+}$ | > 100 | — | > 100 | — | 0.78 | 0.08–3.69 |

| | 1–4 h | | 24 h | | |
|---|---|---|---|---|---|
| AA/8 + N 1/10 HEPES 2mM pH 6 | $EC_{50}$ | Maximum (%) | $EC_{50}$ and boundaries | $EC_{10}$ | Boundaries |
| N10 | > 100 | + 121 | > 100 | 0.062 | 0.032–0.218 |
| N25 | > 100 | + 127 | > 100 | 0.008 | 0.004–0.047 |
| N50 | > 100 | + 124 | > 100 | 0.057 | 0.038–0.103 |
| N60 | > 100 | + 119 | > 100 | 0.089 | 0.029–0.046 |
| B5000 | > 100 | + 117 | > 100 | 0.021 | 0.003–0.030 |
| $Ce^{3+}$ | > 100 | + 121 | 1.51 (0.91±2.06) | 0.003 | 0.002±0.007 |

**TABLE 4**
**Dose-Effect Relationship Parameters for the Growth Rate of *Pseudokirchneriella subcapitata* Using Different Surrogates. $EC_{50}$ in milligrams per liter of Particles or Dissolved Metal. The Growth Time Was 72 h Except for OD Readings in the Case of N10, N60, and B5000 for Which It Was Extended to 96 h**

| | OD | | Cell counting | | ATP | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ | Boundaries | $EC_{50}$ | Boundaries | $EC_{50}$ | Boundaries |
| N10 | 12.8 | 11.1–13.9 | 29.6 | 26.3–38.1 | 12.3 | 9.6–15.2 |
| N25 | 0.95 | 0.88–1.02 | 9.7 | 8.5–11.5 | 5.2 | 3.2–7.4 |
| N50 | 0.88 | 0.49–1.25 | 4.4 | 3.2–5.9 | 2.4 | 1.5–3.6 |
| N60 | 8.96 | 8.20–9.72 | 16.4 | 13.6–19.9 | 8.5 | 5.6–11.7 |
| B5000 | 16.5 | 15.4–17.7 | 56.7 | 45.9–67.9 | 20.3 | 16.5–24.4 |
| $Ce^{3+}$ | 0.79 | 0.74–0.85 | 4.25 | 3.97–4.55 | 1.35 | 0.77–1.93 |

the OD of algae cultured in a microplate. The results, listed in Table 4, showed good reproducibility and indicated a high toxicity for dissolved cerium and two of the oxides (N25 and N50) and a relatively high effect for the rest, including the bulk ceria N5000. Both in microplate and in higher volume cultures, we observed a tendency for ceria particles to induce the flocculation of cells. Comparing $EC_{50}$ values obtained from OD, with the growth rate obtained by direct cell counting, quantified this effect and allowed a direct surrogate to be obtained for biomass density. The results for microplate tests using direct cell counting are also listed in Table 4. A set of assays were also performed in 25-ml cultures in well-aerated shaken flasks with an initial cell load of $5 \times 10^5$ to $7 \times 10^5$ cells per milliliter. The results show considerably lower toxicity than that found in microplate cultures, with $EC_{50}$ of 29.2 ± 3.0 mg/l for N50 and 35.7 ± 2.3 mg/l for N25, the boundaries representing 95% confidence intervals. Finally, the results obtained at 72 h using quantitative ATP detection show a higher toxic effect than that observed using daily cell counting, the highest toxicity corresponding to the dissolved $Ce^{3+}$.

The toxicity for the green alga assessed either by cell counting or by quantitative ATP measurement yields the same order of toxicity, ranging from the most toxic N50 to the micron-sized particles: N50 > N25 > N60 > N10 > B5000. The order of toxicity was almost the same as that found for *Anabaena* with N50 being the most toxic nanoparticle for both organisms.

In order to explore further the cellular mechanisms of the observed nanoparticle toxicity, we took both bright-field and TEM micrographs in pure water, pH 6, bioassay of *Anabaena*

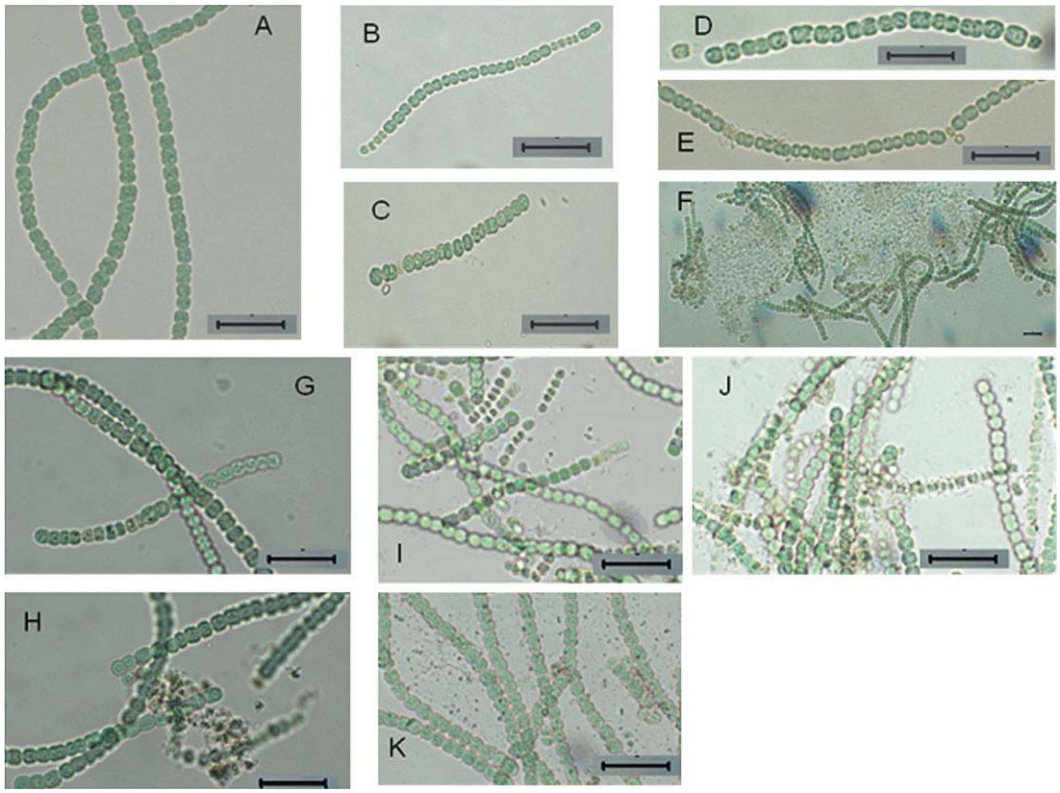


**FIG. 2.** Bright-field micrographs of *Anabaena* CPB4337 exposed to increasing concentrations of ceria nanoparticles. (A) Control *Anabaena* filaments. (B and C) *Anabaena* filaments exposed to 1 mg/l N10 for 48 h and 80 mg/l N10 for 72 h. (D, E, and F) *Anabaena* filaments exposed to 0.1 mg/l N25 for 72 h, 1 mg/l N25 for 24 h, and 80 mg/l for 24 h. (G and H) *Anabaena* filaments exposed to 0.01 mg/l N50 for 48 h and 50 mg/l N50 for 48 h. (I, J, and K) *Anabaena* filaments exposed to 1 mg/l N60 for 24 h, 50 mg/l N60 for 72 h, and 80 mg/l N60 for 24 h. Bars, 20 μm.

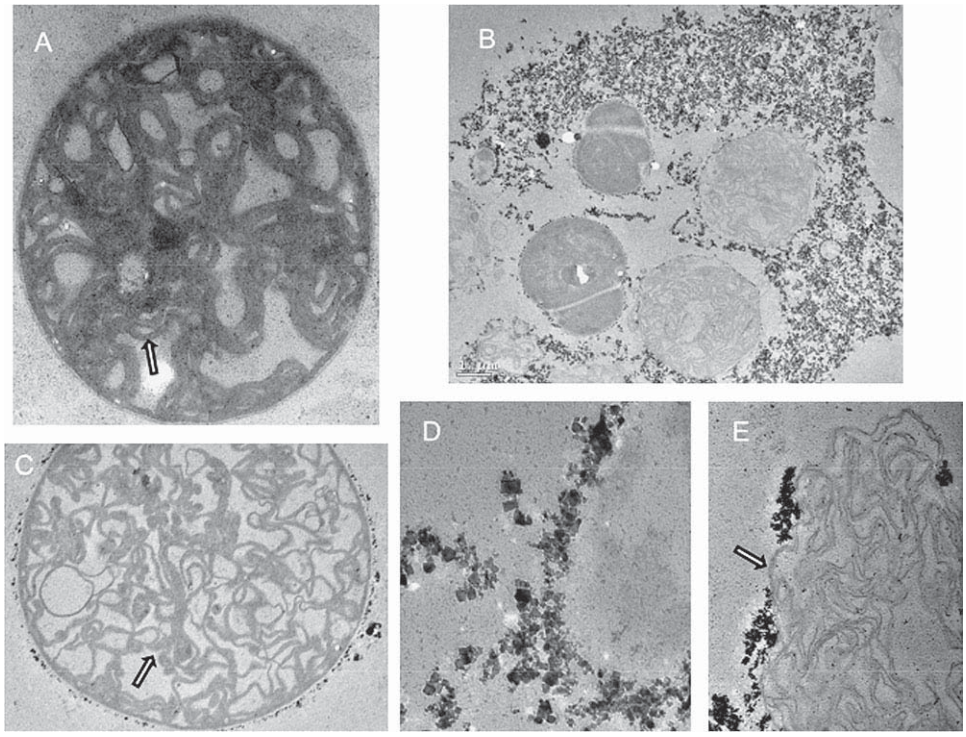


**FIG. 3.** TEM micrographs of *Anabaena* CPB4337 exposed to 80 mg/l N25 ceria nanoparticles for 24 h. (A) Control cell. (B) *Anabaena* cells in close contact to N25 aggregates. (C) *Anabaena* cell coated with N25 nanoparticles. (D) Detail of a highly damaged *Anabaena* cell coated with nanoceria showing the crystalline nature of the nanoparticles. (E) *Anabaena* cell that has lost cell envelopes with nanoparticle clusters attached to the thylakoids. Arrows indicate thylakoids. Bars: 0.5 μm (A), 1.0 μm (B), 1.0 μm (C), 0.1 μm (D), and 0.2 μm (E).

and *P. subcapitata* cells in AGM exposed to nanoparticle suspensions. Figure 2 shows that nanoparticle exposure generally resulted in a shortening and narrowing of *Anabaena* filaments, many of which manifested lysed or highly damaged cells; cell damage was already evident at low concentrations (0.01 and 0.1 mg/l, Fig. 2D, N25 and Fig. 2G, N50) of the most toxic nanoparticles, N25 and N50; for N10 and N60, damage to filaments and cells could be seen clearly at the higher concentration of 1 mg/l (Fig. 2B, N10 and Fig. 2I, N60). At high concentrations of most tested nanoparticles (Fig. 2F, N25; Fig. 2H, N50; and Fig. 2K, N60), what seemed to be large aggregates of nanoparticles could be seen. The filaments in contact with these aggregates were particularly damaged, and cell lysis debris was evident; curiously, at these high concentrations, apparently healthy filaments, which were not in direct contact with these large aggregates, could be found (e.g., filaments outside the large cell debris-nanoparticle aggregates in Fig. 2F). These observations suggest that direct contact between the nanoparticles/nanoparticle aggregates and cells could be relevant to the observed toxicity. In this regard, TEM images revealed cell damage whenever cells came into close contact with the nanoparticles; a clear example of this is Figure 3, which shows images at different magnifications of cells surrounded by large aggregates of N25 nanoparticles at 80 mg/l after 24 h of exposure. Figure 3C shows clearly an *Anabaena* cell whose cellular surface is completely coated by what seems to be a layer of nanoparticles; the crystalline structure of the nanoparticle coating is clearly visible in Figure 3D where layers of these nanoparticles cover the whole cell. The next micrograph (E) shows the remainder of a cell that has completely lost its cell wall and membrane; the nanoparticles aggregated and attached tightly to the cytoplasmatic photosynthetic membranes of cyanobacteria, the thylakoids. The observed strong adsorption of the nanoparticles to the cell wall may impair the surface architecture resulting in the complete disruption of the cell wall and membrane and leaving loose photosynthetic membranes where nanoparticles also attached themselves. Such strong adsorption followed by membrane disruption is most probably the cause of the observed toxicity that leads to cell lysis; Also, it is highly likely that when cells are completely trapped inside the shell of $CeO_2$ nanoparticles as in Figures 3C and 3D, nutrient transport is severely impaired with a resultant lack of essential nutrients and energy. As ceria nanoparticles are positively charged in the water bioassay medium and the cyanobacterial surface is negatively charged under these conditions, electrostatic attraction favors the observed strong adsorption of nanoparticles onto the cell surface that subsequently triggers cell damage.

The optical microphotographs for the green alga exposed to ceria (Fig. 4) show clearly the formation of particle aggregates that include algal cells. Figure 4B, which corresponds to a culture exposed to 40 mg/l N50 after 72 h, plainly shows how

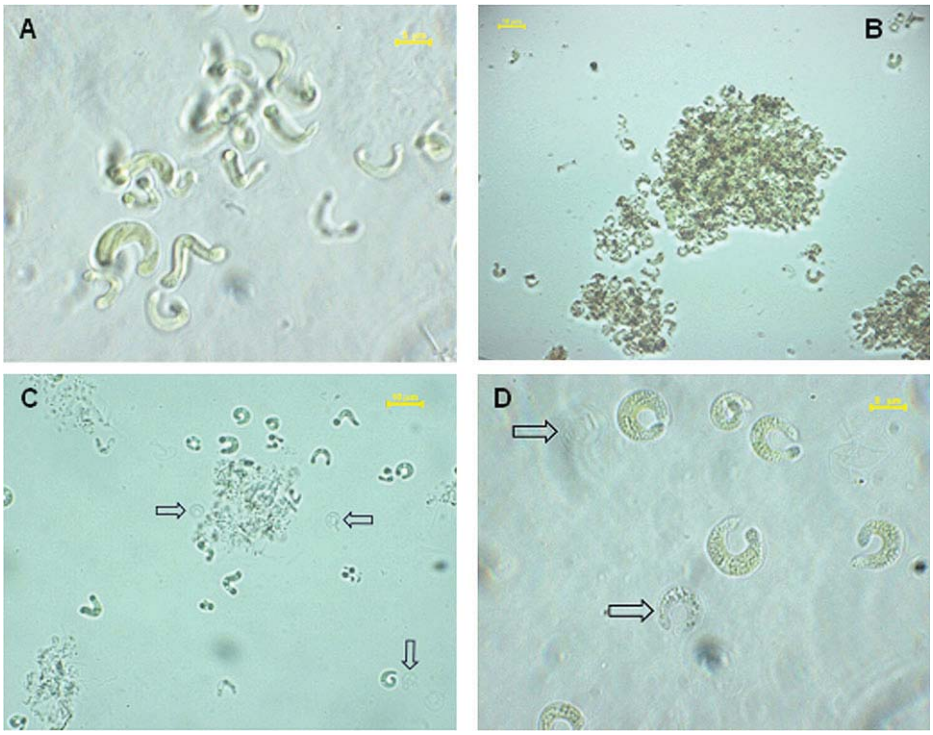


**FIG. 4.** Bright-field micrographs of *Pseudokirchneriella subcapitata* exposed to N50. (A) Control *Anabaena* filaments. (B) Algae exposed to 40 mg/l N50 for 72 h. (C) Algae exposed to 5 mg/l N50 for 48 h. (D) Cells exposed to 5 mg/l N50 for 72 h. Arrows indicate highly damaged cells. Bars: 5 μm (A), 10 μm (B), 5 μm (C), and 10 μm (D).

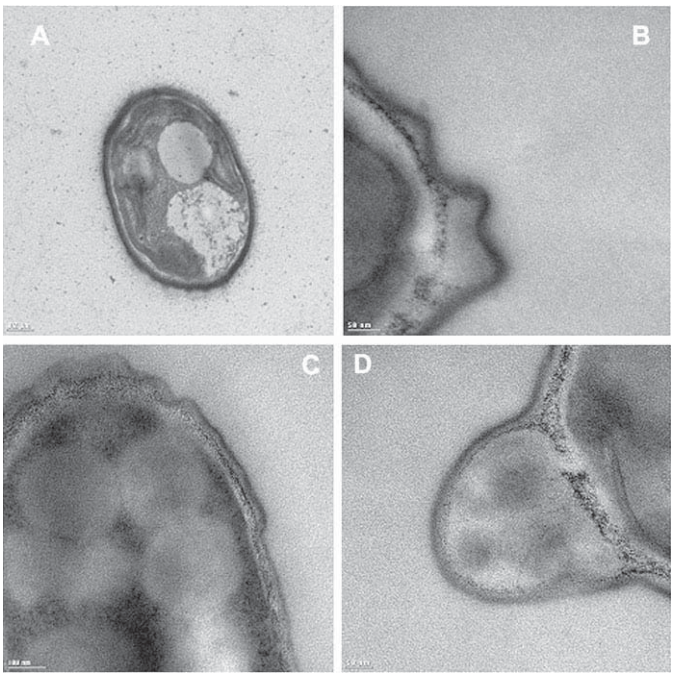


**FIG. 5.** TEM micrographs of *Pseudokirchneriella subcapitata* exposed to N50. (A) Control cells. (B, C, and D) Algae in contact with 2 mg/l for 48 h. Bars: 0.2 μm (A), 50 nm (B), 100 nm (C), and 50 nm (D).

particles and cells come into close contact even though particles at the bioassay pH are negatively charged as indicated in Table 2. For lower particle concentrations, the aggregates were looser, this is to say with a more extended conformation, but also contained cells in close contact with particles as shown in Figure 4C, which corresponds to a culture exposed for 48 h to 5 mg/l of N50. In this case, it can also be seen how aggregates coexisted with free cells that showed no apparent damage. Inside the looser aggregates and in the vicinity of many others, colorless cells could be seen quite clearly, which suggests damage to their internal structure. Figure 4D, taken at higher magnification after algae were kept during 72 h in contact with 5 mg/l N50, shows almost transparent cells that contrast sharply with the dark green ones whose chloroplast is easy to see. As far as TEM micrographs shown in Figure 5 are concerned, the occurrence of highly damaged cells, which otherwise coexisted with other apparently normal or with a minor degree of harm, may be observed. The TEM images of algae exposed to nanoceria plainly show damage to cell membranes. The disrupted cell wall appeared separated from the cell membrane with cytoplasm leaking into the vacuity (Figs. 5B and 5D). We further observed a parallel profusion of cytoplasmic vesicles, whereas chloroplast and the rest of the cell structures disappeared or became highly damaged (Fig. 5C). These highly damaged cells are supposed to

correspond to those that appeared as empty or transparent in bright-field images (Fig. 4D). Neither in the cyanobacterium nor in the alga did we find any evidence of nanoparticle uptake and internalization by cells.

## DISCUSSION

The aggregation of metal oxides in water depends on particle charge and has a significant effect on their bioavailability. We have showed that cerium oxide nano powders form aggregates in water, taking a few hours or days to reach stable size distribution (Supplementary fig. S4). This is probably a consequence of the hydration of surface and the adsorption of ions from solution, both of them processes that reach equilibrium slowly. Nanoceria exhibited a certain decrease in ζ-potential during the first few hours or days after being put in suspension. Subsequent changes were negligible, and bulk oxide was observed to behave similarly. Berg *et al.* (2009) have reported a change in ζ-potential for nanoceria samples during the first few days after suspension. We found the largest aggregates in AGM and undiluted AA8 + N, most of them in the micron-sized region, the rest being mostly around 200 nm (Table 1). These differences should be because of the interaction of inorganic ions or particles from the medium with the surface of ceria. For the bulk material B5000, a clear peak in the few hundred nanometers was obtained in all cases according to the TEM images.

The detection of zinc by TXRF was significant because of its toxicity and ability to dissolve (Aruoja *et al.*, 2009). Franklin *et al.* (2007) have reported that $EC_{50}$ was near 60 μg/l for the growth inhibition of *P. subcapitata*, whereas Rodea-Palomares *et al.*, (2009b) have reported $EC_{50}$ values of dissolved zinc of 90 μg/l (1 h) and 79 μg/l (24 h) for *Anabaena* CPB4337. If all the zinc in the samples dissolved, the final concentration would be about 0.20 μg $Zn^{2+}$/mg $CeO_2$. We found a maximum concentration of 24 μg/l zinc for a suspension of 100 ppm (B5000). This would represent an amount of zinc of 2–13 μg/l at their $EC_{50}$, and consequently, the contribution of dissolved zinc to bulk oxide toxicity was not negligible. For the most toxic nanoceria, N25 and N50, the dissolution of all zinc in samples would yield a concentration of just 0.4–1.0 μg/l $Zn^{2+}$ at their $EC_{50}$, and consequently, zinc dissolution cannot explain the observed toxicity in this case. The dissolution of ceria was negligible in all cases (Table 2).

Despite their role as primary producers in aquatic ecosystems, the toxicity of nanoparticles for cyanobacteria has only seldom been explored. Recently, Zeyons *et al.* (2009) have studied the effect of ceria nanoparticles on the unicellular cyanobacterium *Synechocystis* PCC6803 and observed a decrease of cell viability only at low concentrations of nanoparticles and in ultrapure water, but not in a synthetic salt medium. Our results (Table 3) show the clear toxicity of all tested ceria nanoparticles for the filamentous cyanobacterium *Anabaena* CPB4337. The effect was much more significant in the pure water assay at pH 6 than in the growth medium. As already mentioned, the presence of salts considerably decreased the toxicity of the particles. This could be the consequence of an aggregation effect because of the presence of other particles and dissolved electrolytes. However, the fact that toxicity also decreased in saline medium for dissolved cerium seems to indicate that the cells were inherently less vulnerable in the presence of salts. The fact that microorganisms are more susceptible to the toxic effect of nanoceria in salt-depleted media has also been reported by Thill *et al.* (2006) and Zeyons *et al.* (2009) who attribute it to the aggregation of nanoparticles in high–ionic strength media. It has been pointed out how, as ionic strength increases, the nanoparticle charge is neutralized more effectively (Keller *et al.*, 2010).

$EC_{50}$ values for the green alga were in the range of those reported by Rogers *et al.* (2010) and Van Hoecke *et al.* (2009). Cell counting led to $EC_{50}$ values approximately twice those obtained from ATP determinations, probably as a consequence of the lower ATP content of cells exposed to nanoparticles, an effect already described by Mortimer *et al.* (2010). The effect observed on the growth rate of *P. subcapitata* when using OD as surrogate was particularly high for the most toxic nanoparticles, N25 and N50. In this case, the suspensions showed a marked tendency to form aggregates and flocculate, an effect that was plain to see in larger volume cultures after shaking ceased. These low $EC_{50}$ values reflect the clarification because of the formation of aggregates and are mainly a consequence of the physical effect of nanoparticles on cells.

To gain further insights into the way nanoparticles act, we studied the effect of ceria nanoparticles on the integrity of the cells. Optical micrographs of *Anabaena* CPB4337 exposed to ceria showed shortened and yellowed filaments with a significant percentage of highly damaged/lysed cells (Fig. 2). At higher nanoparticle concentrations, cell toxicity was clearly related to the presence of nanoparticle aggregates, with damaged cells concentrating in the neighborhood or adhering to the aggregates. As shown in Figure 3, we observed a high load of nanoparticles attached as a layer to the cell walls; this adsorption was most probably driven by the electrostatic attraction between the positively charged nanoparticles in the water assay and the negatively charged cell wall of the cyanobacterium. At higher magnifications, the crystalline nature of the nanoparticles coating the cells could be seen. Strong adsorption of ceria nanoparticles to cell walls has also been found for *E. coli* (Thill *et al.*, 2006; Zeyons *et al.*, 2009) and biological sludge (Limbach *et al.*, 2008); however, Zeyons *et al.* (2009) found no toxicity for the unicellular cyanobacterium *Synechocystis* but attributed it to the production of cyanobacterial exopolymeric substance (EPS), which may adsorb nanoparticles thus preventing their interaction with cell walls. *Anabaena* CPB4337 is a planktonic organism that does not produce EPS (Pereira *et al.*, 2009); this explains why ceria nanoparticles attach so strongly to it.

Van Hoecke *et al.* (2009) found no evidence of either the uptake/strong adsorption of $CeO_2$ nanoparticles to *P. subcapitata* or ultrastructural damage. However, although we found no evidence of particles anchored to the algal cell surface, we did observe membrane rupture, cytoplasm leakage, and intracellular damage (Figs. 5B, 5C, and 5D), all of which suggested direct contact between nanoparticles and algal cell envelopes. In this regard, the formation of aggregates between particles that included algal cells in close contact was plain to see (Figs. 4B and 4C). This aggregation effect was evidenced in the tendency to flocculate cells exposed to particle suspensions and could be a consequence of the alteration of the particle charge because of the release of cell material. As for the interaction of cells with negatively charged particles, Wilhelm *et al.* (2003) have suggested that these particles bind cationic sites on the cell surface to form clusters favored by the repulsive interactions with negatively charged domains. Once bound to the cell, these nanoparticles have a lower charge density that favors the adsorption of other particles and the formation of large clusters.

There could be several explanations of how attached nanoparticles exert toxicity on the cyanobacterial/algal cell; in the case of highly damaged *Anabaena* cells trapped inside the nanoparticle shell (Fig. 3D), the transport of nutrients and metabolites across cell wall and membrane could be affected, leading to cell death because of prolonged starvation as also pointed out by Zeyons *et al.* (2009) for *E. coli* cells tightly coated by ceria nanoparticles. In addition, tightly attached nanoparticles may cause mechanical damage to the cell membrane because of the numerous edges, corners, and reactive sites present in the crystal structure of the nanoparticle as suggested by Rogers *et al.* (2010). In the end, this mechanical damage may result in membrane disruption as we have found in both the optical and the TEM images of both organisms; alternatively, direct interaction between nanoparticles and the cell membrane may potentially generate ROS, thus inducing oxidative stress and cell toxicity. Thill *et al.* (2006) and Zeyons *et al.* (2009) have found a reduction of cerium(IV) to cerium(III) when the ceria nanoparticles were firmly adsorbed onto the outer membrane of *E. coli* and have linked it to a strong cytotoxicity that could be because of lipid/protein oxidation; in mammalian cells (Park *et al.*, 2008b), the cell toxicity of ceria nanoparticles was also linked to oxidative stress.

As indicated by our results and those of others (Rogers *et al.*, 2010; Thill *et al.*, 2006; Zeyons *et al.*, 2009), the direct contact of the ceria nanoparticles with the cell wall/membranes is essential for cytotoxicity to occur. The mechanisms underlying nanoceria toxicity seem to be mediated by direct contact with nanoparticles, thus resulting in cell wall and membrane disruption, which ultimately leads to cell lysis. It would be interesting to investigate whether nutrient transport is impaired in the nanoparticle-coated *Anabaena* cells and whether oxidative stress plays a role in membrane disruption and cytotoxicity, with special emphasis on the effect of nanoceria in photosynthesis, the main bioenergetic process of both organisms, which could also be involved in the generation of ROS when photosynthetic reactions are not well balanced. As far as dose metrics are concerned, our data did not support the view that surface area might work better than mass concentration as a dose variable.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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# Physicochemical characterization and ecotoxicological assessment of $CeO_2$ nanoparticles using two aquatic microorganisms

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## Supplementary material

This section contains additional data on the particle size and properties of powders and suspensions. Fig. S1, S2, and S3 show TEM micrographs of suspensions of N10, N25, N60, respectively. Fig. S4 indicates the evolution of DLS particle size distribution of a suspension of 100 mg/L of N50 after being prepared. Fig. S5 gives the evolution of DLS particle size of N10 at different concentrations. Fig. S6 shows several TEM pictures from B5000. Fig. S7 shows ζ-potential and PZC evolution for N50 and Fig. S8 plots the Nitrogen adsorption isotherm of N50.

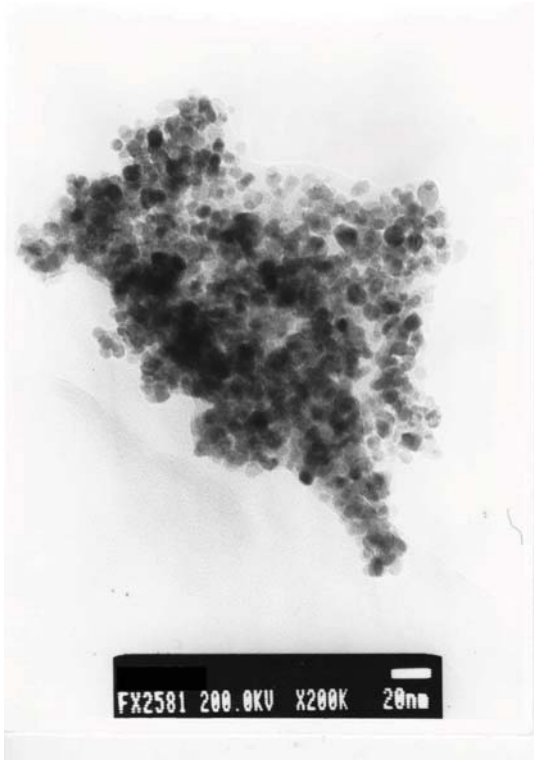


Fig. S1. TEM micrograph of a suspension of 100 mg/L of N10 particles in water

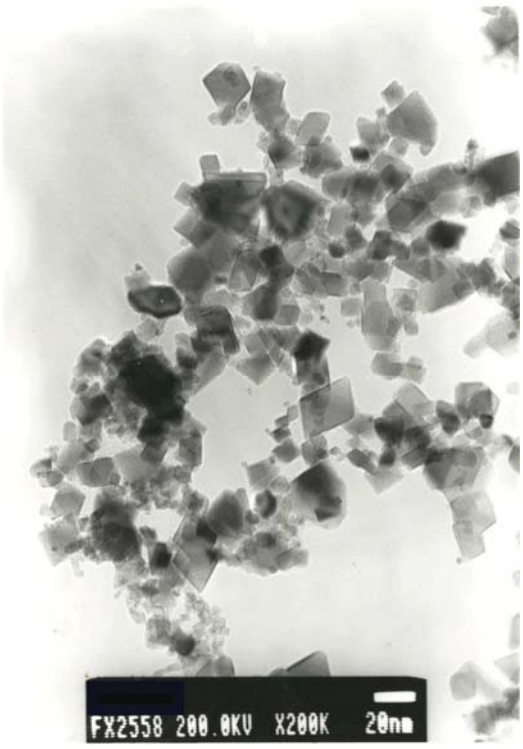


Fig. S2. TEM micrograph of a suspension of 100 mg/L of N25 particles in water

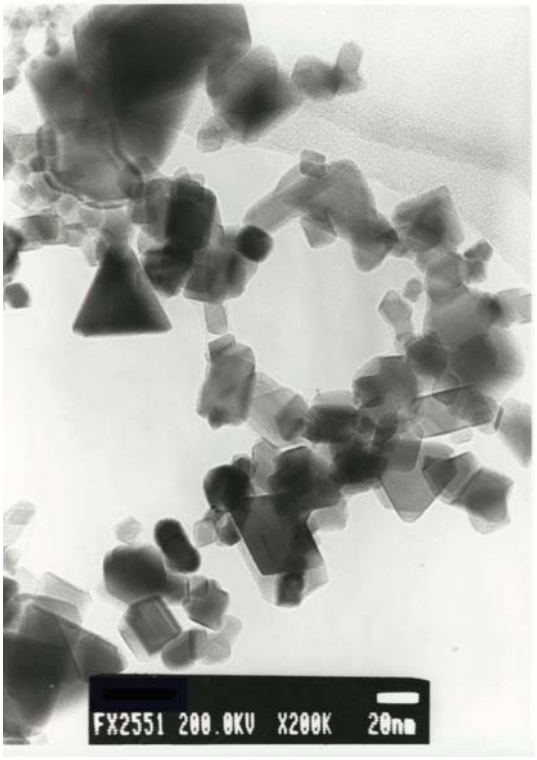


Fig. S3. TEM micrograph of a suspension of 100 mg/L of N60 particles in water

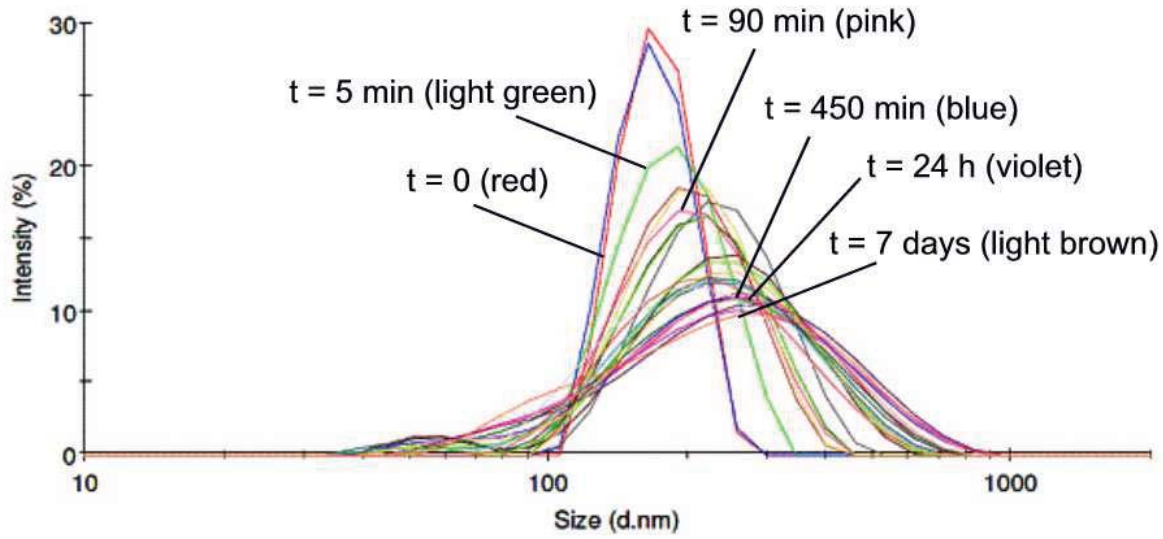


Fig. S4. Evolution of DLS particle size distribution of a suspension of 100 mg/L of N50 for one week after preparation.

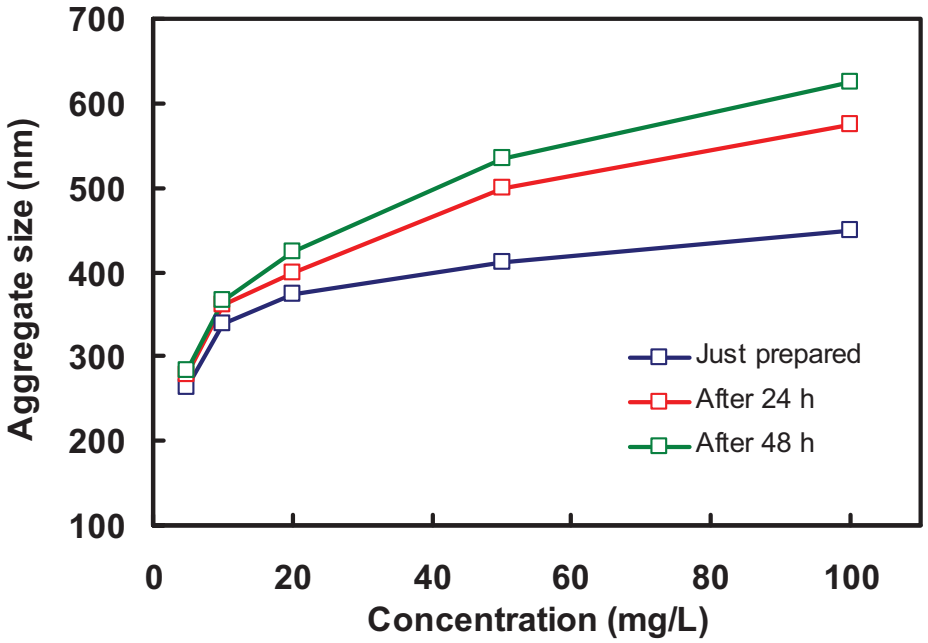


Fig. S5. Evolution of DLS particle size of suspensions of N10 in water at different concentrations from 5 to 100 mg/L.

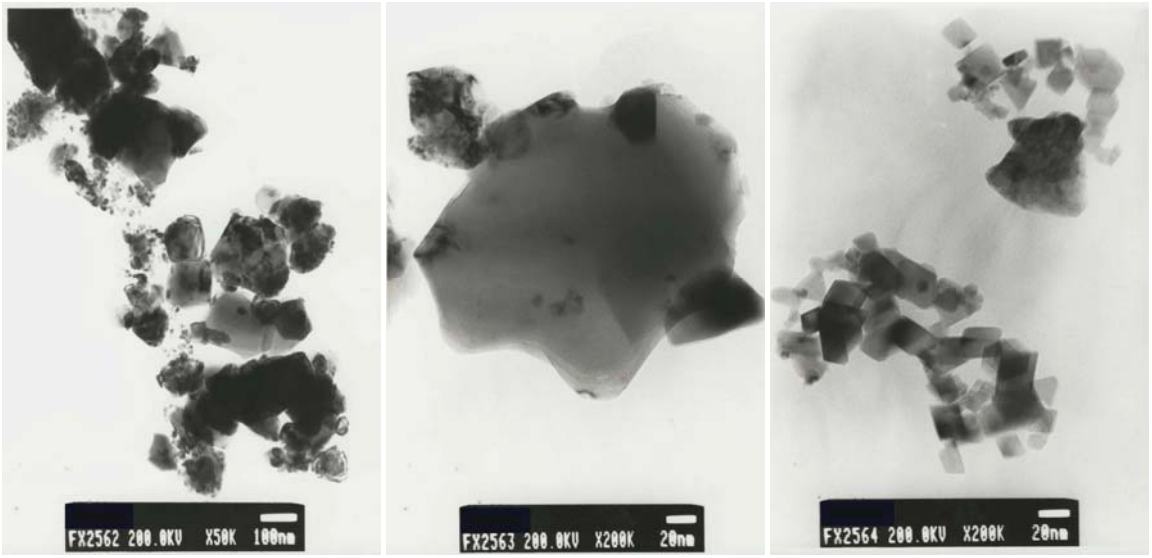


Fig. S6. TEM micrograph of suspensions of 100 mg/L of B5000 particles in water

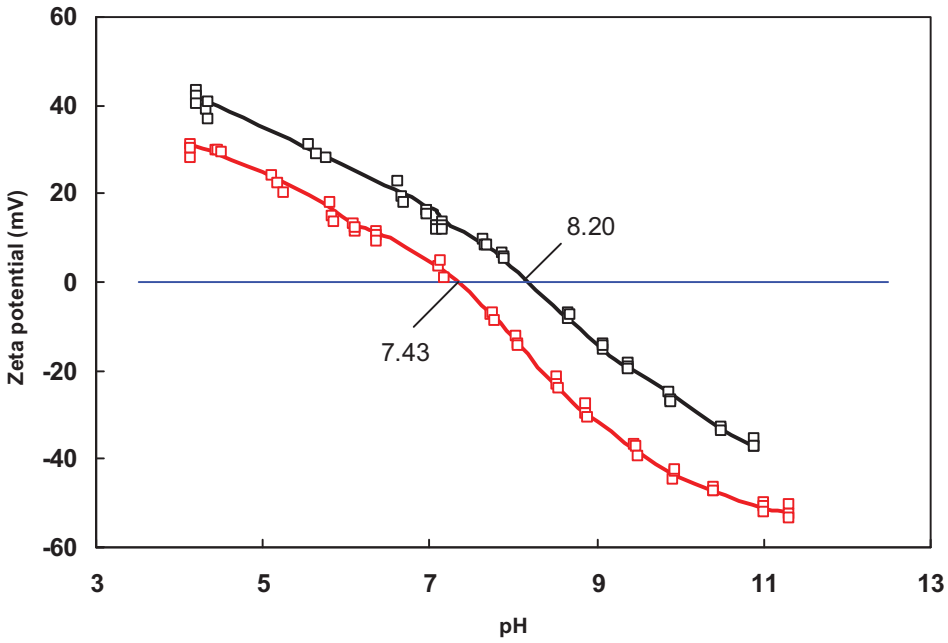


Fig. S7 ζ-potential and PZC evolution for N50. Black symbols/line correspond to a newly prepared suspension; red symbols/line to the same suspension after one week. The measurements were conducted in 10 mM aqueous KCl solutions at 25°C.

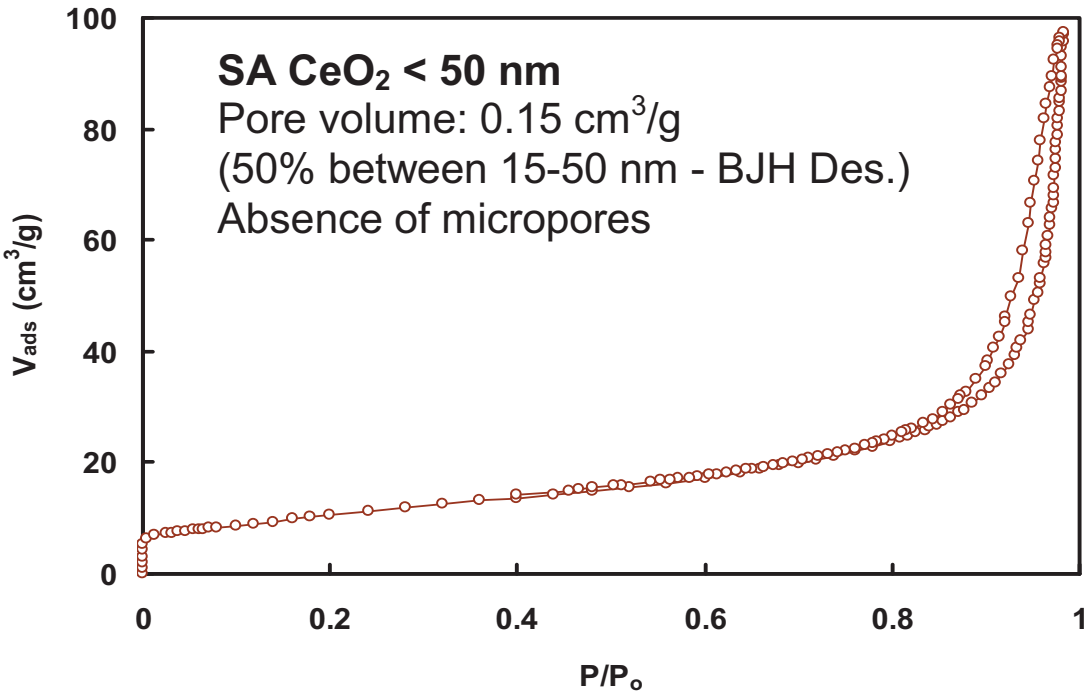


Fig. S8. Adsorption isotherm of N50 and some data calculated from it.

# CHAPTER VI
# General Discussion

## GENERAL DISCUSSION

The aim of the present thesis was to evaluate the applicability of a new general toxicity test based on the freshwater filamentous cyanobacterium *Anabaena sp.* PCC7120 CPB4337 in environmental toxicology. To get this, a general characterization of the performance of its bioluminescence in different aqueous media has been performed, the bioassay has been calibrated against a reference toxicant, and the toxicity of different priority and emerging pollutants singly and in mixture has been assessed. Its general performance and sensitivity has been compared with the commercial bioluminescent bioreporter *Vibrio fischeri* and with classical toxicity tests: *Pseudokirchneriella subcapitata* (green algae) and *Daphnia. magna* (macroinvertebrate).

### *Anabaena sp.* PCC7120 CPB 4337 as a novel toxicity biorreporter.

*Anabaena sp.* PCC7120 CPB4337 (hereinafter *Anabaena* CPB4337) carries a chromosomal integration of *luxCDABE* from *Photorhabdus luminescens*. Its luminescence is high and stable in a range of temperatures between 20ºC and 30°C (Fernandez Pinas and Wolk, 2000; Szittner and Meighen.1990). Cell viability and growth were not affected by chromosomal integration or by the generation of endogenous aldehyde (Fernandez Pinas and Wolk, 1994). In the present thesis, this novel toxicity test proved to have a series of advantages which made it attractive to be used as a complement to classical environmental toxicity tests: It is a novel generation toxicity test based on a physiological cellular endpoint (bioluminescence inhibition) which made it a very simple, sensitive and quick toxicity bioassay. In fact, these three characteristics have been the main causes of the success of the *Vibrio fischeri* based toxicity assays, such as Microtox®. This kind of bioassays makes the difference when a high number of samples are need to be processed or in experiments where a high number of parallel treatments are required to get a valid conclusion, such as in the investigation of mixture toxicity. Furthermore, not special skills are needed to perform the bioassay due to the easiness of the procedure. In fact, although the number of criticisms over its use in freshwater samples due to its lack of ecological relevance (since *Vibrio fischeri* is a marine organism) and the manipulations of samples which are needed to perform the bioassay (due to the addition of 2% NaCl to the sample) (Deheyn et al. 2004), it is still one of the most used toxicity test when assessing the effect of aquatic pollutants and specially their combined effects when a large number of components and mixtures is studied (Quin et al, 2011; Parvez et al, 2009; Kortenkamp et al, 2009; Wang et al. 2009). However, until now it has not been accepted in environmental regulation as a toxicity test likely due to the above mentioned reasons. In this regard the new cyanobacterial toxicity test *Anabaena* CPB4337 presented in this thesis has the advantages of the *Vibrio fischeri* based

toxicity tests but none of their disadvantages since it is based on a ecologically relevant freshwater organism, and it needs a minimum sample manipulation to perform the assay (just to keep constant the pH over the different treatments) enabling it to a future possible implementation in environmental regulations. Another advantage of *Anabaena* CPB 4337 is the source of its *luxCDABE* genes: they were cloned from the terrestrial luminescent bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) which is the bacterial luciferase with the highest thermal stability (Fernandez Pinas and Wolk, 2000; Szittner and Meighen.1990), so that, this toxicity test does not require to ensure a tight control of the assay temperature, as *Vibrio fischeri* assay, where temperature must be kept constant between 15 ºC – 17 ºC, otherwise serious problems of bioluminescence inhibition may occur (ISO 11348). Probably, the only disadvantage of this new cyanobacterial toxicity test with respect to *Vibrio fischeri* is the assay time: since with *Vibrio fischeri* one can get reproducible result with 5 min of time of exposure, and the cyanobacterial toxicity test requires at least 30 min to 1h of exposure to get the highest reproducibility. Nevertheless, both tests are complementary: one based on a heterotrophic bacterium and the cyanobacterial test is a photosynthetic bacterium, representing different levels in the trophic webs.

Speaking of cyanobacteria in general, an important advantage of using them as toxicity biorreporters is that they fill up an important ecological gap when assessing the toxicity of pollutants at the level of primary producers: The environmental risk of pollutants to primary producers is usually assessed by green algae or plants (ECHA, 2008; EC/2008/1272; EC/EC/2000/60), but cyanobacteria are a relevant and abundant group of primary producers (dominant in some aquatic and terrestrial ecosystems) which are prokaryotic in nature and are at the very base of the trophic webs. Furthermore, some species such as this *Anabaena* strain can fix atmospheric nitrogen into bioavailable forms, ability which being only prokaryotic is not shared with green algae and plants so that they are an important source of bioavailable nitrogen for many ecosystems (especially in oligotrophic aquatic ecosystems) and a natural source of nitrogenous nutrients in some agricultural crops such as rice (Leganés and Fernández Valiente, 1992). Cyanobacteria play a special key ecological role also in the marine environment, where they can be dominant especially in the open oceans and where they are responsible of the fixation of up to the 50% of bioavailable N forms (Sohm et al, 2011). In this case, a bioluminescent test based on a recombinant marine cyanobacterium such as *Synechococcus elongatus* strain PCC7002 whose genome is fully sequenced would be really interesting.

**Toxicity of priority and emerging pollutants**

In the second chapter of this thesis, the toxicity of heavy metals was assessed in detail by using the novel *Anabaena* CPB4337 toxicity test: The toxicity of 13 heavy metals and metalloids was assessed at short and long times of exposure, and the sensitivity of the bioreporter was compared with that of some other *luxAB* and *luxCDABE* general toxicity biorreporters, the sensitivity of *Anabaena* CPB4337 was similar or even higher than that of the majority of these biorreporters (several species of *Vibrio*, some *luxAB* and *luxCDABE* marked heterotrophic bacteria and a *luc* marked strain of the cyanobacterium *Synechoccous sp. PCC7942*) (see Chapter III; Table 3 of *Use of lux-marked filamentous cyanobacterial biorreporters for assessment of individual and combined metal toxicities in aqueous samples*). We found that *Anabaena* CPB4337 was extremely sensitive to heavy metals such as Cu, Zn and Ag at 1h of exposure and that it became very sensitive also to Hg and Ni at 24 h of exposure. In the case of Cu, the $EC_{50}$ at 24 h of exposure was as low as 5 μg/L, a concentration which can be found in rivers receiving industrial and agricultural run-offs especially from vineyards and orchards (Hsu et al, 2011; Masson et al, 2006; He et al, 2005). Curiously, *Anabaena* CPB4337 resulted to be less sensitive to $AsO_4^{3-}$ with $EC_{50}$ of one or two orders of magnitude higher with respect to the rest of heavy metals and metalloids including other anionic metalloids such as $CrO_4^{2-}$ or $VO^{3-}$ even after 24 h of exposure; it could be interesting to investigate the *Anabaena* $AsO_4^{3-}$ efflux system to construct an arsenic biosensor based on its promoter system. In the second part of this chapter we tried to link heavy metal speciation with toxicity, in order to identify which heavy metal species might exert the observed toxicity to the cyanobacterium. For this reason, the toxicity of Hg, Zn, Cd and Cu was examined in the presence of several possible modifying factors of metal speciation: pH, organic ligands (EDTA), carbonates, phosphates and chlorides. We made computer simulations of heavy metal speciation and performed correlation analyses between bioluminescence inhibition and the different metal species predicted by the speciation program. We found that in general heavy metal toxicity is exerted by free ions but some fraction of the data variability was not explained by the amount of free ion such as in the case of Zn and Hg in the presence of EDTA, and Cd and Cu in the presence of phosphate. These findings implied that other species besides the free ion may exert toxicity to *Anabaena* CPB4337 or that the toxicity is modulated by differences in the metabolic status of the cyanobacterium in the presence of these modifying factors.

In the third and fourth chapters, the ecotoxicity of emerging pollutants and real wastewater samples were assessed by using the new *Anabaena* CPB4337 toxicity test together with others standard toxicity tests in order to study the suitability of the novel biorreporter to be applied

with compounds whose toxicological mode of action is unknown and to whom cyanobacteria are non-target organisms, and to compare its performance and sensitivity to that of standard toxicity tests. The results of these experiments were really interesting since *Anabaena* CPB4337 proved to be sensitive to both, lipid regulators (even apparently lacking their known pharmacological receptor (Schoonjans et al. 1996), and fluorinated surfactants. Furthermore, the sensitivity of *Anabaena* CPB4337 was in general higher or comparable to that of any of the used standard toxicity tests (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Daphnia magna)*. The sensitivity of *Anabaena* CPB4337 was higher than that of *V. fischeri* to all the fluorinated surfactants assessed, and comparable to that of another primary producer, the green alga *P. subcapitata,* even when the green alga assays were chronic (72 h of exposure) and *Anabaena* was exposed only for 1 h. When assessing the toxicity of lipid regulators, *Anabaena* proved to be a key organism: the four fibrates could be considered at least harmful to *Anabaena* CPB4337 even in the case of bezafibrate or clofibric acid that did not evidence any toxicity when using classical tests. Even more surprisingly were the results when the toxicity of a real wastewater sample collected from an STP was assessed: *Anabaena* CPB4337 proved to be a key organism since no toxicity was found for *V. fischeri* and *D. magna* and a severe toxicity was found for the cyanobacterium. Once again, the use of this novel toxicity test allowed detecting unwanted toxic effects that might become overlooked when using conventional bioassays.

**Combination Index and the ecotoxicity of mixtures**

In the present thesis, the Combination Index method (CI) was applied by the first time in ecotoxicology. The Combination Index is a fractional approach method used in pharmacology to analyze the combined effect of drugs (Bovill, 1998), it was developed and mathematically derived from enzyme-substrate kinetics by Chou and Talalay (1984) and their definition of additivity is essentially that of Loewe (1953) which is derived from the classical definition of isobologram. This method is independent of any consideration on the mode of action of the substances under analysis (Chou, 2006). It can be used to analyze and quantify the degree of the deviation from additivity (that is, synergism or antagonism) of two to *n* substances with similar, dissimilar or unknown mode of action at any level of effect exerted on any organism (Chou, 2006). Through the chapters two, three and four of the thesis, the Combination Index method was applied to study the interaction exhibited by several mixtures of pollutants in several organisms (*Anabaena* CPB4337, *V .fischeri* and *P. subcapitata*). Some of these pollutants had the same mechanism of action (MOA) (heavy metals), the same pharmacological MOA but unknown effect on non-target organisms (fibrates), and some of them were mixtures of pollutants with similar, dissimilar and unknown MOAs (Perfluorinated surfactants, pesticides, heavy metals and pharmaceutical products). Main results of this work have been deeply

discussed in the articles of the different chapters of the thesis but some key aspects can be inferred from the whole information: Firstly, the nature of the interaction of two compounds is variable along the levels of the toxic effect (*fa*, or $EC_x$) exerted on the organism. It can greatly vary, going from synergism to antagonism, as demonstrated in the case of the ternary mixture of fenofibric acid, gemfibrozil and bezafibrate to *Anabaena* CPB4337 which went from highly synergistic (CI < 0.1) to strongly antagonistic (CI > 2). It is not very easy to explain the causes underlying this fact, but it can be imagined that there exists a gradient of direct and indirect effects and changes in the adaptive, defensive and toxicological responses of the organism depending on the general health status of the cell as indicated by the effect level ($EC_x$) making the response of the organism to this mixture to vary even from synergism to antagonism or inversely depending on the total concentration of the mixture.

Another important finding on the described work on mixtures is the fact that the nature of the interaction of the pollutants also varied with the test organism. Even in similar organisms sharing common physiological functions (as green algae and cyanobacteria), an opposite behavior can be found in the interaction of the same chemicals, as in the case of the docusate and TCP/triclosan mixtures which were strongly antagonistic for the green algae and mainly synergistic for *Anabaena* CPB4337. Similar findings have been already reported by other authors (Cedergreen et al, 2007; Teuschler et al, 2007; Cleuvers et al, 2003; Ince et al, 1999). So that in general, we cannot speak about a *synergistic*, *antagonistic or additive* mixture in an absolute way since the "interaction" is, as bioavailability, a biological function and not an intrinsic property of the chemicals.

Finally, two key points for the risk assessment of complex mixtures are: firstly, which degree of deviation from additivity is significant enough to need to be taken into account in risk assessment? Or in other words: When Concentration Addition (CA) and Independent Action (IA) models are not good enough to predict the toxicity of certain mixtures?; And secondly, does the increase of complexity of mixtures make them more synergistic or antagonistic, or on the contrary, make them to approach additivity? By using the CI method we found statistically significant departures from additivity (synergism and antagonism) in a large number of mixtures, but in many cases the degree of interaction was not very large (less than a factor of 2 from additivity) (Boobis et al, 2011; Kortenkamp et al, 2009). In the other hand, some mixtures produced synergistic effects with CI < 0.1 (that is more than 10 times more toxic than expected under the additive assumption) specially at low effect levels ($EC_{10}$), such as the mixtures of fenofibric acid plus gemfibrozil (Fn + Gm) and fenofibric acid plus gemfibrozil plus bezafibrate (Fn + Gm + Bz) (Chapter III). Recently, Boltes et al, (2011) reported a CI as low as 0.01 at low levels of effect for a PFOS + Clophibric acid mixture to *P. subcapitata*. Some recently reviews

about this issue state that deviations from additivity lesser than a factor of 2 should not be considered as a significant deviation from additivity for risk assessment purposes, but it is still a matter of discussion (Boobis et al, 2011; Spurgeon et al, 2010; Kortenkamp et al, 2009). With respect to the effect of the increasing complexity on the nature of the interaction, some conclusions can also been inferred from the whole results of the present Thesis: Figure 14 summarizes the tendency of CI values at three representative levels of effect *fa* ($EC_{10}$, $EC_{50}$, $EC_{90}$) in mixtures ranging from binary to multicomponent mixtures including all the mixtures tested in the Thesis for *Anabaena* CPB4337. As can be seen in the figure, we found that in general, the higher degree of departure from additity is found in binary and ternary mixtures. An increase in the number of components above four in equitoxic concentrations normally resulted in a reduction of the departure from additivity. Similar results have been found by other authors for multicomponent mixtures in equitoxic concentrations (Faust et al, 2003; Alterburger et al, 2001)

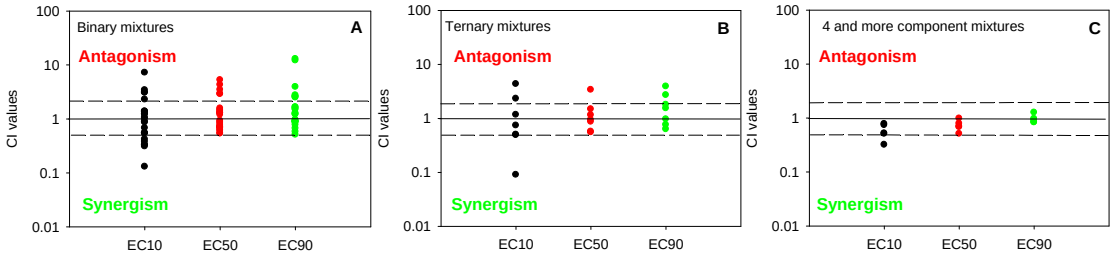


Figure 14. CI values of binary (A), ternary (B) and 4 and more component mixtures (C) at three representative levels of effect ($EC_{10}$, $EC_{50}$, $EC_{90}$) of all the mixtures used in the thesis (heavy metals, fibrates, PFCs and organic pollutants). Straight lines represent additivity, broken lines indicate suggested limits for additivity in risk assessment (CI = 2 upper limit for antagonism, CI = 0.5 upper limit for synergism).

This doesn´t mean that this tendency to additivity in complex mixtures in laboratory would be the general rule in real mixtures; in fact, multicomponent mixtures dominated by one to three main contaminants (that is mixtures with components with concentrations of one or two orders of magnitude higher, in dose or potency, than those of the rest of components) are not rare in industrial effluents (Botalova et al, 2011; Parvez et al, 2009). In those mixtures in which few main contaminants are dominant, the whole mixture may be considered essentially as a binary or ternary mixture, and therefore synergistic interactions may appear especially at low levels of effect ($EC_{10}$). In fact, in the chapter III of the thesis, mixtures of fibrates and wastewater were analyzed and the resulting interactions were synergistic; in these case, just one dominant component in the resulting mixture (the fibrate) was able to interact synergistically with the background components of the wastewater all of them present in concentrations really low (in the micro and nano molar range) (Rosal el al, 2008). So that, additivity (considering the window of deviation of 2-fold from the CA assumption) seems to be the apparent common rule (Boobis et al, 2011; Kortenkamp et al, 2009), at least in the commonly used equitoxic mixture

experimental design (whether based on $EC_x$ or NOECs) but possible departures from additivity must be investigated in non-equitoxic mixtures in the future, especially at low levels of effect, as well as compounds with a common tendency to result in non-additive interactions (Cedergreen et al, 2006), in this regards, fibrates which interacted synergistically at low levels of effect, and perfluorinated surfactants, as PFOS and PFOA which interacted antagonistically to *Anabaena* CPB4337 and displayed an inverse interaction with some organics, may receive special attention. Regarding the Combination Index itself, a key point is whether it is possible to use it in a predictive way as Concentration addition (CA) and Independent Action (IA), allowing correcting the predictions of additivity models in function of the degree of interaction of the components of the mixture. In principle, the CI equation could be rearranged in a predictive form, but the CI values would remain as an unknown term in the equation. To assign values to the CI term is not a trivial issue at all, since they are *a priori* unknown and in theory they should be calculated for each organism, each mixture, each level of effect and each mixture ratio. However, the possibility of at least identifying compounds with a general tendency to synergy in several taxa, and their mean CI value for low levels of effect; and also to identify complex mixtures in which the resulting CI values are kept more or less constant at all effect levels can be interesting ideas, and not so difficult to implement in the additivity models (CA and IA) or in a predictive CI equation.

**Ecotixicology of $CeO_2$ nanoparticles**

In the last chapter of the thesis we analyzed the toxicological effects of $CeO_2$ nanoparticles on two aquatic organisms: the green alga *P. Subcapitata* and the cyanobacterial bioreporter *Anabaena* CPB4337. In this experimental approach, we went a step forward: we linked toxicity with the physiochemical characteristics of the nanoparticles suspensions in different aqueous media, and tried to identify the mechanisms by which the toxicity of $CeO_2$ nanoparticles is exerted to the cells, for this we made an extensive physicochemical characterization of the nanoparticles (ζ-potential, aggregation state and size distribution of aggregates, TEM images), and an extensive toxicological characterization (several toxicological endpoints, bright field and TEM images). As discussed in chapter five, toxicity of nanoparticles was strongly affected by the pH and salinity of the assay media in the case of the cyanobacterium with effects which went from an increase in luminescence [hormesis, an increase in the toxicological endpoint under study which has been reported at low toxicant concentrations; (Calabrese et al, 2008)] in AA/8 + N cyanobacterial growth medium to $EC_{50}$ values of 0.27 for N50 ($CeO_2$ nanoparticles of nominal size less than 50 nm) in $H_2O$ at 24h of exposure. In this case, similar to the study on heavy metal speciation (chapter II), a good knowledge of the physicochemical behavior of the toxicant in different media allowed us to better understand the toxicological effects. When we

analyzed bright field images, we realized that at very low nanoparticle concentrations morphologically alterated *Anabaena* cells could be found, and that even at the maximum concentration of nanoparticles, intact cells could be found together with really damaged cells both for the cyanobacterium and green algae, it made us think that a direct contact between nanoparticles and cells is required to produce toxicity and that even at very low concentrations nanoparticles could exert toxicity if the cell got in contact with a nanoparticle.

When we analyzed TEM images we focused in two main facts: firstly, if nanoparticles were able or not to attach firmly and to produce changes in the cellular envelope; and secondly if they were able to be internalized into cells or if structural intracellular alterations could be seen even without nanoceria internalization. We didn´t find $CeO_2$ nanoparticles attached to the cell envelope of *P. subcapitata* in agreement with Van Hoecke et al. (2009), but on the contrary, we found a severe disruption of *P. subcapitata* envelope. In *Anabaena* CPB4337 we found nanoparticles firmly attached to the cell envelope and we found also a disrupting effect of $CeO_2$ nanoparticles on its cell wall and membrane, in agreement with the just published findings of Cherchi et al. (2010) and Cherchi and Gu (2011) who found changes on cell surface smoothness and topology in *Anabaena variabilis* exposed to $TiO_2$ nanoparticles. Regarding the internalization or the occurrence of intracellular alterations, we didn´t found internalization of the nanoparticles in any of the two organisms, this result is similar to that found by Van Hoecke et al. (2009) for *P. subcapitata* exposed to $CeO_2$ nanoparticles; but on the other hand, we found a profusion of intracellular cytoplasmic vesicles in *P. subcapitata* cells exposed to $CeO_2$, indicating no need of cell uptake of nanoparticles to induce intracellular effects. Regarding *Anabaena* CPB4337 we didn´t find internalization or apparent intracellular alterations in cells with intact envelope, which disagrees with Cherchi and Gu (2010) who found in *Anabaena variabilis* cells exposed to $TiO_2$ nanoparticles internalization of $TiO_2$ and the occurrence of intracellular cyanophycin granules in treated cells without apparent membrane breakage or disruption. These differences may be duo to different toxicological effects of $CeO_2$ and $TiO_2$ nanoparticles which could display different effects on exposed organisms. General conclusions of this work were that a direct contact between nanoparticles and cells seems to be necessary for cytotoxicity to occur and that the intracellular effects can be seen even without internalization of $CeO_2$ nanoparticles. An important step forward would be to investigate whether ROS (reactive oxygen species) induction is behind the alteration and disruption of cell envelope and if alteration in photosynthesis can be observed in cells treated with nanoceria since photosynthesis is the main bioenergetic process of both organisms (green algae and cyanobacteria) and an impairment of their redox reactions could also be involved in the generation of intracellular ROS.

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# CONCLUSIONES/CONCLUSSIONS

## CONCLUSIONES

1. La respuesta bioluminiscente de *Anabaena* sp. PCC 7120 CPB4337 fue caracterizada en diferentes medios de ensayo y pH y se establecieron las condiciones estándar para realizar los ensayos de toxicidad con *Anabaena* sp. PCC 7120 CPB4337: las condiciones óptimas para realizar el bioensayo en términos de máxima emisión de luminiscencia fueron agua destilada o medio de cultivo a un pH de 5,8 a 6. El bioensayo puede realizarse en un rango de pH de 5 a 8. El bioensayo es simple y rápido y muy sensible, y requiere una manipulación mínima de las muestras. La calibración del bioensayo con Cu como tóxico de referencia ha demostrado que el bioensayo se encuentra en los niveles de reproducibilidad aceptados por la US EPA para ensayos de toxicidad para muestras líquidas.

2. Los metales pesados resultaron ser muy tóxicos para *Anabaena* sp. PCC 7120 CPB4337, y su toxicidad aumentó con el tiempo de exposición. Se pueden obtener resultados del test de toxicidad desde unos pocos minutos de tiempo de exposición (30 minutos), hasta 24 h. Los factores modificantes tuvieron un efecto importante sobre la toxicidad de los metales pesados, y el uso de programas de especiación para modelizar el comportamiento de los metales pesados y el análisis de correlaciones demostraron ser muy útiles para relacionar toxicidad y biodisponibilidad. En general se observó una buena correlación entre la cantidad de ión libre en solución y la toxicidad. La combinación del uso de programas de modelización química y los análisis de correlación han probado ser una herramienta útil para explicar datos complejos de toxicidad de muestras ambientales.

*3.* El bioensayo con *Anabaena* sp. PCC 7120 CPB4337 ha demostrado ser igual o incluso más sensible que los ensayos estándar de toxicidad con los que fue comparado (*Daphnia magna,* Microtox® y *Pseudokirchneriella subcapitata*) en el análisis de toxicidad de los contaminantes emergentes ensayados (fibratos, compuestos orgánicos clorados y surfactantes perfluorados). El hecho de que los tests estándar de toxicidad basados en la inhibición del crecimiento de productores primarios como el de *Pseudokirchneriella subcapitata* tengan un tiempo de resolución de al menos 48 – 72 h es especialmente remarcable, ya que *Anabaena* sp. PCC 7120 CPB4337 mostró en general una mayor sensibilidad que éste a tiempos de exposición más cortos (1 h – 24 h). En este sentido, cuando se estudió la toxicidad de los fibratos, la introducción de *Anabaena* sp. PCC 7120 CPB4337 en la batería de test de toxicidad permitió reclasificar el bezafibrato y el ácido clofíbrico de *no tóxico* ($EC_{50}$ > 100 mg/l) a *dañino para los organismos acuáticos* (10 mg/l < $EC_{50}$ < 100 mg/l). El agua residual ensayada resultó ser muy tóxica para la cepa de *Anabaena* CPB4337 y sin embargo no presentó ninguna toxicidad en los ensayos de

toxicidad estándar. Estos resultados enfatizan la necesidad de desarrollar nuevos ensayos de toxicidad que presenten mayor sensibilidad para poder detectar efectos adversos de contaminantes que podrían pasar desapercibidos usando bioensayos convencionales.

4. El método del Índice de Combinación (IC) permite la cuantificación de la interacción (sinergia y antagonismo) de *n* compuestos químicos de forma simultánea en todo el rango de niveles de efecto. Puede ser usado con cualquier parámetro de toxicidad (bioluminiscencia, inhibición del crecimiento, etc.,). El tipo de interacción puede ser estudiada independientemente del mecanismo de acción de los componentes en organismos diana y no diana ya que el método no requiere ninguna información previa sobre el mecanismo de acción farmacológico o toxicológico del contaminante. Las conclusiones generales sobre la toxicidad de mezclas de contaminantes son:

   I. El tipo de interacción puede variar con cada organismo, y dentro de un mismo organismo puede también variar con el nivel de efecto ($EC_x$) producido.
   II. Las mayores desviaciones de la aditividad fueron encontradas en mezclas binarias y ternarias para todos los organismos estudiados.
   III. De forma general, el aumento de la complejidad de las mezclas (con cuatro componentes o más) tuvo como resultado una reducción en el grado de interacción de los componentes, haciendo que el resultado de la mezcla se aproximara a la aditividad. Sin embargo se encontró una tendencia a la sinergia en las mezclas más complejas en los niveles bajos de efecto.

5. En el estudio de la toxicidad de nanopartículas de óxidos de cerio, la combinación del uso en paralelo de distintas medidas de toxicidad y de una extensa caracterización físico-química de las nanopartículas resultó ser una combinación muy poderosa para determinar la toxicidad de los nanomateriales. El efecto de las nanopartículas de $CeO_2$ sobre los organismos acuáticos fotosintéticos ensayados varió en gran medida en función de su estado de agregación y potencial-Z. El escenario más tóxico para *Anabaena* CPB4337 fue en agua destilada donde la agregación es mínima y las nanopartículas están cargadas positivamente. El contacto directo entre las nanopartículas de cerio y las paredes o membranas celulares parece imprescindible para que muestren citotoxicidad. Dicho contacto produce disrupción de la pared celular y la membrana plasmática que conduce finalmente a la lisis celular. En lo concerniente a los parámetros empleados para medir las dosis, nuestros datos no evidencian que el área superficial de la nanopartícula sea un parámetro más adecuado que la masa a la hora de explicar los efectos toxicológicos detectados.

## CONCLUSIONS

1. The bioluminescent response of *Anabaena* sp. PCC7120 CPB4337 in different assay media, and pH values was characterized. Standard biotest conditions to perform toxicity assays with *Anabaena* sp. PCC7120 CPB4337 were established: A pH value of 5.8-6 with cells resuspended in ddH2O or growth media are the optimum conditions in terms of maximum bioluminescence emission; however the bioassay may be performed in a pH range from 5 to 8. The assay is simple, rapid and highly sensitive and a minimum sample manipulation is required; calibration of the assay using copper as reference toxicant has demonstrated that the levels of test reproducibility are within those reported by USEPA.

2. Heavy metals proved to be very toxic to *Anabaena* sp. PCC7120 CPB4337 and its toxicity increased with time of exposure; results from the bioassay can be obtained from few minutes (15 min) to 24 h. Modifying factors had a marked effect on metal toxicity. Chemical modeling and correlation analyses proved very useful to link toxicity and bioavailability. In general, there was a good correlation between the observed toxic effects and free-ion metal concentration. This approach of combining toxicity studies with chemical modeling to predict changes in metal speciation may help to interpret complex toxicity data when testing real environmental samples.

3. When assessing the toxicities of emerging pollutants (fibrates, chlorinated compounds and surfactants), *Anabaena* sp. PCC 7120 strain CPB4337 proved to be as sensitive as or even more sensitive than the standard toxicity tests (*Daphnia magna,* Microtox® *and Pseudokirchneriella subcapitata*). Especially remarkable is the fact that standard toxicity tests based on growth inhibition of aquatic primary producers such as *Pseudokirchneriella subcapitata* had a resolving time of 48-72 h; the *Anabaena* sp. PCC 7120 strain CPB4337 assay, based on luminescence inhibition, was in general more sensitive at shorter times of exposure. In this respect, when assessing the individual fibrate toxicity, the inclusion of *Anabaena* sp. PCC 7120 strain CPB4337 test allowed to reclassify bezafibrate and clofibric acid from "non-toxic" ($EC_{50}$> 100 mg/l) to "harmful to aquatic organisms" (10 mg/l<$EC_{50}$<100 mg/l); the wastewater sample was not toxic to the standard toxicity tests, but proved to be highly toxic only to the *Anabaena* strain. These results emphasize the need to develop new and more sensitive toxicity tests for the detection of unwanted toxic effects that might become overlooked using conventional bioassays.

4. The Combination Index (CI) method allows calculation of *n* chemical interactions (synergism or antagonism) at all effect levels simultaneously. It may be used with any

toxicity endpoint of interest (bioluminescence, growth …). Using the CI method, the nature of the interaction can be studied independently of any consideration on the mechanism of action (MOA) on target and non-target organisms as no previous knowledge on the pharmacological/toxic mechanism of action of the pollutants is needed. General conclusions on the toxicity of mixtures of pollutants are:

I. The nature of the interaction varies with organisms and, within a given organism; it may also vary with the level of effect of the pollutants.
II. The higher degree of deviation from addivity was found in binary and ternary mixtures for all the organisms studied.
III. An increase in complexity of mixtures (four and more components) leads in general to a reduction of the degree of interaction approaching additivity; however a synergistic tendency was found in the more complex mixtures at the lowest levels of effect.

5. When assessing the toxicity of CeO2, a parallel physicochemical characterization and ecotoxicological assessment proved to be a very powerful tool to understand the toxicological effects of nanoparticles. The effect of CeO2 nanoparticles on photosynthetic aquatic organisms varied with their aggregation state and ζ-potential, finding the most toxic situations for *Anabaena* CPB4337 in pure water where the aggregation is minimal and particles are charged positively. The direct contact of ceria nanoparticles with the cell wall/membranes seems to be essential for cytotoxicity to occur, resulting in cell wall and membrane disruption, which ultimately leads to cell lysis. As far as dose metrics are concerned, our data did not support the view that surface area might work better than mass concentration as a dose variable.

# FUTURE PROSPECTS

## FUTURE PROSPECTS

In the present work, the potential applications of a cyanobacterial bioreporter in environmental toxicity have been assessed. *Anabaena* sp. PCC 7120 strain CPB4337 has proved to be a robust, useful and polyvalent bioreporter which has been applied with single contaminants, mixtures and real wastewater samples. Future prospects of the present work include:

1. To explore different methods of immobilization of the cyanobacterial bioreporters in order to be able to integrate the bioreporters in a biosensor configuration, this will allow on-site real time recording or miniaturized chips reading systems for high-throughput screening.

2. In the field of mixture toxicity, work is needed to be undertaken in order to determine whether the CI method can be a useful tool for regulatory/monitoring purposes. The CI method can be used to identify compounds with high tendency to result in synergistic interactions which may be a problem in the environment when these interactions are found at low concentrations of pollutants. Work on the prediction potential of CI in multicomponent mixtures of pollutants may be highly relevant.

3. With respect to nanoparticle toxicological evaluation, a good physicochemical characterization and a multi end-point toxicity approach has proved to be really useful in order to explain toxicological observations. It would be interesting to investigate whether nutrient transport is impaired in the nanoparticle-coated *Anabaena* cells and whether oxidative stress plays a role in membrane disruption and cytotoxicity; also, it could be of interest to investigate the effect of nanoceria in photosynthesis, which could also be involved in the generation of ROS when photosynthetic reactions are not well balanced. Also, work with other engineered nanoparticles which eventually may be released to the aquatic environment will be needed in order to better understand their potential toxicity.

4. In our group, work on the development of novel filamentous cyanobacterial bioreporters like that of the aequorin-expressing *Anabaena* strain or those able to detect bioavailable phosphate has been done; however, we wish to expand the battery of cyanobacterial strains (including other species like unicellular ones) exploring the potential of the cyanobacterial SOS and ROS- scavenging systems in order to identify cyanobacterial gene promoters which may be of high interest in order to develop bioreporters able to detect specific stresses affecting primary producers.

# ANNEXE I
# Other Published Articles

# A PARADOX IN ECOTOXICOLOGY: 2 + 2 DOES NOT ALWAYS EQUAL 4

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# A paradox in ecotoxicology: 2 + 2 does not always equal 4

••• This article is a study on the interaction of pollutants in a complex mixture using the combination index (CI)-isobologram equation

**Francisca Fernández-Piñas[1], Ismael Rodea-Palomares[1], Alice L. Petre[2], Karina Boltes[2], Francisco Leganés[1], José Antonio Perdigón-Melón[2], Roberto Rosal[2]**

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## Introduction

Biota is usually exposed to complex mixtures of pollutants; however, due to simplicity, most ecotoxicity studies and risk assessment strategies focus on hazard of individual chemicals only and may underestimate the risk associated with the toxic action of mixtures.

The toxicity of a mixture depends on the toxicity of the components and how the components interact with each other in a dose-dependent way.

Chemicals in a mixture may show zero interaction or may interact in two ways:

- Synergistically: The effect of the combination is greater than that expected from the sum of their individual effects (more than an additive effect)
- Antagonistically: The effect of the combination is less than that expected from the sum of their individual effects (less than an additive effect)

Synergism and antagonism may be defined as departures (deviations) from zero interaction (additive effect) between chemicals in a mixture.

But, how to define zero interaction or additive effect between chemicals in a mixture?

An additive effect is not just the simple arithmetic sum of the effects of the individual chemicals.

Definition of additivity is usually based on the classical isobolograms of the combination of two chemicals that follows next equation:

$$\frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} = I$$

Where $(D)_1$ and $(D)_2$ are the doses of chemicals 1 and 2 that in combination produce some specified effect (i. e. 50% inhibition of luminescence) and $(D_m)_1$ and $(D_m)_2$ are the doses of the chemicals that when applied singly also have the same effect (50% inhibition of luminescence)

An isobologram (Fig. 1) can graphically display chemical interactions, the x and y axes representing the doses of drugs 1 and 2. The lines of the isobologram show dose combinations of the two chemicals, 1 and 2 which yield the same effect:

An isobologram is then a dose-oriented graphic that can give information on the interaction of two drugs at any effect level ($EC_{10}$, $EC_{20}$, $EC_{50}$….) but has some practical limitations as it is designed for two-three drugs.

The isobologram is independent of the nature of the effects (toxicity endpoints) under consideration; it does not depend on the mechanism of action or the shapes of the dose response-curves.

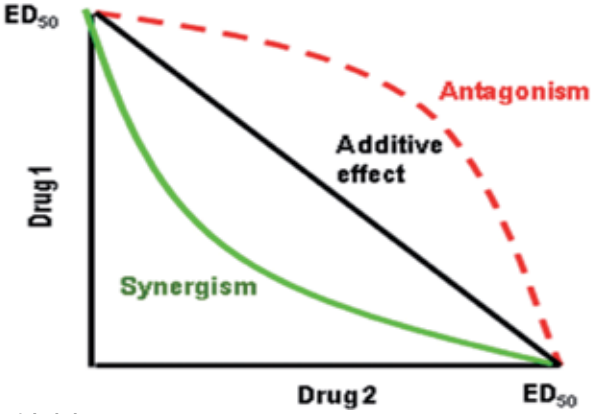


**Fig. 1. Isobologram**

1. A straight line connecting dose of chemical 1 (D1) and dose of chemical 2 (D2) on the respective x and y axes that yield 50% effect ($D_m$, $EC_{50}$, $ED_{50}$) - ZERO INTERACTION OR ADDITIVE EFFECT (Isobologram equation = 1)
2. When the line connecting both doses lie below and to the left of the line of additiviy (concave-up line)- SYNERGISM (Isobologram equation < 1)
3. When the line connecting both doses lie above and to the right of the line of additivity (concave-down)– ANTAGONISM (Isobologram equation > 1)

The Combination Index-Isobologram Equation generalizes the isobologram equation for *n* drugs, is independent of the mechanism of action but takes into account both the potency of each drug and combinations of these drugs and the shapes of their dose-effect curves.

For each effect level, combination index (CI) values are calculated according to the general combination index equation for n-chemical combination at *x%* inhibition:

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1\text{-}n}\{D\}}{(D_m)_j\{(f_{ax})_j\}\{1-(f_{ax})_j\}^{1/mj}}$$

Where $^{n}(CI)_x$ is the combination index for *n* chemicals (e.g., fibrates) at *x%* inhibition (e.g., bioluminescence inhibition); $(D_x)_{1\text{-}n}$ is the sum of the dose of *n* chemicals that exerts *x%* inhibition in combination, is the proportionality of the dose of each of n chemicals that exerts *x%* inhibition in combination; and $(D_m)_j \{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts *x%* inhibition; *fa* is the fraction affected by dose D (e.g.; 0.9 if bioluminescence is inhibited by 90%). From the equation, CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, respectively.

The combination index (CI) allows for quantitative determination of the interactions of *n* chemicals in a mixture at all effect levels simultaneously.

## Materials and Methods

We are currently working on environmental applications

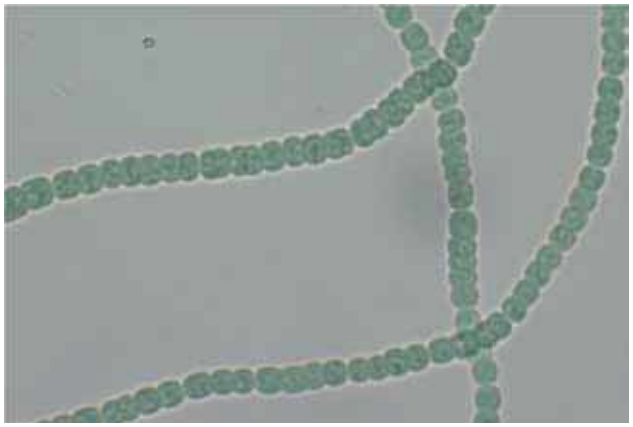

**Fig. 2.** *Anabaena* CPB4337

of the combination index (CI)-isobologram equation, a method widely used in pharmacology, to study the nature of the interaction of selected group of chemicals (heavy metals, lipid regulators, chlorinated pollutants and surfactants) in three aquatic organisms: naturally bioluminescent *Vibrio fischeri*; a recombinant bioluminescent freshwater filamentous cyanobacterium, Anabaena CPB4337 (Fig. 2), and the unicellular freshwater green alga *Pseudokirchneriella subcapitata*. The toxicity endpoints for the luminescent cyanobacterium and *Vibrio* was luminescence inhibition and that of the green alga, cell growth inhibition. The heavy metals used were copper, zinc and cadmium; the lipid regulators were three fibrates: gemfibrozil (Gm), bezafibrate (Bz) and fenofibric acid (Fn); the chlorinated pollutants were two chlorophenols: trichlorophenol (TCP) and triclosan (tri) and the surfactant was the sulfosuccinate docusate sodium (Doc).

## Results and Discussion

The *fa*-CI plot depicted in Fig. 3 shows the CI (combination index values) versus *fa* (the fraction of bioluminescence inhibited by a toxicant with respect to the control). The *fa*-CI plot is an effect-oriented plot that shows the evolution of the kind of interaction (synergism, antagonism, additive effect) as a function of the level of the effect (*fa*) of a particular toxicant on the reference organism (*fa*, where $EC_a$ = *fa* x 100; i.e., $EC^{10}$ = f10 x 100). Fig. 3 shows the *fa*-CI plots for heavy metals (Fig. 3a), lipid regulators (fibrates) (Fig. 3b); and the surfactant docusate sodium + chlorinated pollutants (Fig. 3c) interactions in *Anabaena* CPB4337.

**Heavy metals (Fig. 3a)**: In heavy metal combinations, the nature of the interaction was mainly antagonistic, but it turned additive or synergistic at the higher levels of effect (*fa*).

**Lipid regulators (Fig. 3b)**: In lipid regulator (fibrates) combinations, the nature of the interaction was more complex, from strong synergism at low *fa* values to strong antagonism at the higher levels of effect.

**Docusate sodium and chlorinated contaminants (Fig. 3c)**: Docusate sodium (an anionic surfactant widely used in pharmaceutical formulations) exhibited mainly strong synergism with both TCP and Triclosan in binary and ternary combinations.

Fig. 3 clearly indicates that the nature of the interaction between pollutants varied with the effect levels (*fa*).

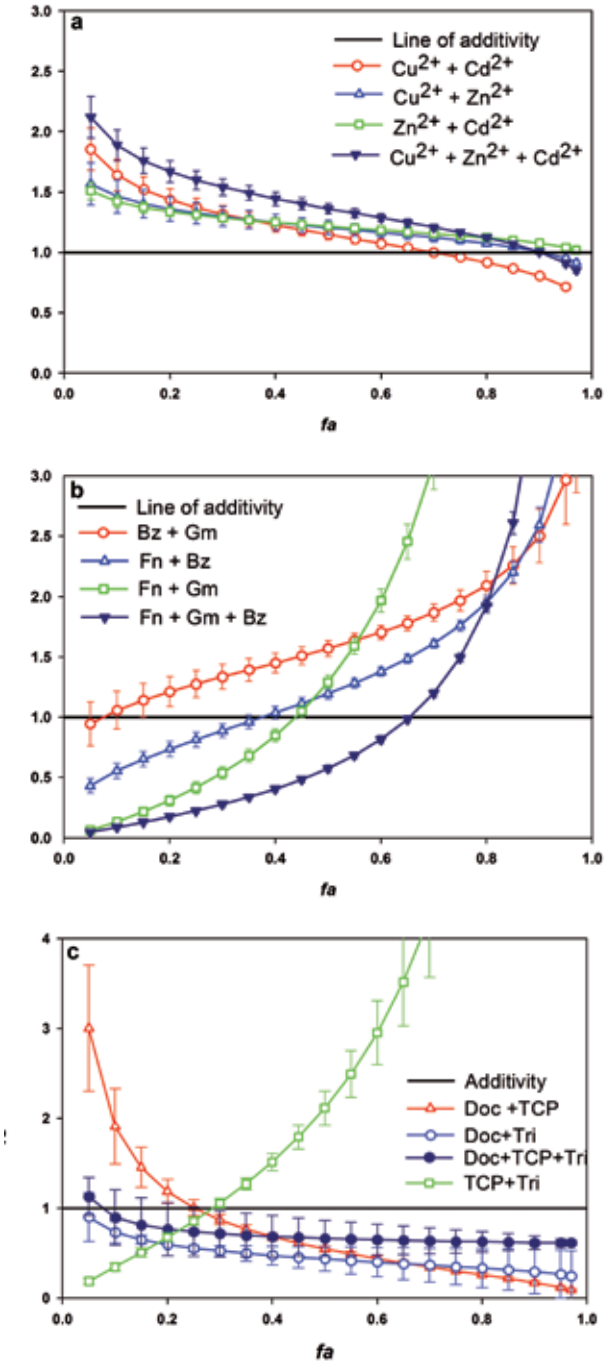


**Fig. 3.** *fa*-CI plots of binary and ternary combinations of heavy metals (a); fibrates [gemfibrozil (Gm), bezafibrate (Bz) and fenofibric acid (Fn)] (b) and docusate sodium (Doc) + chlorophenols (Tri and TCP) (c) in the cyanobacterium *Anabaena* CPB4337

The nature of the interaction also varied with the test organism; for example, in the case of the surfactant docusate sodium and the two chlorophenols, the toxicity interaction pattern of the binary mixtures of docusate with any of the chlorophenols for the two prokaryotic organisms, *Anabaena* CPB4337 and *Vibrio fischeri*, was quite similar but totally different to the pattern observed in the eukaryotic green alga.

We wanted to test the CI

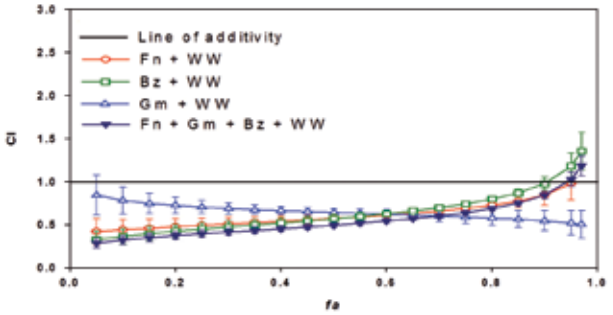


**Fig. 4.** *fa*-CI plots of binary and quaternary combinations of fibrates [gemfibrozil (Gm), bezafibrate (Bz) and fenofibric acid (Fn)] and waste water (WW) in the cyanobacterium *Anabaena* CPB4337

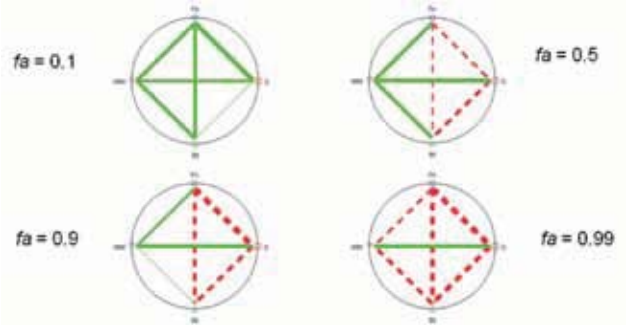


**Fig. 5.** Green solid lines indicate synergism
- Red broken lines indicate antagonism
- Thickness of the line represents the strength of the interaction

method with real environmental samples; for that, we used a wastewater sample collected from a local sewage treatment plant (STP) which in previous studies resulted very toxic for Anabaena CPB4337. As the analysis of the wastewater sample revealed the presence of many pharmaceuticals and fibrates were present, we investigated the nature of the interactions between fibrates and the wastewater itself (Fig. 4).

As shown in the figure, the three lipid regulators showed strong synergism when combined with wastewater (WW) from the effluent of the STP almost at all effect levels except at very high ones where slight antagonism appeared; however, the gemfibrozil + wastewater binary mixture showed a synergistic behavior in the whole range of effect levels. The quaternary mixture exhibited the strongest synergism although it also became slightly antagonistic at the higher effect levels. This fact poses the following question: Does the increasing complexity of mixtures enhance synergism? Future research is needed with more complex mixtures and compounds different from the ones we have already assayed before answering such a question.

A polygonogram is a semi-quantitative method of representing interactions between three or more compounds at a determined *fa* level allowing rapid interpretation of interactions. Fig. 5 shows poligonograms at four *fa* levels for fibrate (fenofibric acid, gemfibrozil and bezafibrate) + toxic wastewater (ww) interactions in *Anabaena* CPB4337:

Fig.5. Polygonogram indicating the nature and strength of the interactions between the three fibrates and a wastewater (ww) in Anabaena CPB4337

As expected, both the *fa*-CI plot (Fig. 4) and polygonograms (Fig. 5) yielded identical results with synergism being the predominant interaction at almost all effect levels which turned into antagonism at higher levels of effect except for the gemfibrozil + wastewater mixture which was synergistic at all effect levels.

## Conclusions

- The Combination Index (CI) method allows calculation of n chemical interactions (synergism, additive effect, antagonism) at all effect levels simultaneously. Knowledge of mechanisms of action is not needed
- The nature of the interaction varies with organisms and, within a given organism, it may also vary with the level of effect of the pollutants
- The proposed method can be applied in environmental toxicology as a general method to define interactions of potential toxicants in mixtures toward target and non-target organisms as no previous knowledge on the pharmacological/toxic mechanism of action of the pollutants is needed
- Although we have used bioluminescence inhibition as toxicity endpoint, we propose that the method may be used with other endpoints of interest (growth, chlorophyll fluorescence, pigment profiles…)
- The method may be especially useful for risk assessment strategies that take into account the toxicological interactions of substances in a mixture. This may be particularly of concern if synergism is found at low/very low effect levels of pollutants
- The method could be considered for further implementation of the EU Water Framework Directive as exposure towards multi-component mixtures is a characteristic situation in aquatic environments
- The application of the method to community level bioassays rather than single test organisms could increase environmental realism

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**FREE $CA^{2+}$ AS AN EARLY INTRACELLULAR BIOMARKER OF EXPOSURE OF CYANOBACTERIA TO ENVIRONMENTAL POLLUTION.**

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**(PAPER IN FOREFRONT [1])**

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PAPER IN FOREFRONT

# Free $Ca^{2+}$ as an early intracellular biomarker of exposure of cyanobacteria to environmental pollution

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**Abstract** Calcium functions as a versatile messenger in a wide variety of eukaryotic and prokaryotic cells. Cyanobacteria are photoautotrophs which have a great ecological impact as primary producers. Our research group has presented solid evidence of a role of calcium in the perception of environmental changes by cyanobacteria and their acclimation to these changes. We constructed a recombinant strain of the freshwater cyanobacterium *Anabaena* sp. PCC 7120 that constitutively expresses the calcium-binding photoprotein apoaequorin, enabling in-vivo monitoring of any fluctuation in the intracellular free calcium concentration of the cyanobacterium in response to any environmental stimulus. The "$Ca^{2+}$ signature" is the combination of changes in all $Ca^{2+}$ signal properties (magnitude, duration, frequency, source of the signal) produced by a specific stimulus. We recorded and analyzed the $Ca^{2+}$ signatures generated by exposure of the cyanobacterium to different groups of environmental pollutants, for example cations, anions, organic solvents, naphthalene, and pharmaceuticals. We found that, in general, each group of tested chemicals triggered a specific calcium signature in a reproducible and dose-dependent manner. We hypothesize that these $Ca^{2+}$ signals may be related to the cellular mechanisms of pollutant perception and ultimately to their toxic mode of action. We recorded $Ca^{2+}$ signals triggered by binary mixtures of pollutants and a signal induced by a real wastewater sample which could be mimicked by mixing its main constituents. Because $Ca^{2+}$ signatures were induced before toxicity was evident, we propose that intracellular free $Ca^{2+}$ may serve as an early biomarker of exposure to environmental pollution.

**Keywords** Aequorin · Biomarker · $Ca^{2+}$ signature · Cyanobacterium · Environmental pollution · Pollutant interaction

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## Introduction

Cyanobacteria constitute a phylogenetically diverse group of photosynthetic prokaryotes [1]. They are globally widespread organisms that occupy a diverse range of habitats and may in fact be a dominant feature of microbial populations in many ecosystems, including terrestrial, aquatic, and polar [1]; in these habitats, they have a great ecological impact as primary producers and in global $CO_2$ sequestration.

During the course of evolution, cyanobacteria have developed signal-transduction systems that enable them to sense and respond to any change in their extracellular or internal milieu; in fact, these organisms are able to sense and give the adequate cellular response to diverse environmental stresses, for example pollutant-related stress [2, 3]. Intracellular messengers are basic components of signaling systems; among these, calcium has arisen as probably the most versatile in eukaryotes [4–6] and, as increasing evidence indicates, also in prokaryotes [7]. This versatility is probably derived from the existence of diverse calcium signaling systems with characteristic spatial and temporal properties [5, 6]. In different cell types, a variety of abiotic and biotic stimuli generate intracellular calcium signals, the specificity

of the signal relies not only in the change of the intracellular calcium concentration. A combination of changes in all $Ca^{2+}$ properties of the signal, for example amplitude, duration, frequency, rise time, final $Ca^{2+}$ resting levels, recovery time, and source of the signal induced by a specific stimulus, is referred to as a "Ca signature" [5, 6, 8–11]. $Ca^{2+}$ signatures encode, in their spatio–temporal dynamics, information relating to the nature and strength of stimuli [10, 11].

$Ca^{2+}$-sensitive fluorescent indicators, for example Fura-2, Fluo-3, or Quin-2, have been extensively used to monitor changes in intracellular $Ca^{2+}$ concentrations in different cell types but not without problems such as dye loading and autofluorescence, which limit their application [12, 13]; besides, most of these fluorescent probes bind $Cd^{2+}$ and other divalent cations which modify the absorption spectrum and cause increases in fluorescence as intense as those of $Ca^{2+}$ [14]. As an alternative to fluorescent probes, the $Ca^{2+}$-sensitive photoprotein apoaequorin can be expressed in animal, plant, and bacterial cells, enabling quantification of intracellular $Ca^{2+}$ fluxes [15–17]. Functional recombinant aequorin can be successfully reconstituted on addition of the hydrophobic luminophore coelenterazine; the reconstituted protein has three $Ca^{2+}$ binding sites and once $Ca^{2+}$ ions are bound, aequorin catalyses the oxidation of the substrate coelenterazine by oxygen, resulting in blue light emission that can be measured with a luminometer. Aequorin is very sensitive to $Ca^{2+}$ changes with a dose response curve that begins at approximately 50 nmol $L^{-1}$ free $Ca^{2+}$ and is saturated well above 10 μmol $L^{-1}$ free $Ca^{2+}$ [18].

Our research group has constructed a recombinant strain of the freshwater nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 constitutively expressing apoaequorin, *Anabaena* sp. PCC7120 (pBG2001a), which enables continuous and in-vivo monitoring of the intracellular free Ca concentration. Any fluctuation in response to any stimulus is easily detected and recorded; we have been able to record and analyze a variety of calcium signatures induced by specific environmental stimuli in this strain and in a unicellular cyanobacterium also expressing apoaequorin [18–22].

Pollutants are a class of environmental stressors that have been found to interfere with $Ca^{2+}$ homeostasis/signaling in a number of cells [23–29]. Most studies have dealt with animal cells [25, 26] and a few have considered eukaryotic microorganisms [27, 29, 30]; however, such studies on prokaryotes seem to be lacking.

In this study we report systematic recording and analysis of the $Ca^{2+}$ signatures generated by exposure of the apoaequorin-expressing *Anabaena* strain to different groups of potential environmental toxicants, for example cationic and anionic heavy metals, the metalloid As, naphthalene (polycyclic aromatic hydrocarbon, PAH), organic solvents (acetone, ethanol, toluene), and pharmaceuticals, for example lipid regulators (fibrates) and antibiotics (fluoroquinolones). These pollutants were selected on the basis of their occurrence and persistence in the environment and on their toxic characteristics [31–38].

To link the $Ca^{2+}$ responses with toxicity caused by the pollutants to the cyanobacterial cells, we also compared the recorded $Ca^{2+}$ signatures with the toxicity values of some of the tested pollutants toward a recombinant bioluminescent strain of *Anabaena* sp. PCC 7120, denoted *Anabaena* CPB4337 that our group has constructed and have previously used in toxicity bioassays [39–41].

For the first time we also present data on $Ca^{2+}$ signatures triggered by binary mixtures of pollutants and a real wastewater sample which could be mimicked by mixing its main constituents at environmental concentrations. We propose that monitoring of intracellular free $Ca^{2+}$ signals induced by pollutants could be useful as an early biomarker of exposure to environmental pollution.

## Materials and methods

### Chemicals

Chemicals were of analytical grade and purchased from Sigma–Aldrich if not otherwise stated. Stock solutions were prepared by dissolving these compounds in MilliQ water, except naphthalene and toluene that were dissolved in 1% (*v*/*v*) dimethyl sulfoxide (DMSO). Injection of water or DMSO at 1% (*v*/*v*) induced a $Ca^{2+}$ transient much smaller in amplitude (peak height 0.55±0.08 μmol $L^{-1}$) and duration (11±1 s) which has been attributed to a small mechanically induced $Ca^{2+}$ increase [18].

The fibrate fenofibric acid (Fn) was produced from fenofibrate (Sigma–Aldrich, +99% purity) by hydrolysis, as described elsewhere [41].

### Organism and growth conditions

The strain of *Anabaena* sp. PCC7120 (pBG2001a) expressing apoaequorin [18] was routinely grown in 100-mL conical flasks containing 50 mL BG11 medium with 25 mmol $L^{-1}$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.2 and 2.5 μg $mL^{-1}$ spectinomycin, with the standard calcium concentration (0.25 mmol $L^{-1}$). Cell cultures were incubated on a rotatory shaker at 28°C under 65 μE $m^{-2}s^{-1}$ fluorescent white light.

### In vivo aequorin reconstitution and luminescence measurements

For aequorin luminescence measurements, in-vivo reconstitution of aequorin was performed by addition of 2.5 μmol $L^{-1}$ coelenterazine to cell suspensions (15 μg $mL^{-1}$

chlorophyll) and incubation for 15 h in darkness and shaking at 18°C. Excess coelenterazine was removed before $Ca^{2+}$ measurements were made [18].

Luminescence measurements were made using a digital luminometer with a photomultiplier (BioOrbit 1250). Reconstituted cell suspensions (0.5 mL) in a transparent polystyrene cuvette were placed in the luminometer and luminescence was recorded every 1 s over the duration of the experiment.

Calibration of the $[Ca^{2+}]_i$ changes requires knowledge of the total amount of reconstituted aequorin available in cell suspensions ($L_{max}$) at any one point in time of the experiment, and the running luminescence ($L_0$). For estimation of total aequorin luminescence, the remaining aequorin was discharged at the end of the experiment by addition of 0.5 mL 500 mmol $L^{-1}$ $CaCl_2$ and 5 % (*v*/*v*) Triton X-100. Rate constants of luminescence ($L_0\ L_{max}^{-1}$), and $[Ca^{2+}]_i$ were calculated by using calibration curves obtained for aequorin extracted from the recombinant strain of *Anabaena* sp PCC7120 (pBG2001.a), according to Torrecilla et al. [18].

### $Ca^{2+}$ chelator treatment

When ethylene glycol tetraacetic acid (EGTA) was used, aequorin reconstitution was performed as described above, followed by incubation with 1 mmol $L^{-1}$ EGTA for 1 h. After the incubation, treated cells were challenged with the different pollutants individually or in mixtures and used for luminescence measurements.

### Cell lysis check

For each of the treatments used in this work, the occurrence of cell lysis was checked by use of three methods:

1. examination by optical microscopy;
2. measurement of luminescence after addition of $Ca^{2+}$ to the medium in which reconstituted cells were present after removing the cells by centrifugation at 23,000 g at room temperature for 15 min; and
3. measurements of phycobiliproteins in the medium in which reconstituted cells were present after removing the cells by centrifugation at 23,000 g at room temperature for 15 min.

### Cyanobacterial toxicity bioassay

Toxicity bioassays using self-bioluminescent *Anabaena* CPB4337 are based on inhibition of constitutive luminescence caused by the presence of a toxic substance and were carried out essentially as described elsewhere [39–41] except that cells were resuspended in the same assay medium as that used for aequorin analysis. *Anabaena* CPB4337 was grown as *Anabaena* sp. PCC7120 (pBG2001a) expressing apoaequorin but supplemented with 10 μg $mL^{-1}$ neomycin sulfate (Nm).

### Wastewater source and chemical analysis

Wastewater samples were collected from the secondary clarifier of a sewage-treatment plant (STP) located in Alcalá de Henares (Madrid) [37, 42]. Quantitative analysis of the elemental composition of the wastewater sample was performed by inductively coupled plasma–mass spectrometry (ICP–MS; Perkin–Elmer Sciex Elan 6000 equipped with an AS 91 autosampler) by the ICP–MS laboratory of the Universidad Autonoma de Madrid; the results are listed in Table 1. More detailed information about organic pollutants has been given elsewhere; individual organic pollutants were detected in higher amounts than are commonly encountered in biologically treated wastewater, with high contributions from stimulants, anti-inflammatories, antibiotics, β-blockers, and lipid regulators [37, 42].

### Statistical analysis

All tests of statistically significant differences between data sets were performed using Student's *t*-tests or analysis of variance at $P<0.05$ with the software SigmaStat. All data were obtained from a minimum of three replicates for each assay. The $Ca^{2+}$ traces presented in the tables and figures were chosen to best represent the average result of at least three replicates. Toxicity response of the cyanobacterium was estimated as $EC_{50}$ values, the median effective concentration of a toxicant that causes 50% of bioluminescence inhibition compared with an untreated control. $EC_{50}$ and confidence intervals (CI) were estimated using the linear interpolation method [43].

## Results

### $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to different classes of environmental pollutants

Cell suspensions of *Anabaena* sp. PCC 7120 (pBG2001a) were first incubated with coelenterazine in order to reconstitute aequorin. Reconstituted cells were then placed in the luminometer cuvette, increasing concentrations of pollutants were injected and the subsequent changes (if any) in intracellular free $Ca^{2+}$ concentrations were recorded. Cations, anions, pharmaceuticals, and naphthalene were tested at concentrations ranging from 0.5 to 50 mg $L^{-1}$; the concentration range for organic solvents was from 0.05 to 5%. All the pollutants tested triggered a calcium transient

**Table 1** ICP–MS analysis of total element concentrations in wastewater from an STP located in Madrid (Spain)

| Element | Concentration (μg $L^{-1}$) | Element | Concentration (μg $L^{-1}$) |
|---|---|---|---|
| Ag | 0.21 | La | 0.024 |
| Al | 0 | Li | 18.833 |
| As | 3.7 | Mg | 19111.016 |
| B | 175.276 | Mn | 28.296 |
| Ba | 2.885 | Mo | 3.796 |
| Be | 0.003 | Na | 82242.981 |
| Br | 394.003 | Ni | 4.163 |
| Ca | 44803.918 | Pb | 0.334 |
| Cd | 0 | Pd | 0 |
| Ce | 0.03 | Rb | 11.441 |
| Co | 0.415 | Sb | 0.563 |
| Cr | 0 | Se | 0 |
| Cs | 0.134 | Sn | 0 |
| Cu | 2.025 | Sr | 919.549 |
| Fe | 38.158 | Ti | 7.319 |
| Hg | 0.153 | V | 2.158 |
| I | 62.257 | Zn | 0 |
| K | 17502.394 | Zr | 0.143 |

(Tables 2 and 3), the response in most cases was very quick, within seconds of injection of the compound. Calcium signature properties assessed were transient shape, amplitude, rise time (time from application of the stimulus to maximum amplitude of the response), total transient duration (length of the transient from zero time to recovery of the resting intracellular free $Ca^{2+}$ level), and source of the signal, to determine whether extracellular or intracellular $Ca^{2+}$ or both were responsible for the observed signature. Although with the lowest concentration tested for most compounds, 0.5 mg $L^{-1}$ or 0.05% for the solvents, a clear calcium signal was induced (Electronic Supplementary Material Fig. S1, Table 3), in order to facilitate comparison, for cations, anions, and pharmaceuticals, the transient shape and other properties shown in the tables correspond to a given concentration which showed a well defined and sufficiently measurable $Ca^{2+}$ signal—5 mg $L^{-1}$; because naphthalene and organic solvents induced quite different $Ca^{2+}$ signatures depending on the concentration, two or three concentrations that induced different $Ca^{2+}$ signatures are shown (Table 3); for $Hg^{2+}$ and the fibrates fenofibric acid and gemfibrozil, higher concentrations which induced $Ca^{2+}$ signatures that differed in shape are also shown (Tables 2 and 3).

As shown in Table 2, all tested cations triggered calcium transients with similar shapes characterized by a quick burst of intracellular free $Ca^{2+}$, followed by a quick decline, reaching a plateau that brought the level of intracellular free $Ca^{2+}$ back to the resting value (100 nmol $L^{-1}$ $Ca^{2+}$) [18] the major difference between the induced $Ca^{2+}$ signatures was the total transient duration ranging from 3.85 min ($Hg^{2+}$) to 24.5 min ($Cd^{2+}$). Interestingly, $Hg^{2+}$ at concentrations ≥50 mg $L^{-1}$ triggered a $Ca^{2+}$ signature consisting of two consecutive $Ca^{2+}$ transients—one quick and steep $Ca^{2+}$ spike followed by a bell-shaped transient with lower amplitude that lasted almost an hour.

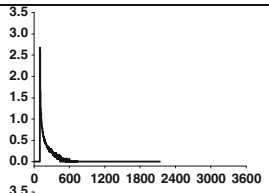
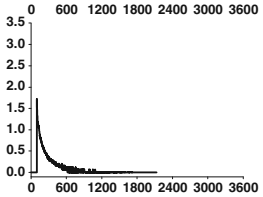
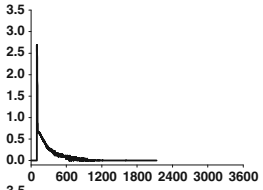
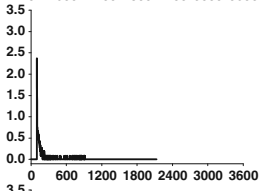
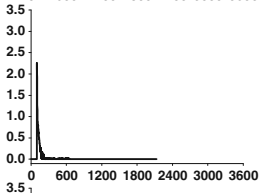
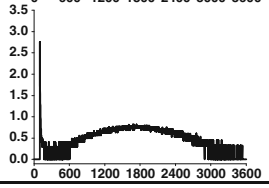
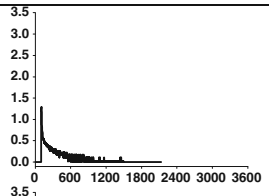
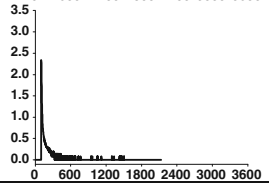
The cations were tested as sulfate or chloride salts; however, no effect of the counteranion was found on the induced $Ca^{2+}$ signature. As examples, the $Ca^{2+}$ signatures triggered by 5 mg $L^{-1}$ $CuCl_2$ and $Cu(SO_4)_2$ are shown in Electronic Supplementary Material Fig. S2; these imply that the observed $Ca^{2+}$ signals were triggered specifically by the tested cation.

The arsenate and chromate anions triggered $Ca^{2+}$ signatures with a shape similar to that of cations (Table 2). The amplitude of the $Ca^{2+}$ signature induced by arsenate was significantly lower (Student's $t$ test, $P < 0.05$) than that of chromate and cations.

Within the tested pharmaceuticals (Table 3), the four fibrates induced quick $Ca^{2+}$ spikes with longer recovery times than those of cations and anions; the transients had larger durations with the exception of that induced by gemfibrozil. Similar to $Hg^{2+}$, fenofibric acid and gemfibrozil at concentrations of 25 mg $L^{-1}$ and above induced a biphasic transient with a first quick spike and a second bell-shaped phase that lasted more than 60 min.

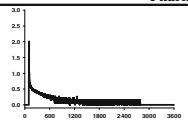
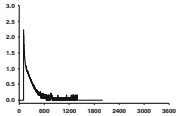
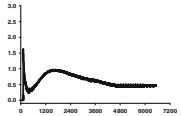
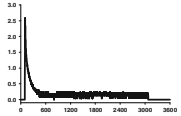
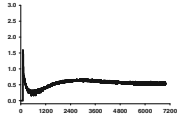
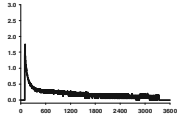
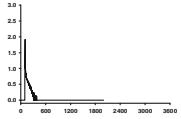
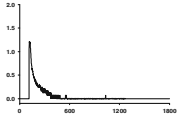
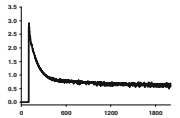
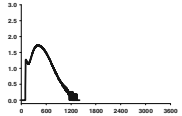
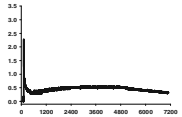
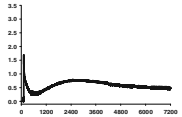
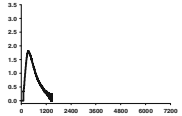
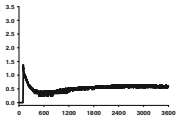
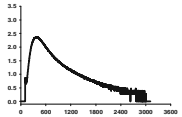
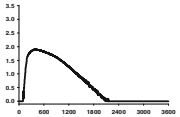
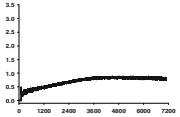
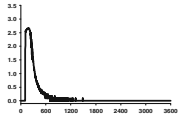
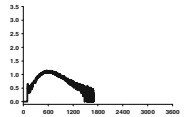
The fluoroquinolone antibiotics ciprofloxacin and ofloxacin induced very similar $Ca^{2+}$ transients with a quick and short $Ca^{2+}$ spike although that of ofloxacin was of lower amplitude.

**Table 2** Properties of calcium signatures induced by cations and anions in *Anabaena* sp. PCC7120 (pBG2001a)

| Stimulus | Transient Shape (x-axis:Time (s); y-axis: µM [$Ca^{+2}$]) | Amplitude µM [$Ca^{+2}$] | Rise Time (s) | Total transient duration (min) | Source of $Ca^{2+}$ |
|---|---|---|---|---|---|
| **Cations** | | | | | |
| $Zn^{+2}$ (5 $mgL^{-1}$) |  | 2.61±0.20 | 2-3 | 8.89±0.9 | Extracellular |
| $Pb^{+2}$ (5 $mgL^{-1}$) |  | 1.80±0.21 | 2-3 | 9.64±0.95 | Extracellular |
| $Cd^{+2}$ (5 $mgL^{-1}$) |  | 2.59±0.30 | 2-3 | 24.50±2.5 | Extracellular |
| $Cu^{+2}$ (5 $mgL^{-1}$) |  | 2.55±0.16 | 2-3 | 5.09±0.07 | Extracellular |
| $Hg^{+2}$ (5 $mgL^{-1}$) |  | 2.23±0.23 | 2-3 | 3.85±0.07 | Extracellular |
| $Hg^{+2}$ (50 $mgL^{-1}$) |  | 2.53±0.20<br>0.74±0.05 | 2-3<br>1537±54 | 59.28±0.70 | Extracellular |
| **Anions** | | | | | |
| $(AsO_4)^{-3}$ (5 $mgL^{-1}$) |  | 1.30±0.17 | 2-3 | 12.34±0.04 | Extracellular |
| $(CrO_4)^{-2}$ (5 $mgL^{-1}$) |  | 2.49±0.28 | 2-3 | 9.97±0.10 | Extracellular |

The only PAH tested, naphthalene (Table 3), induced different $Ca^{2+}$ signatures when applied at different concentrations: when tested at concentrations between 0.5 and 12.5 mg $L^{-1}$, it induced a quick spike with an amplitude similar to that of other tested pollutants and a long recovery time. The resulting transient lasted more than 60 min. When applied at concentrations ≥50 mg $L^{-1}$, it induced a biphasic transient with a short and quick first

**Table 3** Properties of calcium signatures induced by pharmaceuticals, naphthalene and organic solvents in *Anabaena* sp. PCC7120 (pBG2001a)

| Stimulus | Transient Shape (x-axis:Time (s); y-axis: µM [$Ca^{+2}$]) | Amplitude µM [$Ca^{+2}$] | Rise Time (s) | Total transient duration (min) | Source of $Ca^{2+}$ |
|---|---|---|---|---|---|
| **Pharmaceuticals** | | | | | |
| Bezafibrate (5 $mgL^{-1}$) |  | **1.86±0.24** | **2-3** | **37.2±3.5** | Extracellular |
| Gemfibrozil (5 $mgL^{-1}$) |  | **2.18±0.19** | **2-3** | **12.75±1.3** | Extracellular |
| Gemfibrozil (25 $mgL^{-1}$) |  | **1.59±0.2**<br>**0.68±0.26** | **2-3**<br>**1570±74** | **>60** | Extracellular |
| Fenofibric acid (5 $mgL^{-1}$) |  | **2.48±0.17** | **2-3** | **42.08±2.2** | Extracellular |
| Fenofibric acid (25 $mgL^{-1}$) |  | **1.64±0.2**<br>**0.98±0.1** | **2-3**<br>**2250±68** | **>60** | Extracellular |
| Clofibric acid (5 $mgL^{-1}$) |  | **2.32±0.16** | **2-3** | **46.4±4.5** | Extracellular |
| Ciprofloxacin (5 $mgL^{-1}$) |  | **2.03±0.20** | **2-3** | **4.7±0.34** | Extracellular |
| **Pharmaceuticals** | | | | | |
| Ofloxacin (5 $mgL^{-1}$) |  | **1.20±0.09** | **2-3** | **5.98±0.52** | Extracellular |
| **PAH** | | | | | |
| Naphtalene (≤12,5 $mgL^{-1}$) |  | **2.81±0.21** | **2-3** | **>60** | Extracellular |
| Naphtalene (≥50 $mgL^{-1}$) |  | **1.53±0.27**<br>**1.75±0.10** | **2-3**<br>**281±46** | **20±0.63** | Extracellular |
| **Organic Solvents** | | | | | |
| Acetone 0.05% |  | **2.29±0.25**<br>**0.57±0.0.06** | **2-3**<br>**2880±200** | **>60** | Extracellular |
| Acetone 0.5% |  | **1.69±0.2**<br>**0.79±0.1** | **2-3**<br>**2224±150** | **>60** | Extracellular |
| Acetone 5% |  | **1.64±0.24** | **257±11** | **21.67±2.2** | Extracellular |
| **Organic Solvents** | | | | | |
| Ethanol 0.05% |  | **1.36±0.2**<br>**0.64±0.1** | **2-3**<br>**2243±150** | **>60** | Extracellular |
| Ethanol 0.5% |  | **1.84±0.19** | **366±25** | **41±66** | Extracellular |
| Ethanol 5% |  | **1.75±0.28** | **249±25** | **40±4** | Extracellular |
| Toluene 0.05% |  | **0.5±0.05**<br>**1.32±0.25** | **2-3**<br>**43122±50** | **>60** | Extracellular |
| Toluene 0.5% |  | **2.58±0.25** | **46±5** | **20±2** | Extracellular |
| Toluene 5% |  | **0.68±0.4**<br>**1.32±0.27** | **2-3**<br>**482±18** | **28.33±3** | Extracellular |

phase and a second, slower bell-shaped phase; however, for these high concentrations, the total transient duration was shorter.

The organic solvents acetone, ethanol, and toluene induced $Ca^{2+}$ signatures very different from those triggered by the other pollutants tested; in fact, the signatures clearly changed with increasing concentration of the solvents (Table 3). At the lowest concentration tested, 0.05%, acetone and ethanol induced a similar biphasic transient; at 0.5%, the $Ca^{2+}$ transient induced by acetone was also biphasic. The ethanol-induced transient was totally different—a bell-shaped transient with a large rise time. At the highest concentration tested, 5%, for both ethanol and acetone, the rise time of the recorded transients was significantly larger (Student's $t$ test, $P < 0.05$) than that of the previously tested pollutants (Tables 2 and 3). Both solvents induced a bell-shaped transient, that by ethanol of longer duration. Toluene (5%) induced a biphasic transient with a first rapid and short spike and a second slower bell-shaped phase similar to that of acetone and ethanol that lasted almost 30 min. Toluene at the lowest concentration, 0.05%, triggered a biphasic transient with a shape clearly different from that of the other organic solvents. Ethanol at 0.5% triggered a bell-shaped transient that was also different from that triggered by ethanol.

The calcium signatures induced by all the compounds tested were highly reproducible; as an example, Electronic Supplementary Material Fig. S3 shows three different additions of the same concentration of $Zn^{2+}$, arsenate, bezafibrate, and naphthalene. The amplitude and/or total transient duration of $Ca^{2+}$ signatures were dose-dependent; as examples, the $Ca^{2+}$ signatures induced by increasing concentrations of $Zn^{2+}$, chromate, fenofibric acid, and bezafibrate are shown in Electronic Supplementary Material Fig. S4. Only at the highest concentration tested did some of these pollutants ($Hg^{2+}$, fenofibric acid, bezafibrate) induce a $Ca^{2+}$ signature with a different shape, as already shown in Tables 2 and 3; for naphthalene and the organic solvents, dose-dependency was manifested by $Ca^{2+}$ transients which changed shapes at increasing concentrations (Table 3).

At the concentrations tested for all the pollutants, immediate toxicity did not occur, because cell lysis was not observed, indicating that the recorded $Ca^{2+}$ signatures were not caused by aequorin released to the assay medium.

The $Ca^{2+}$ chelator EGTA at 1 mmol $L^{-1}$ completely abolished the $Ca^{2+}$ signatures triggered by all the pollutants tested, indicating that the observed $Ca^{2+}$ transients arise mainly from extracellular sources; as examples the effect of EGTA on the $Ca^{2+}$ signatures induced by 5 mg $L^{-1}$ $Zn^{2+}$ and 50 mg $L^{-1}$ $Hg^{2+}$ are shown in Electronic Supplementary Material Fig. S5.

## Relationship between $Ca^{2+}$ responses and toxicity measured as bioluminescence inhibition in a recombinant self-bioluminescent strain of *Anabaena* sp. PCC 7120

Our group has constructed a self-bioluminescent strain, *Anabaena* CPB4337, of the same species, *Anabaena* sp. PCC 7120, that expresses the bacterial *lux* operon. We have developed a toxicity assay based on luminescence inhibition by pollutants, using as endpoint of toxicity the $EC_{50}$ (concentration of the pollutant that reduces bioluminescence by 50%) [39–41, 44]. Thus, we may compare the $Ca^{2+}$ signatures induced by different pollutants with the $EC_{50}$ calculated for the bioluminescent derivative of the same species which is a direct toxic response. Table 4 shows the $EC_{50}$ values after one hour of exposure (time where acute toxicity may be easily measured with this strain) for some of the chemicals tested in this study; these $EC_{50}$ values were determined for cells resuspended in the same assay medium as that used for aequorin analysis. For anionic and cationic heavy metals, as the speciation and subsequently toxicity of many metals is modified by the composition of the assay medium, the $EC_{50}$ values shown in Table 4 are, in general, higher than those previously reported, which were determined in distilled $H_2O$ at pH 5.8 [39]. Comparing Table 4 with Electronic Supplementary Material Fig. S1 and Tables 2 and 3, it can be observed that for most of the chemicals (cations, arsenate, chromate, pharmaceuticals, naphthalene, or organic solvents) a calcium signature is induced within seconds at concentrations lower than their $EC_{50}$ values.

## $Ca^{2+}$ signatures induced by binary mixtures of pollutants in *Anabaena* sp. PCC 7120 (pBG2001a)

Pollutants in the aquatic environment do not appear individually and usually occur as complex mixtures, whose combined effect may be toxic to the biota. Chemicals in a mixture may show zero interaction or may interact in two ways, synergistically, when the effect of the combination is greater than that expected from the sum of their individual effects (more than an additive effect), or antagonistically, when the effect of the combination is less than that expected from the sum of their individual effects (less than an additive effect).

We examined how several of the compounds tested interacted in binary mixtures to induce specific $Ca^{2+}$ signatures in order to compare them with their individually induced $Ca^{2+}$ signatures. Binary mixtures of $Zn^{2+}$ plus $Cu^{2+}$, arsenate and fenofibric acid (Fn), and fenofibric acid plus bezafibrate (Bz) were made and the induced $Ca^{2+}$ signatures recorded (Figs. 1, 2, 3 and 4); the proportion of both pollutants in the mixtures was 1:1 and increasing mixture concentrations were tested. For comparison, the $Ca^{2+}$ signatures induced by the pollutants when applied individ-

**Table 4** Acute toxicity (1 h of exposure) response as $EC_{50}$ values in mg $L^{-1}$ (cations, anions, pharmaceuticals, and naphthalene) and in % (*v*/*v*) (organic solvents), with 95% confidence limits, to several pollutants in the cyanobacterium *Anabaena* sp. PCC7120 CPB 4337 toxicity assay

| Pollutant | $EC_{50}$ (1h)[a] (mg $L^{-1}$) | CI 95% |
|---|---|---|
| Cations | | |
| $Hg^{2+}$ | 0.31 | 0.28–0.33 |
| $Cu^{2+}$ | 0.26 | 0.26–0.27 |
| $Zn^{2+}$ | 2.93 | 1.64–4.07 |
| $Cd^{2+}$ | 2.27 | 1.81–4.29 |
| $Pb^{2+}$ | 88.13 | 77.64–94.14 |
| Anions | | |
| $(AsO_4)^{3-}$ | >100 | – |
| $(CrO_4)^{2-}$ | >100 | – |
| Pharmaceuticals | | |
| Bezafibrate | 37.28 | 32.60–41.79 |
| Clofibric acid | 48.08 | 45.82–55.92 |
| Fenofibric acid | 10.82 | 8.46–13.35 |
| Gemfibrozil | 8.44 | 7.81–9.24 |
| Ciprofloxacin | >80 | – |
| Ofloxacin | >80 | – |
| PAH | | |
| Naphthalene | > 100 | – |
| Organic solvents | $EC_{50}$ (1h)[a] (% *v*/*v*) | CI 95% |
| Acetone | 8.45 | 7.71–9.16 |
| Ethanol | 6.37 | 6.14–6.55 |
| Toluene | 0.94 | 0.91–0.95 |

[a] $EC_{50}$=effective concentration (mg $L^{-1}$ ; % *v*/*v*) of a toxicant that causes a 50% reduction of the self-luminescence emission of the test organism. $EC_{50}$ and 95% confidence limits, where calculated, using the linear interpolation method [34]

ually at the same concentration as that in the mixture are also shown in Figs. 1, 2, 3 and 4.

As shown in Fig. 1, the $Zn^{2+}$ plus $Cu^{2+}$ binary mixture at the lowest concentration tested, 2.5 mg $L^{-1}$, induced a $Ca^{2+}$ signature whose amplitude and duration were lower than the sum of the $Ca^{2+}$ signatures when both pollutants were applied individually, suggesting a less-than-additive effect or antagonism; the antagonistic interaction was also evident at the higher concentrations tested.

The $Ca^{2+}$ signatures induced by the $Zn^{2+}$ plus arsenate mixture, at the concentrations tested, also had a lower amplitude and more transient duration than the individual signatures (Fig. 2), again suggesting that antagonism was the predominant interaction between these two pollutants at such concentrations.

The $Zn^{2+}$ plus Fn mixture at the lowest concentration tested (Fig. 3) induced a $Ca^{2+}$ signature whose amplitude/duration were almost equal to the sum of the individual signatures, suggesting an additive effect; however, at higher mixture concentrations, the interaction between these pollutants seemed to be antagonistic.

The Fn and Bz binary mixture (Fig. 4), at the lowest concentration, induced a $Ca^{2+}$ signature whose amplitude/duration were clearly higher than those of the individual signatures suggesting a more-than-additive effect or synergism. When the mixture concentration was increased to 2.5 mg $L^{-1}$ of each compound, the interaction between both fibrates could be regarded as an additive effect, which changed into antagonism at higher mixture concentrations.

### $Ca^{2+}$ signature induced in *Anabaena* sp. PCC 7120 (pBG2001a) by a real wastewater sample and mimicking of the $Ca^{2+}$ signal by complex mixtures of the main constituents of the wastewater

We found a characteristic $Ca^{2+}$ signature induced by a wastewater sample from an STP (sewage-treatment plant) effluent (Table 5). In order to discover which of the major components of the mixture were responsible for that signature, we made mixtures with the main components of the wastewater in the same concentrations as those found in the chemical analysis of the wastewater (Table 1, Table 5, and Rosal et al. [37]). The concentration of fenobricic acid in the mixture (Table 5) was much higher than that reported for the same wastewater effluent by Rosal et al. [37] but during some sampling periods fenofibric acid was consistently found at concentrations as high as 0.14 mg $L^{-1}$ and the sample used here belongs to one of these sampling times Something similar happened with some other pharmaceuticals, for example ofloxacin, whose concentration in our sample was as high as 16 μg $L^{-1}$; this could be because of a spill of unknown source (Rosal R, personal communication).

First, we used a mixture with the elements found at the highest concentrations in the wastewater sample (mix A: Na, K, Mg, Ca, and Sr); afterwards, we used a second mix with potential toxic elements that were present at lower concentrations (mix B: B, V, Mn, Ni, Cu, As, Rb, Mo, Ba, Pb, and Fe). We also tested a mixture of the main pharmaceuticals found in the wastewater (mix C: ciprofloxacin, erythromycin, ofloxacin, fenofibric acid, and gemfibrozil). Finally, we used a mixture that contained all these elements (mix A + B + C). All the elements of the mixtures were at their environmental concentrations (Table 1 and Table 5).

As seen in the figure, only the more complete mix induced a $Ca^{2+}$ signature similar in amplitude, transient duration and shape to that of the wastewater (Table 5).

## Discussion

In this report, we present a thorough analysis of $Ca^{2+}$ signals induced by exposure of a filamentous nitrogen-

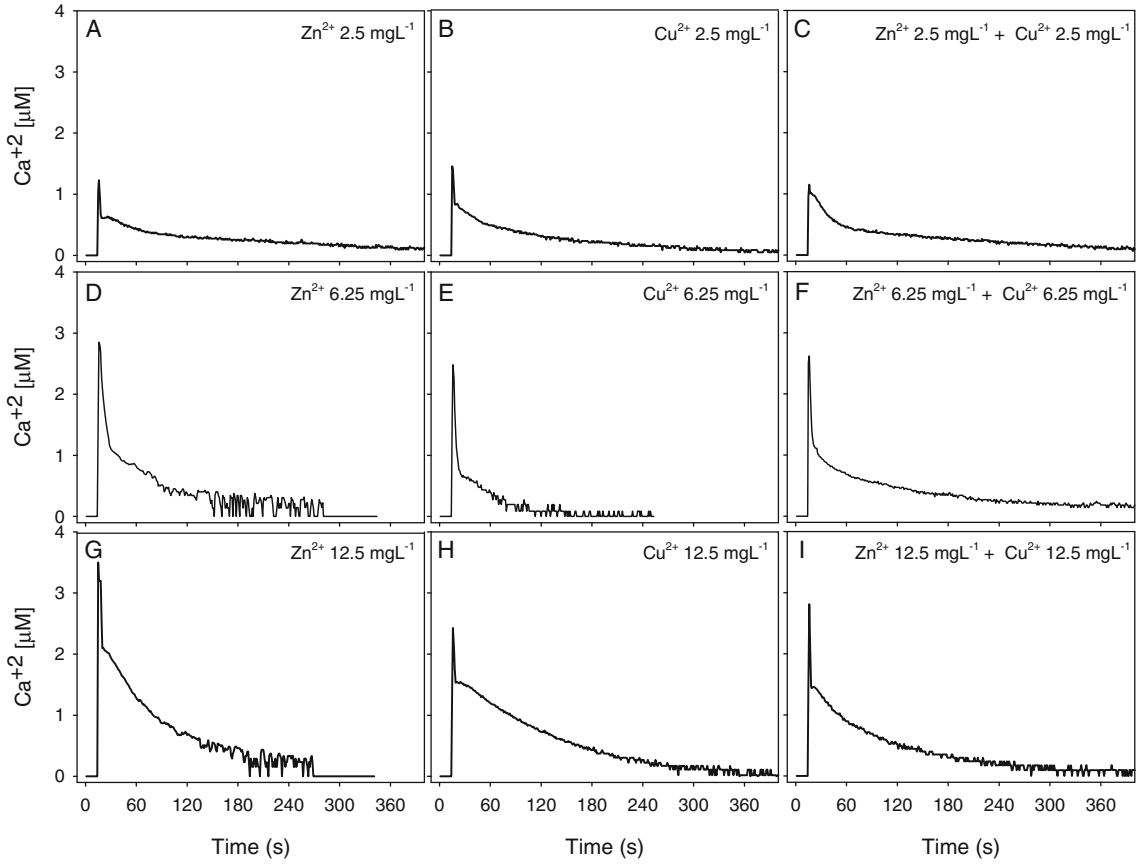



**Fig. 1** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to $Zn^{2+}$ and $Cu^{2+}$ applied individually and in binary mixtures at constant ratio 1:1. $Zn^{2+}$ 2.5 mg $L^{-1}$ (**A**), $Cu^{2+}$ 2.5 mg $L^{-1}$ (**B**), $Zn^{2+}$ 2.5 mg $L^{-1}$+$Cu^{2+}$ 2.5 mg $L^{-1}$ (**C**); $Zn^{2+}$ 6.25 mg $L^{-1}$ (**D**), $Cu^{2+}$ 6.25 mg $L^{-1}$ (**E**), $Zn^{2+}$ 6.25 mg $L^{-1}$+$Cu^{2+}$ 6.25 mg $L^{-1}$ (**F**); $Zn^{2+}$ 12.5 mg $L^{-1}$ (**G**), $Cu^{2+}$ 12.5 mg $L^{-1}$ (**H**), $Zn^{2+}$ 12.5 mg $L^{-1}$+ $Cu^{2+}$ 12.5 mg $L^{-1}$ (**I**)

fixing cyanobacterium to environmental pollutants at a wide range of concentrations. All the pollutants tested induced a quick (within seconds), significant, and specific calcium signature which was highly reproducible and dose-dependent and could be defined by a series of properties. The dose-dependency varied from changes in amplitude and transient duration (cations, anions, some of the organic pollutants) to changes of shape (organic solvents, $Hg^{2+}$, and some other organic pollutants at the highest concentration tested). During the course of our experiments, cell lysis was not observed, indicating that the observed $Ca^{2+}$ signatures were truly intracellular and that no immediate toxicity was caused by exposure to the pollutants at the concentrations tested.

An important characteristic of $Ca^{2+}$ signatures is the source of the $Ca^{2+}$ involved in the induction of the $Ca^{2+}$ transient. The use of the $Ca^{2+}$ chelator EGTA (zero external $Ca^{2+}$) clearly discriminated between extracellular and intracellular spaces. All the recorded $Ca^{2+}$ signals required an influx from the extracellular space. Direct and indirect evidences indicate the presence of $Ca^{2+}$ channels and $Ca^{2+}$ exchangers in cyanobacteria that could be responsible of the $Ca^{2+}$ fluxes [18, 45–47]. Several groups working with $Ca^{2+}$ signaling and environmental pollutants have investigated the source of $Ca^{2+}$ involved in the disruption of $Ca^{2+}$ homeostasis/signaling in their cell systems; several authors [25, 48–51] have found that for organic pollutants such as bromophenols, polybrominated diphenyl ethers, methylmercury, alkylphenols, and the cationic metal $Cd^{2+}$, the $Ca^{2+}$ source was both extra and intracellular; others [52, 53] found an extracellular source of $Ca^{2+}$ for the effect of butyltins and aluminium on $Ca^{2+}$ signaling.

In general, chemicals of the same group, at least for a given concentration, induced very similar $Ca^{2+}$ signatures. We hypothesize that this similarity may be related to similar mechanism of cellular perception of the pollutants and, ultimately, similar toxic mode of action. If $Ca^{2+}$ as a second messenger is truly involved in signaling of environmental pollution in cyanobacteria, any alteration in the specific $Ca^{2+}$ signatures would result in alteration of the cellular response to the pollutant/class of pollutants. We have previously shown that suppression, magnification, or poor regulation of a $Ca^{2+}$ signature that appeared early after nitrogen starvation in two cyanobacteria expressing apoaequorin, *Anabaena* sp.

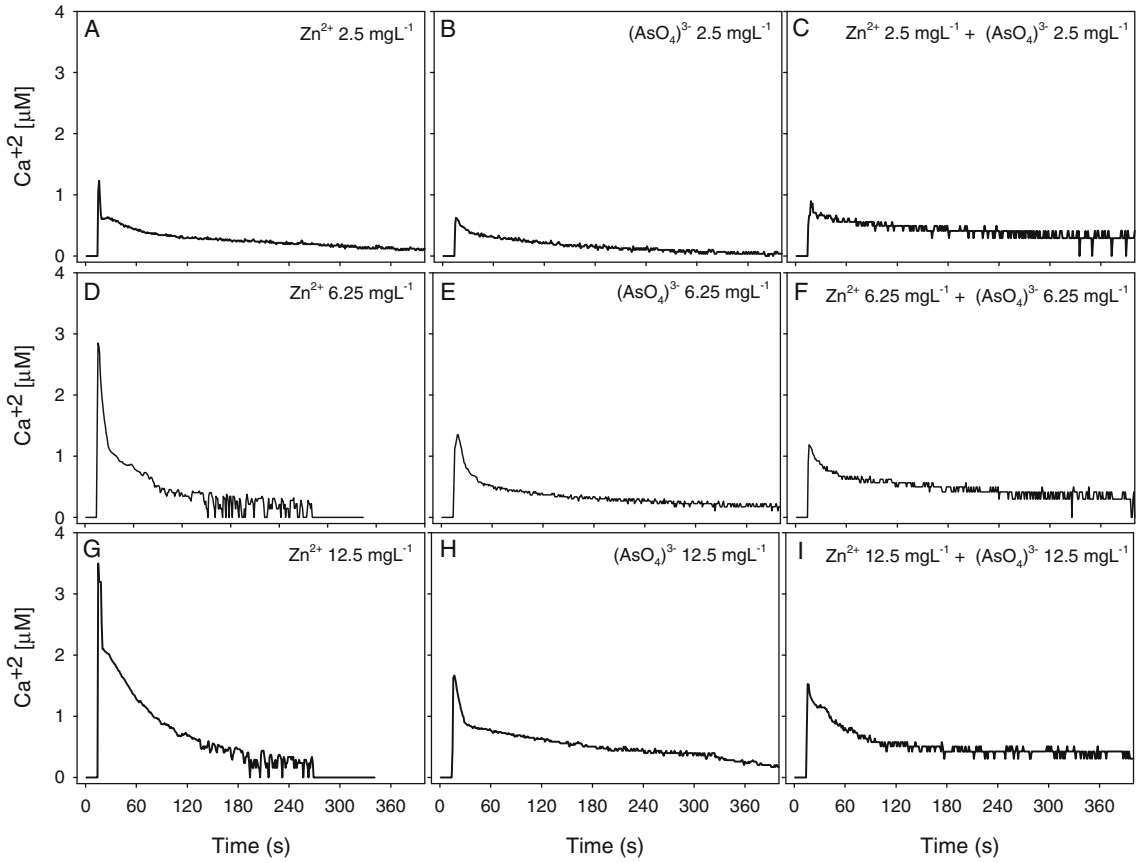



**Fig. 2** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to $Zn^{2+}$ and $(AsO_4)^{3-}$ applied individually and in binary mixtures at constant ratio 1:1. $Zn^{2+}$ 2.5 mg $L^{-1}$ (**A**), $(AsO_4)^{3-}$ 2.5 mg $L^{-1}$ (**B**), $Zn^{2+}$ 2.5 mg $L^{-1}$+$(AsO_4)^{3-}$ 2.5 mg $L^{-1}$ (**C**); $Zn^{2+}$ 6.25 mg $L^{-1}$ (**D**), $(AsO_4)^{3-}$ 6.25 mg $L^{-1}$ (**E**), $Zn^{2+}$ 6.25 mg $L^{-1}$+$(AsO_4)^{3-}$ 6.25 mg $L^{-1}$ (**F**); $Zn^{2+}$ 12.5 mg $L^{-1}$ (**G**), $(AsO_4)^{3-}$ 12.5 mg $L^{-1}$ (**H**), $Zn^{2+}$ 12.5 mg $L^{-1}$+$(AsO_4)^{3-}$ 12.5 mg $L^{-1}$ (**I**)

PCC 7120 (pBG2001a) and *Synechococcus elongatus* PCC 7942, prevented acclimation to N deprivation in both strains [19, 22]. Oxidative stress has been reported to be a first response to different environmental pollutants and has been related to calcium signaling in tobacco cells [52] and brain cells [54]. It would be of interest to check whether oxidative stress is provoked by exposure of our model cyanobacterium to pollutants and if this is so, to relate oxidative stress to calcium signaling by manipulating $Ca^{2+}$ signatures.

Most studies of pollutants and calcium are based on the concept that pollutants disrupt $Ca^{2+}$ homeostasis or $Ca^{2+}$ signaling of a cellular process; this disruption may lead to cellular toxicity, because elevated intracellular $Ca^{2+}$ is known to be cytotoxic in most cellular types [5, 28, 30, 55]. Our approach is different, because, as discussed above, we hypothesize that each pollutant or class of pollutants triggers a specific $Ca^{2+}$ signature which subsequently induces a signaling pathway; this may result in an appropriate cellular response towards the pollutant, not necessarily toxic, for example stress-induced adaptation and survival; only certain circumstances, e.g. high concentration or long exposure to the pollutant, may result in deregulation of $Ca^{2+}$ homeostasis/signaling leading to cellular toxicity.

In this respect, the $Ca^{2+}$ signatures that we have recorded usually have a transient temporal feature: increase of intracellular free $Ca^{2+}$ to form a quick spike or a bell-shaped signal then decrease to reach resting $Ca^{2+}$ levels (approx. 100 nmol $L^{-1}$ in *Anabaena* sp. PCC 7120 [18]) restoring intracellular $Ca^{2+}$ homeostasis; these transient increases are most probably related to a regulatory/signaling role of $Ca^{2+}$. At very high concentrations of some of the pollutants, however, for example fenofibric acid and bezafibrate, and in a wider range of concentrations for organic solvents, the $Ca^{2+}$ transient did not return to basal levels and remained high for more than an hour. In these cases, a high and sustained level of intracellular free $Ca^{2+}$ leads to a significant alteration of $Ca^{2+}$ homeostasis; this may activate major cellular pathways leading to toxicity and, eventually, cell death.

Domingues et al. [56], emphasize that a challenge for biomarkers is the ability to relate the presence of a chemical in the environment with a valid prediction of a subsequent hazard to the organisms or populations; so that biomarker

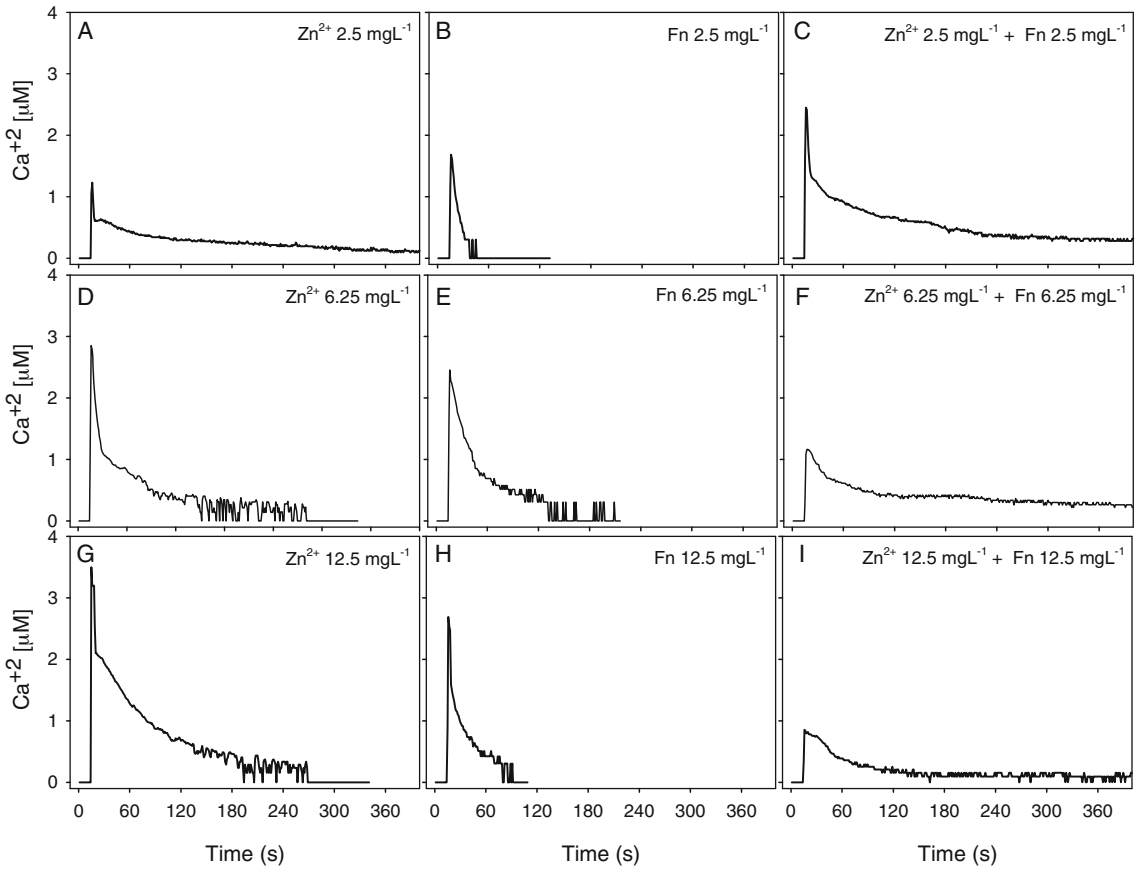



**Fig. 3** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to $Zn^{2+}$ and fenofibric acid (Fn) applied individually and in binary mixtures at constant ratio 1:1. $Zn^{2+}$ 2.5 mg $L^{-1}$ (**A**), Fn 2.5 mg $L^{-1}$ (**B**), $Zn^{2+}$ 2.5 mg $L^{-1}$+Fn 2.5 mg $L^{-1}$ (**C**), $Zn^{2+}$ 6.25 mg $L^{-1}$ (**D**), Fn 6.25 mg $L^{-1}$ (**E**), $Zn^{2+}$ 6.25 mg $L^{-1}$+Fn 6.25 mg $L^{-1}$ (**F**), $Zn^{2+}$ 12.5 mg $L^{-1}$ (**G**), Fn 12.5 mg $L^{-1}$ (**H**), $Zn^{2+}$ 12.5 mg $L^{-1}$ +Fn 12.5 mg $L^{-1}$ (**I**)

responses might correlate with toxicity because of these pollutants. We have compared the $Ca^{2+}$ signatures induced by pollutants at different concentrations with the $EC_{50}$ values of the toxicity bioassay using a recombinant bioluminescent strain of the same species, *Anabaena* sp. PCC 7120. For most chemicals a significant calcium signature is induced within seconds at concentrations well below their $EC_{50}$ values (sublethal levels), highlighting its role as an early biomarker of exposure to pollutants.

The $Ca^{2+}$ signatures induced by pollutants in the cyanobacterium are clearly different from those induced by environmental factors that may affect a photosynthetic organism, for example temperature shocks, salinity, osmotic stress, light–dark transitions, pH changes, or nitrogen starvation [18–21]. This emphasizes the specificity of $Ca^{2+}$ signatures, reinforcing again its potential use as a biomarker of environmental pollution.

A relevant issue is the biomarker responses to chemical mixtures; a realistic scenario in natural environments is exposure of the biota to complex mixtures of pollutants. For simplicity, most ecotoxicological studies and risk-assessment strategies focus on the hazard of individual chemicals; this may underestimate the risks associated with the toxic action of mixtures. The toxicity of a mixture depends on the toxicity of the components and how the components interact with each other in a dose-dependent way.

It would be interesting to find biomarkers that could predict antagonistic or synergistic interactions of the chemicals in a mixture; in this context, our group was the first to work on environmental applications of the combination index (CI) [57], a method widely used in pharmacology to study the nature of interactions of drugs in a mixture. So far, we have applied the method to our bioluminescent strain *Anabaena* CPB4337 exposed to mixtures of heavy metals [39] and fibrates [40]. For the heavy metals $Cu^{2+}$, $Cd^{2+}$, and $Zn^{2+}$, antagonism was the predominant interaction at low effect levels (low concentrations of the cations in the mixture); this turned into synergism at the highest effect levels (high concentrations of the cations in the mixture). For fibrate mixtures, however, synergism was evident at medium to low levels of effects, even at concentrations at which individual pollutants did not induce toxicity.

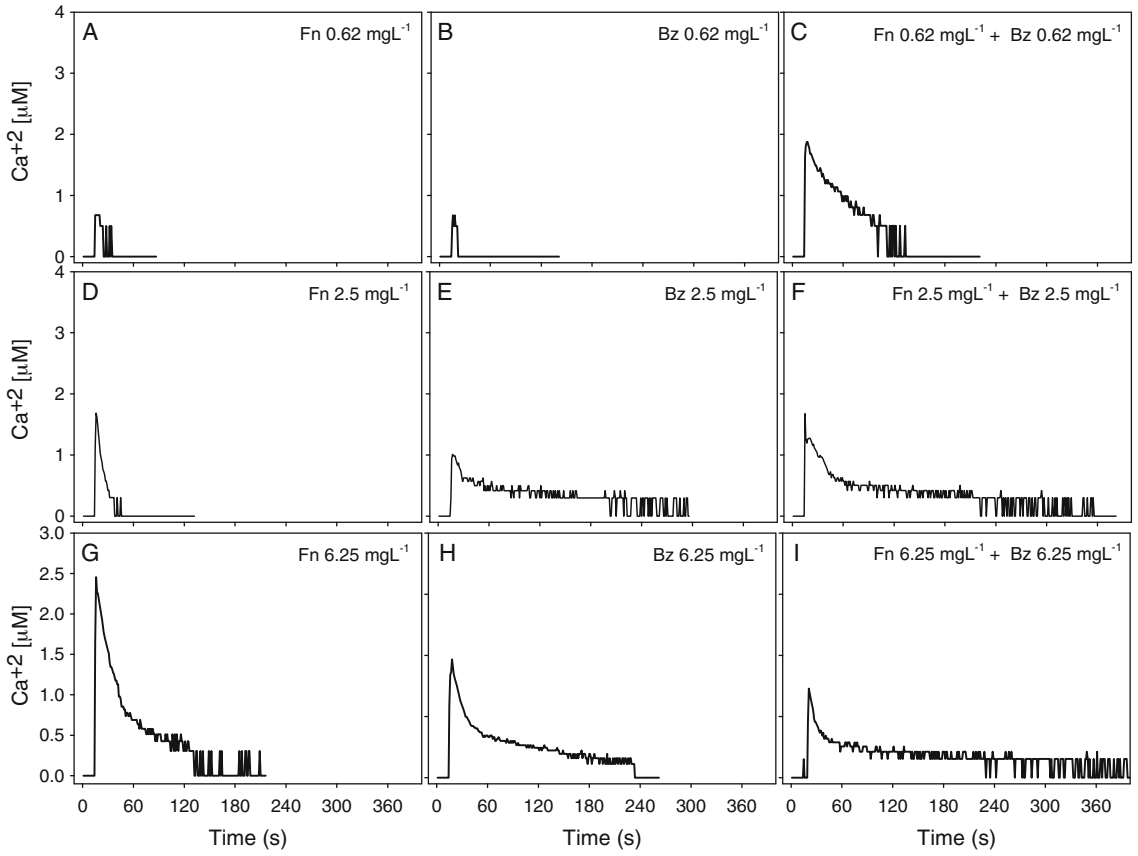



**Fig. 4** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to fenofibric acid (Fn) and bezafibrate (Bz) applied individually and in binary mixtures at constant ratio 1:1. Fn 0.62 mg $L^{-1}$ (**A**), Bz 0.62 mg $L^{-1}$ (**B**), Fn 0.62 mg $L^{-1}$+Bz 0.62 mg $L^{-1}$ (**C**),Fn 2.5 mg $L^{-1}$ (**D**), Bz 2.5 mg $L^{-1}$ (**E**), Fn 2.5 mg $L^{-1}$+Bz 2.5 mg $L^{-1}$ (**F**), Fn 6.25 mg $L^{-1}$ (**G**), Bz 6.25 mg $L^{-1}$ (**H**), Fn 6.25 mg $L^{-1}$+Bz 6.25 mg $L^{-1}$ (**I**)

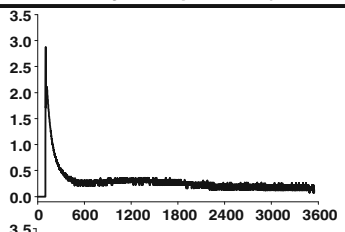
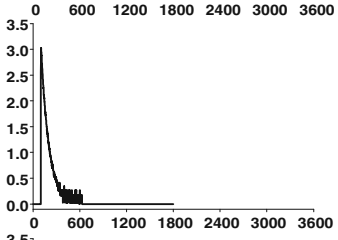
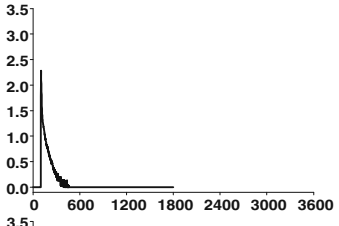
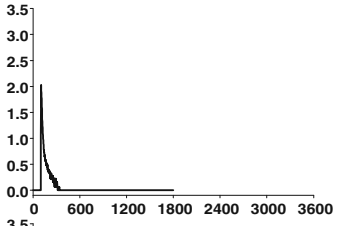
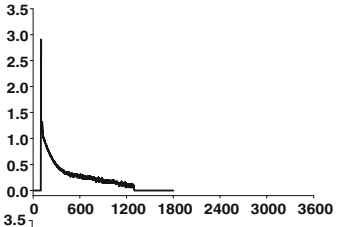
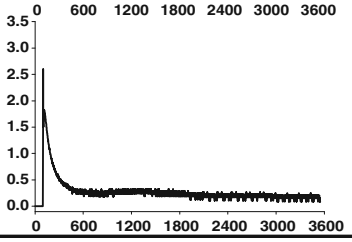
In agreement with our previous results using the combination index with cations [39], according to the induced $Ca^{2+}$ signatures, $Zn^{2+}$ and $Cu^{2+}$ had an antagonistic interaction at the tested mixture concentrations which were in the range of those which resulted in antagonism by CI. The $Ca^{2+}$ signature induced by the binary mixture of the two fibrates, Fn and Bz, indicated synergism at low levels which turned into antagonism when the mixture concentration was increased. This finding completely agrees with our previous results on interaction of fibrate mixtures [40].

$Zn^{2+}$ plus fenofibric acid and $Zn^{2+}$ plus arsenate are two binary mixtures to which we have not applied the CI to discover how they interact; however, the $Ca^{2+}$ signatures induced by both mixtures compared with the individual signatures suggested that antagonism was the predominant interaction in the range of mixture concentrations tested. Although our results are promising, we believe that a thorough and parallel study of the interaction of pollutants in binary and more complex mixtures, applying both CI and recording of $Ca^{2+}$ signatures, is necessary before proposing that $Ca^{2+}$ signatures might predict the nature of interactions of pollutants. Llabjani et al. [58] used infrared spectroscopy as a biomarker to assess biochemical alterations induced by binary mixtures of polychlorinated biphenyls and polybrominated diphenyl ether congeners in a breast carcinoma cell line and could predict the nature of the interactions between pollutants on the basis of the increased or decreased level of observed alterations compared with the chemicals applied individually.

Regarding complex mixtures of pollutants, we have been able to record a $Ca^{2+}$ signature induced by a real wastewater sample whose chemical analysis is available. More interestingly, we have been able to mimic the $Ca^{2+}$ signature by mixing its main constituents at their environmental concentration. We intend to continue the recording and analysis of $Ca^{2+}$ signatures that may be induced by water samples from polluted sites to compare them with those that may be obtained from clean reference sites.

We propose that intracellular $Ca^{2+}$ signatures could be envisaged as an early biomarker of exposure to pollutants as they show a dose-response relationship in a defined range of concentrations in which rapid induction/rapid recovery to basal levels is observed; if, moreover, at a given concentration of the pollutant, return to resting levels of intracellular free $Ca^{2+}$ does not happen or is delayed, a

**Table 5** Properties of calcium signatures induced in *Anabaena* sp. PCC7120 (pBG2001a) by a wastewater from an STP located in Madrid (Spain), and by mixtures of its main constituents[a]

| Stimulus | Transient Shape (x-axis:Time (s); y-axis: µM [$Ca^{+2}$]) | Amplitude µM [$Ca^{+2}$] | Rise Time (s) | Total transient duration (min) | Source of $Ca^{2+}$ |
|---|---|---|---|---|---|
| Wastewater |  | 2.86.±0.25 | 2-3 | >60 | Extracellular |
| Mix A |  | 2.78±0.19 | 2-3 | 10.20±0.24 | Extracellular |
| Mix B |  | 2.14±0.18 | 2-3 | 5.27±0.55 | Extracellular |
| Mix C |  | 2.54±0.25 | 2-3 | 6.04±0.12 | Extracellular |
| Mix A+Mix B |  | 2.68±0.27 | 2-3 | 20.56±0.28 | Extracellular |
| Mix A+Mix B +Mix C |  | 2.61.±0.33 | 2-3 | >60 | Extracellular |

[a]Mix A: $Na^{+}$ 80 mg $L^{-1}$, $Mg^{2+}$ 20 mg $L^{-1}$, $Ca^{2+}$ 45 mg $L^{-1}$, $K^{+}$ 20 mg $L^{-1}$, and $Sr^{2+}$ 1 mg $L^{-1}$

Mix B: $B^{3+}$ 175 µg $L^{-1}$, $V^{5+}$ 2 µg $L^{-1}$, $Mn^{2+}$ 27 µg $L^{-1}$, $Ni^{2+}$ 5 µg $L^{-1}$, $Cu^{2+}$ 2 µg $L^{-1}$, $(AsO_4)^{3-}$ 4 µg $L^{-1}$, $Rb^{1+}$ 11 µg $L^{-1}$, $Mo^{6+}$ 4 µg $L^{-1}$, $Ba^{2+}$ 3 µg $L^{-1}$, $Pb^{2+}$ 1 µg $L^{-1}$, and $Fe^{2+}$ 38 µg $L^{-1}$

Mix C: Ciprofloxacin 6 µg $L^{-1}$, erythromycin 6 µg $L^{-1}$, ofloxacin 16 µg $L^{-1}$, gemfibrozil 5 µg $L^{-1}$, and fenofibric acid 0.14 mg $L^{-1}$

cytotoxic response will probably occur. The fact that the $Ca^{2+}$ signature appears very early after exposure to concentrations of pollutants which do not provoke immediate toxicity, as observed by the lack of cellular lysis during our experiments, make intracellular free calcium a sensitive indicator; it might, in fact, be one of the first detectable and quantifiable responses to environmental pollution.

## Conclusions

All the tested pollutants induced a quick and specific $Ca^{2+}$ signature which was highly reproducible and dose-dependent. Chemicals of the same group, at least for a given concentration, gave very similar $Ca^{2+}$ signatures, suggesting a similar cellular mechanism of pollutant perception and, ultimately, a similar toxic mode of action. Monitoring of $Ca^{2+}$ signatures induced by binary mixtures could be a promising tool to predict the nature of the interactions between the pollutants; complex mixtures such as real wastewater mixtures also induced specific $Ca^{2+}$ signatures which may be mimicked by mixing its main constituents at the environmental concentrations. The fact that $Ca^{2+}$ signatures are induced at concentrations at which toxicity is still not evident makes monitoring of intracellular free $Ca^{2+}$ changes an early and sensitive biomarker of exposure to pollutants either individually or in combination.

**Acknowledgements** This research was funded by the Spanish Ministry of Science and Innovation (grant CGL2010-15675, subprogramme BOS) and by the Comunidad de Madrid grant S-2009/AMB/1511 (Microambiente-CM) .

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**Analytical and Bioanalytical Chemistry**

**Electronic Supplementary Material**

# Free $Ca^{2+}$ as an early intracellular biomarker of exposure of cyanobacteria to environmental pollution

Ana Lilia Barrán-Berdón, Ismael Rodea-Palomares, Francisco Leganés, Francisca Fernández-Piñas

**Figure S1:** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to 0.5 $mgL^{-1}$ of $Zn^{2+}$ (A); bezafibrate (Bz, B); $Cu^{2+}$ (C); gemfibrozil (Gm,D), $Hg^{2+}$ (E); fenofibric acid (Fn, F), $Pb^{2+}$ (G), clofibric acid (Clo, H); $Cd^{2+}$ (I); $CrO_4^{2-}$ (J); $AsO_4^{3-}$ (K); naphthalene (Naph,L); ofloxacin (Oflo, M); ciprofloxacin (Cipro, N)

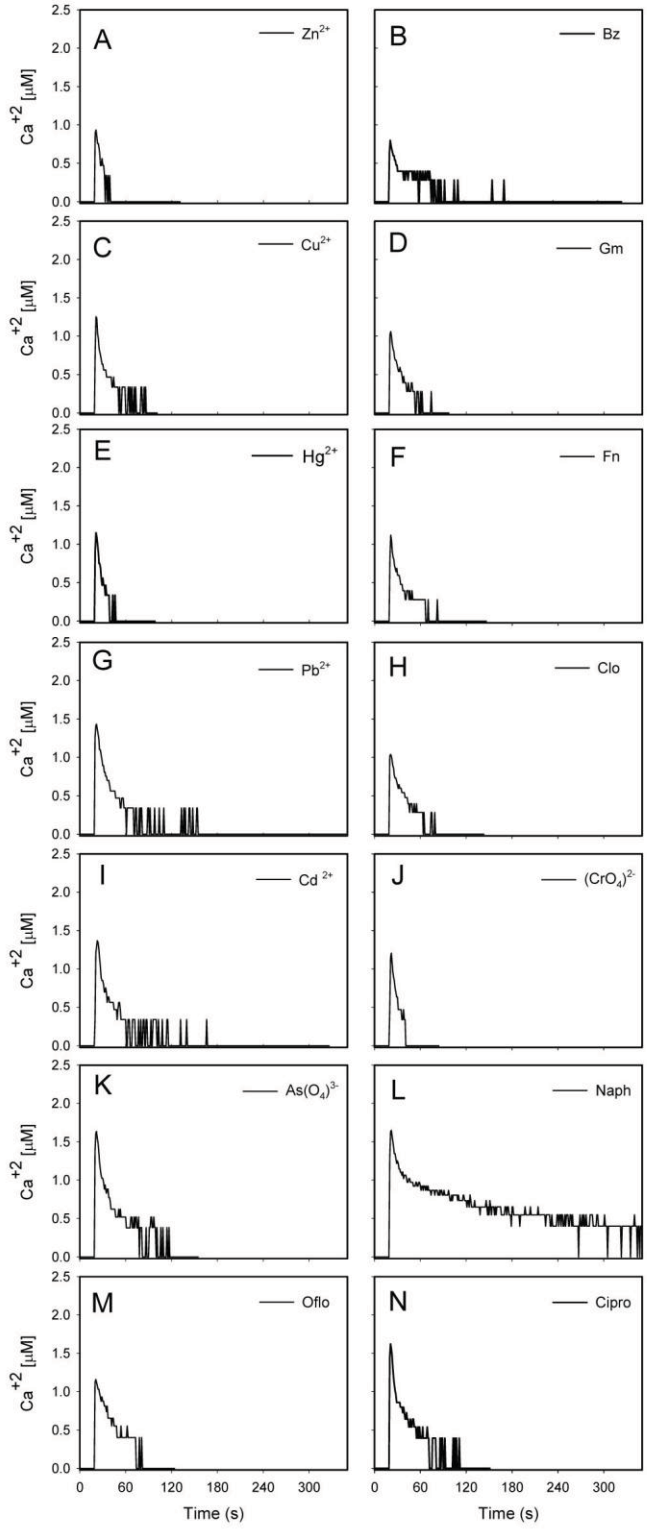


**Figure S2:** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to $CuCl_2$ and $CuSO_4$ at $5mgL^{-1}$

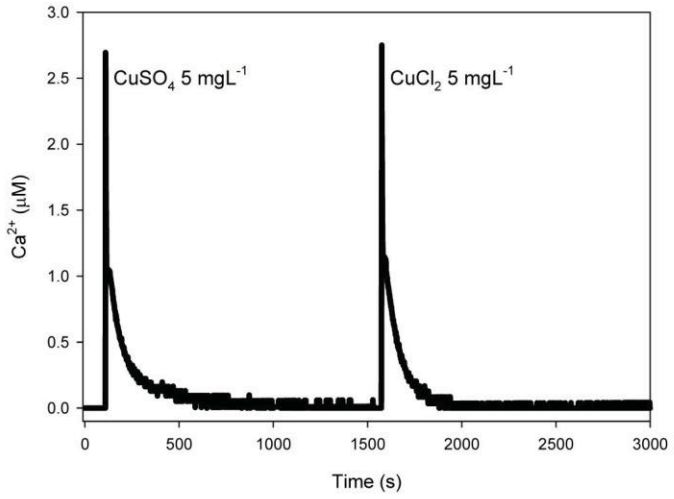


**Figure S3:** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to 3 different injections of $Zn^{2+}$ at 5 $mgL^{-1}$ (A), $(AsO_4)^{3-}$ at 5 $mgL^{-1}$ (B), Bezafibrate at 5 $mgL^{-1}$ (C) and Naphthalene at 50 $mgL^{-1}$ (D)

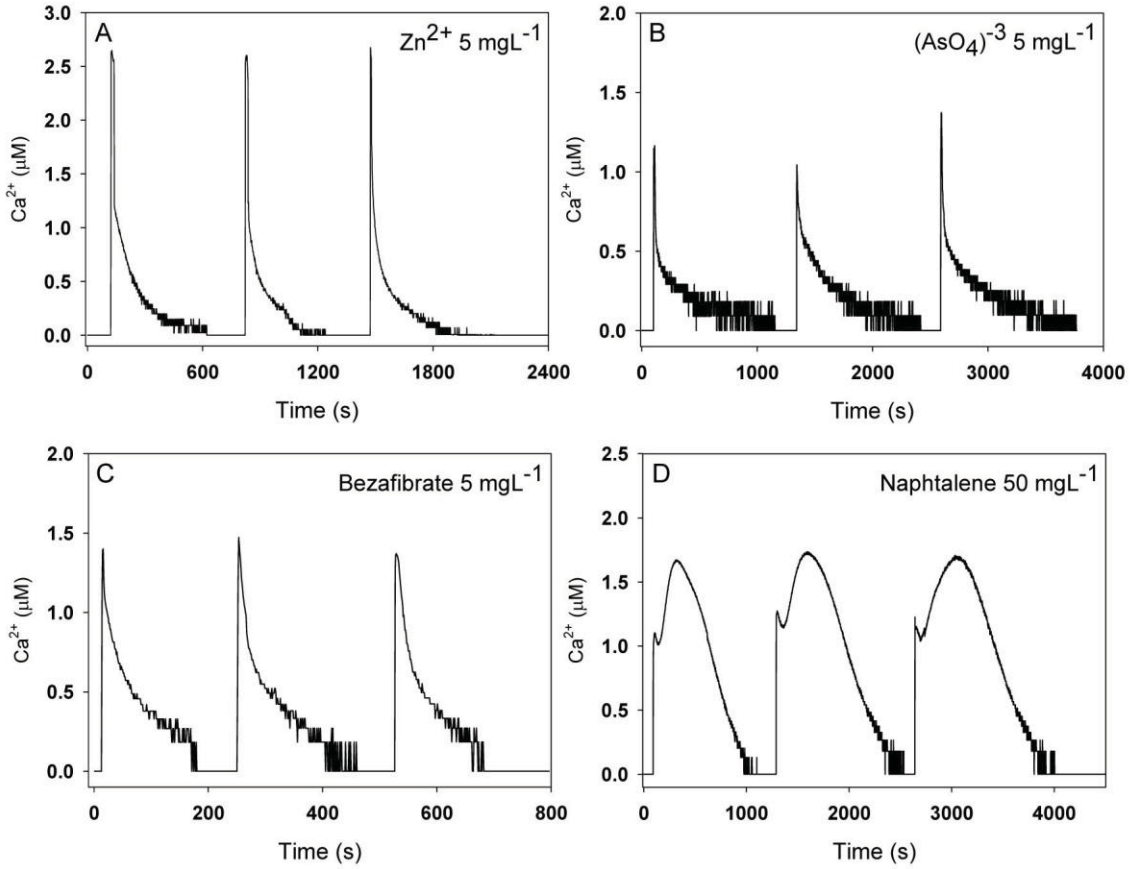


**Figure S4:** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to increasing concentrations of $Zn^{2+}$ (A), $(CrO_4)^{2-}$ (B), Fenofibric acid (C) and Bezafibrate (D)

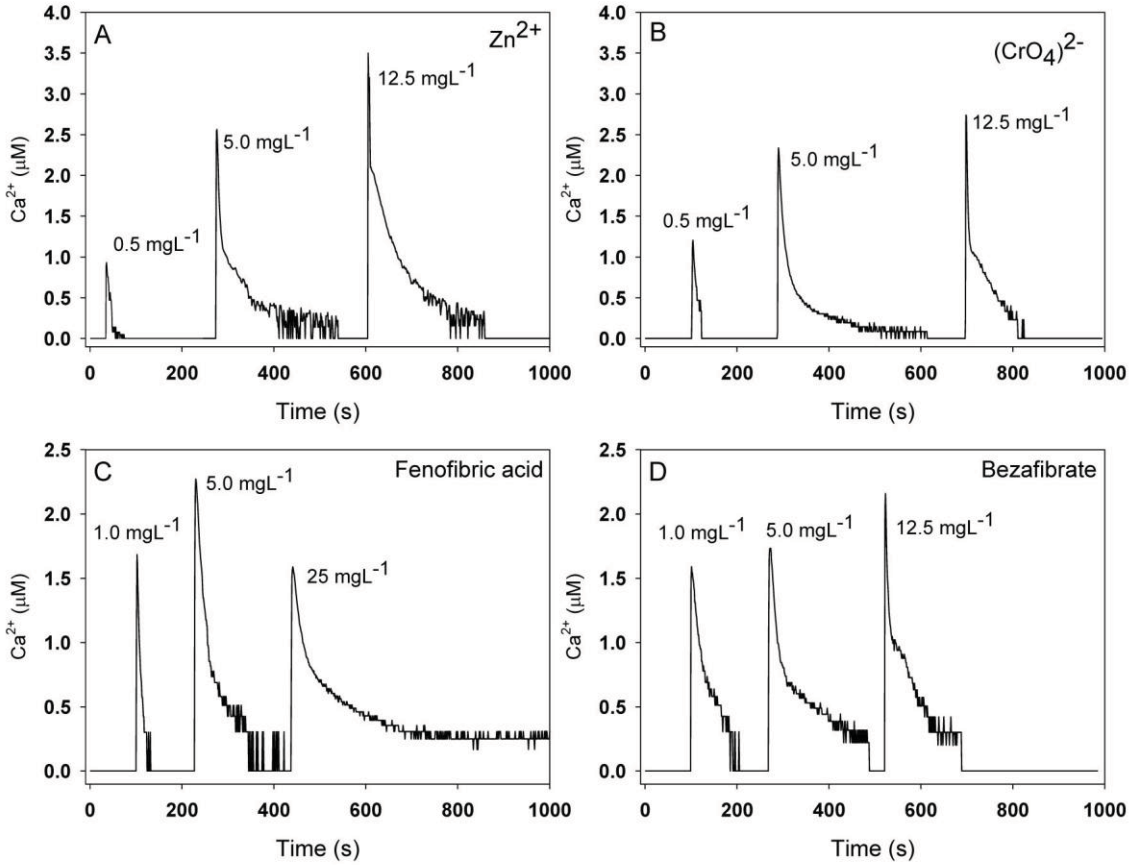
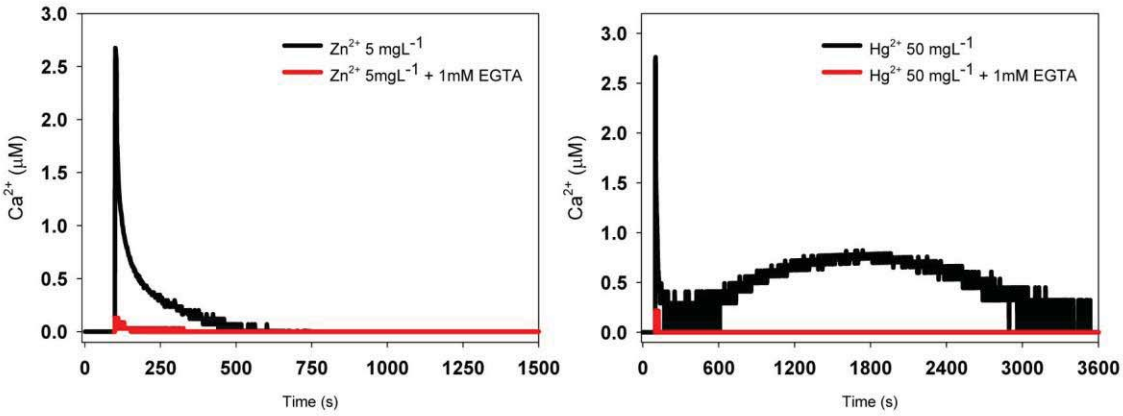


**Figure S5:** $Ca^{2+}$ signatures induced in *Anabaena* sp. PCC 7120 (pBG2001a) in response to $Zn^{2+}$ (A) and $Hg^{2+}$ (B) with and without being previously treated with the extracellular $Ca^{2+}$ chelator EGTA



# ANNEXE II
# Outlines of published articles

## Impacto ambiental de los contaminantes emergentes

*25 de febrero de 2010*

Los investigadores de la Universidad Autónoma de Madrid (UAM) Ismael Rodea-Palomares, Francisco Leganés y Francisca Fernández Piñas, en colaboración con el grupo de investigación del Dr. Roberto Rosal de la Universidad de Alcalá de Henares, han estudiado el impacto de ciertos contaminantes provenientes de medicamentos en agua tanto residual como para consumo humano.

En el trabajo, publicado en *Environmental Science and Pollution Research*, se describe el uso de tres organismos acuáticos para evaluar la toxicidad aguda de cuatro compuestos farmacéuticos de la familia de los fibratos en agua y aguas residuales.

Los fibratos son compuestos derivados del ácido fíbrico empleados para controlar el colesterol y son usados de forma generalizada en medicina humana en países desarrollados. Estos compuestos se integran dentro del grupo de los "contaminantes emergentes", que engloba medicamentos, productos de uso y cuidado cosmético, nanomateriales, etc.

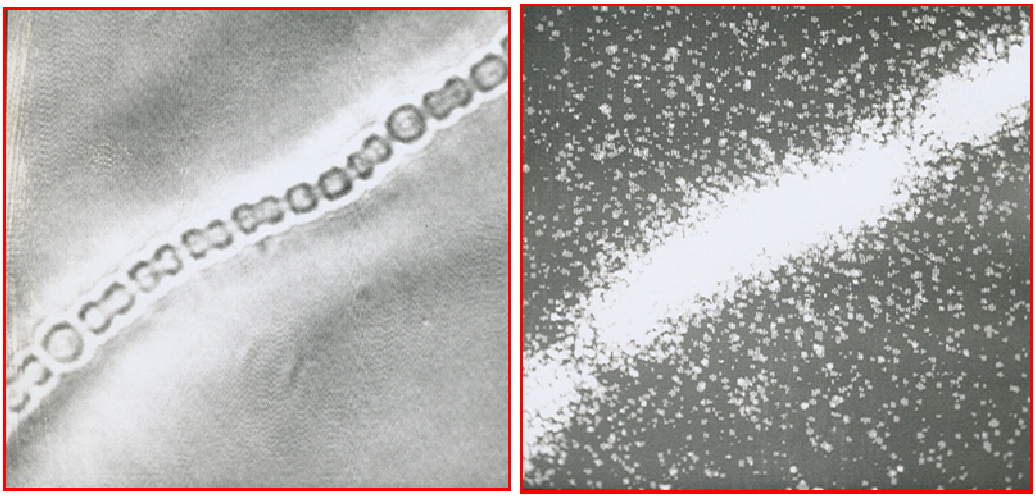
La gran concentración poblacional en grandes urbes y los altos niveles de consumo de dichos compuestos hace que estos contaminantes vayan a parar a las aguas naturales vía aguas residuales urbanas y depuradoras, y ya desde hace varios años vienen siendo detectados en efluentes de depuradoras, aguas continentales, marinas, subterráneas e incluso en agua de consumo humano.

Estos contaminantes han tomado gran interés para la comunidad científica en los últimos años al ser sustancias para los cuales suele conocerse bien su aplicación y mecanismo farmacológico pero cuyo posible efecto sobre el medio ambiente y los ecosistemas puede ser totalmente desconocido.

En el presente trabajo se analizó la toxicidad de varios fibratos (ácido fenofíbrico, ácido clofíbrico, gemfibrozil y bezafibrato) utilizando para ello 3 organismos acuáticos: 2 organismos empleados en métodos estándar de análisis: una bacteria marina (*Vibrio fischeri*) y la pulga de

agua (*Daphnia magna)* y un test de toxicidad novedoso desarrollado por el grupo de investigación de la UAM: una cianobacteria de agua dulce recombinante autoluminiscente denominada *Anabaena* CPB4337. Las cianobacterias tienen una gran relevancia ecológica al ser productores primarios con un papel clave en los ciclos del carbono y del nitrógeno en ambientes acuáticos.

En el estudio pudo comprobarse que todos los compuestos analizados presentaron toxicidad para los tres organismos. Un punto interesante fue que basándose en los resultados de los test comerciales sólo uno de los medicamentos (el ácido fenofíbrico) sería catalogado como "peligroso para los organismos acuáticos" y que el agua residual cumpliría con los requisitos de vertido al no presentar toxicidad en los test estándar. Sin embargo, la introducción de la cianobacteria como test de toxicidad hace que el gemfibrozil y el bezafibrato pasen a considerarse también "peligrosos para los organismos acuáticos" y que el agua residual se catalogue como "muy tóxica".



Cianobacteria bioluminiscente: foto realizada con una cámara fotónica (fotomultiplicador) donde se puede apreciar la luminiscencia del organismo.

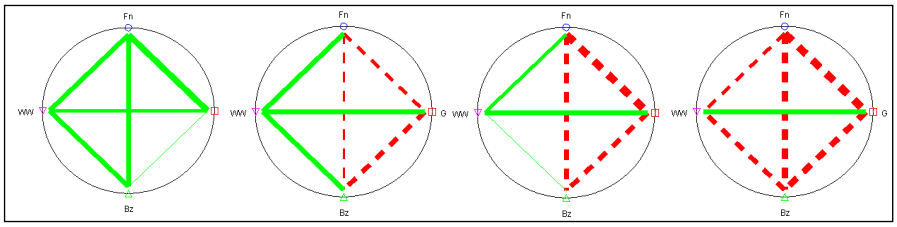
Los resultados obtenidos apoyan la necesidad de desarrollar nuevos y más sensibles test de toxicidad usando especies ecológicamente relevantes para la detección de posibles efectos negativos de contaminantes ambientales que pueden ser infraestimados usando bioensayos convencionales.

## En ecotoxicología 2+2 no siempre son cuatro

*Mayo 21 de 2010*

Un trabajo conjunto llevado a cabo por investigadores de la Universidad Autónoma de Madrid (UAM) junto con investigadores de la Universidad de Alcalá (UAH) aplica, de forma pionera en la evaluación ambiental, el teorema del *Índice de Combinación (CI)-Isobolograma* de Chou y Talalay, una metodología de análisis de interacción de medicamentos. Dicha metodología permite descifrar la naturaleza de la interacción de sustancias en un organismo para saber si sus efectos se potencian, se anulan o son indiferentes y en qué grado.

En el campo de la evaluación de impacto ambiental la acción conjunta de distintos contaminantes es un punto de arduo estudio y discusión, debido a que en las situaciones de contaminación normalmente intervienen un gran número de sustancias cuyo efecto conjunto es difícil de predecir. En muchas ocasiones se producirán efectos denominados “sinérgicos” o “antagónicos” entre los contaminantes, es decir, que el efecto conjunto será mayor que la suma de los efectos individuales, o el caso contrario: la suma de efectos será menor que la de los efectos individuales.




Evolución del patrón de interacción de 3 compuestos farmacéuticos y un agua residual aplicando el índice de combinación en cuatro niveles de efecto: desde un efecto tóxico muy pequeño (10%) hasta un efecto del 99% de toxicidad. Las líneas verdes continuas indican sinergia, las líneas rojas discontinuas indican antagonismo. El grosor de la línea indica la intensidad de la interacción.

Para intentar abordar este problema se han propuesto distintos métodos que van desde considerar que los contaminantes en concentraciones muy bajas no interaccionan entre sí

hasta intentar predecir su posible interacción en función de la similitud de sus modos de acción o en base a modelos teóricos basados en su estructura espacial.

En el presente trabajo, publicado en *Water Research*, se consideró fundamental introducir las bases del uso del Teorema del Índice de Combinación en el análisis de la interacción de contaminantes ambientales basándose en varias ventajas del método frente a otros ya existentes: se fundamenta en los parámetros dosis efecto de los compuestos individuales y sus mezclas, no requiere conocimiento previo de los mecanismos de acción de los compuestos, puede usarse con cualquier organismo y parámetro de toxicidad, permitiendo evaluar la interacción de mezclas de un gran número de contaminantes.

Con estas premisas, Ismael Rodea Palomares, Francisco Leganés y Francisca Fernández Piñas de la UAM junto con Roberto Rosal, Alice Petre, Jose Antonio Perdigón-Melón y Karina Boltes de la UAH, evaluaron la naturaleza de la interacción de la toxicidad de 3 medicamentos, denominados *fibratos* y que se emplean para regular el colesterol, entre ellos y con un agua residual procedente de una depuradora madrileña sobre dos organismos acuáticos: el test comercial de ecotoxicidad *Vibrio fischeri* y un biosensor de toxicidad basado en una cianobacteria recombinante autoluminiscente denominada *Anabaena* CPB4337.

**Los resultados de dicho estudio han permitido establecer varias conclusiones generales de amplio impacto en el análisis ambiental:**

1º La naturaleza de la interacción toxicológica de las sustancias no es absoluta, es decir depende del organismo (puede ser sinérgico para un organismo y antagónico para otro).

2º Incluso en el mismo organismo la interacción puede variar en función de la intensidad del daño sobre el organismo (puede pasar incluso de antagonismo a sinergia o al revés).

3º Los distintos tipos de interacción pueden darse independientemente de la similitud o disimilitud de los mecanismos de acción de las sustancias.

Como conclusión global se obtiene que el índice de combinación-isobolograma se configura como un método general en ecotoxicología para definir cómo interaccionan los tóxicos potenciales que pueda haber en una muestra ambiental y puede ser particularmente útil para

definir estrategias de evaluación de riesgos, en particular, cuando se encuentra sinergia a bajas concentraciones de los contaminantes.

# Application of the combination index (CI)-isobologram equation to study the toxicological interactions of pollutants in aquatic organisms

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*Aquatic biota is usually exposed to complex mixtures of pollutants; however, due to simplicity, most ecotoxicity studies and risk assessment strategies focus on hazard of individual chemicals only and may underestimate the risk associated with the toxic action of mixtures.*

The toxicity of a mixture depends on the toxicity of the components and how the components interact with each other in a dose-dependent way.

Chemicals in a mixture may show zero interaction or may interact in two ways:

· Synergistically: The effect of the combination is greater than that expected from the sum of their individual effects (more than an additive effect)

· Antagonistically: The effect of the combination is less than that expected from the sum of their individual effects (less than an additive effect)

Synergism and antagonism may be defined as departures (deviations) from zero interaction additive effect) between chemicals in a mixture.

But, how to define zero interaction or additive effect between chemicals in a mixture?

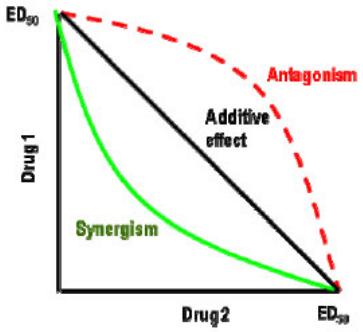
An additive effect is not just the simple arithmetic sum of the effects of the individual chemicals.

Definition of additivity is usually based on the classical isobolograms of the combination of two chemicals:

$$\frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} = 1$$

Where $(D)_1$ and $(D)_2$ are the doses of chemicals 1 and 2 that in combination produce some specified effect (i. e. 50% inhibition of luminescence) and $(D_m)_1$ and $(D_m)_2$ are the doses of the chemicals that when applied singly also have the same effect (50% inhibition of luminescence)

An isobologram can graphically display chemical interactions, the *x* and *y* axes representing the doses of drugs 1 and 2. The lines of the isobologram show dose combinations of the two chemicals, 1 and 2 that yield the same effect:




1.
    1. A straight line connecting dose of chemical 1 (D1) and dose of chemical 2 (D2) on the respective *x* and *y* axes that yield 50% effect ($D_m$, $EC_{50}$, $ED_{50}$) - ZERO INTERACTION OR ADDITIVE EFFECT (Isobologram equation = 1)
    2. When the line connecting both doses lie below and to the left of the line of additiviy (concave-up line)- SYNERGISM (Isobologram equation < 1)
    3. When the line connecting both doses lie above and to the right of the line of additivity (concave-down)– ANTAGONISM (Isobologram equation > 1)

An isobologram is then a dose-oriented graphic that can give information on the interaction of two drugs at any effect level ($EC_{10}$, $EC_{20}$, $EC_{50}$….) but has some practical limitations as it is designed for two-three drugs.

The isobologram is independent of the nature of the effects (toxicity endpoints) under consideration; it does not depend on the mechanism of action or the shapes of the dose response-curves.

THE COMBINATION INDEX-ISOBOLOGRAM EQUATION by Chou (Pharmacol. Rev. 58: 621; 2006) generalizes the isobologram equation for *n* drugs, is independent of the mechanism of action but takes into account both the potency of each drug and combinations of these drugs and the shapes of their dose-effect curves.

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# Doctoral Thesis

UNIVERSIDAD AUTONOMA DE MADRID

## Application of a novel *lux*-based cyanobacterial bioreporter in environmental toxicity: Assessment of individual and combined toxicity of priority and emerging pollutants

The aim of the present study was to evaluate the applicability of a new general ecotoxicity test to determine the individual and combined toxicity of different classes of environmental pollutants. This new ecotoxicity test is based on a freshwater filamentous cyanobacterium of the Order Nostocales: an *Anabaena* PCC 7120 derivative strain named *Anabaena* CPB4337 which carried the whole operon *luxCDABE* of the terrestrial luminescent bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*) which are expressed constitutively, being self-luminescent. Through the thesis, *Anabaena* CPB4337 was applied to study the individual and combined toxicity of priority pollutants such as heavy metals, and emerging pollutants such as pharmaceutical products (fibrates), fluorinated surfactants and metal oxide nanoparticles.

UNIVERSIDAD AUTÓNOMA DE MADRID FACULTAD DE CIENCIAS DEPARTAMENTO DE BIOLOGÍA