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TESIS DOCTORAL

Incorporación de nuevos parámetros subletales para la evaluación de las consecuencias ambientales de contaminantes orgánicos persistentes y emergentes en las emisiones ligadas a la gestión de residuos

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INCORPORACIÓN DE NUEVOS PARÁMETROS SUBLETALES PARA LA EVALUACIÓN DE LAS CONSECUENCIAS AMBIENTALES DE CONTAMINANTES ORGÁNICOS PERSISTENTES Y EMERGENTES EN LAS EMISIONES LIGADAS A LA GESTIÓN DE RESIDUOS

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Madrid, 2014

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CERTIFICAN

Que la Tesis Doctoral titulada "Incorporación de nuevos parámetros subletales para la evaluación de las consecuencias ambientales de contaminantes orgánicos persistentes y emergentes en las emisiones ligadas a la gestión de residuos" presentada por la Licenciada en Veterinaria Dña Federica Martini para optar al título de Doctor, ha sido realizada en el Laboratorio de Ecotoxicología del INIA (Madrid, España) bajo nuestra dirección. Considerando que la misma se encuentra concluida, autorizamos su presentación.

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ABEVIATURAS Y ACRÓNIMOS

ADN	Ácido desoxirribonucleico
AMA	Ensayo de Metamorfosis en Anfibios (por sus siglas en inglés, Amphibian Metamorfosis Assay)
AOP	Adverse Outcome Pathways (por su siglas en inglés)
APRIL:	Señal de inducción de proliferación (por sus siglas en inglés, A Proliferation-Inducing Signal)
AREAG	Grupo de Asesoramiento Ecotoxicológico en Anfibios y Reptiles (por sus siglas en ingles, Amphibian and Reptile Ecotoxicology Advisory Group
ARNm	Ácido ribonucleico mensajero
ASTM	American Society for Testing and Materials (por sus siglas en inglés)
ATP	Adenosín Trifosfato
BAFF	Factor de activación de las células B desde la familia TNF (por sus siglas en inglés, B-cell activation factor from the TNF family)
B[a]P	Benzo[a]pireno
CNTF:	Factor neurotrópico ciliar (por sus siglas en inglés, Ciliary Neurotrophic Factor)
CT-1:	Cardiotropina 1
$CuCl_2$	Cobre II cloruro o Dicloruro de cobre
DQO	Demanda Química de Óxigeno
DTA	Evaluación Directa de la Toxicidad (por su siglas en inglés, Direct Toxicity Assessment)
EC_{50}	Concentración Efecto en el 50% de los casos (por sus siglas en inglés, Effect Concentration)
ECHA	Agencia Europea de Sustancias y Mezclas Químicas (por su siglas en inglés, European Chemical Agency)
ERA	Evaluación de Riesgo Ambiental o Ecológico (por sus siglas en inglés, Environmental or Ecological Risk Assessment)
FETAX	Ensayo de Teratogénesis con Embriones de Rana- *Xenopus* (por sus siglas en inglés, Frog Embryo Teratogenesis Assay-*Xenopus*)
GM-CSF	Factor de estimulación de colonias de los granulocitos y macrófagos (por sus siglas en inglés, Granulocyte-Macrophage Colony-Stimulating Factor)
gp	Glicoproteína El tipo específico se define por el número que sigue la sigla: ej. 96
HSE	Elemento de Choque Térmico (por sus siglas en inglés, Heat Shock Element)
HSF	Factor de Choque Térmico (por sus siglas en inglés, Heat Shock Factor)
HSP	Proteínas de golpe de calor (por sus siglas en inglés, Heat

	Shock Protein). El tipo específico se define por el número que sigue la sigla: 60, 70, 90, etc. Por ejemplo en el caso de la HSP70, si está escrita en minúscula y en cursivo hace referencia a el gen (*hsp70*), mientras en mayúsculas a la proteína (ej: HSP70)
IPCS	Programa Internacional para la Seguridad de las Sustancias Químicas (por sus siglas en inglés, International Programme on Chemical Safety)
IL	Interleuquina. El tipo específico se define por el número que sigue la sigla: 2, 12, 1-β etc. Por ejemplo en el caso de la IL-1β, si está escrita en cursivo hace referencia a el gen (*IL-1β*), si no a la proteína (IL-1β)
ISO	Organización Internacional para la Estandardización (por sus siglas en inglés, International Organization for Standardization)
IT	Índice Teratogénico
JCR	Journal Citation Report (por su siglas en inglés)
LIF:	Factor inhibitorio de leucemia (por su siglas en inglés, Leukemia Inhibitory Factor)
LOEC	Concentración más baja en la que se observan los efectos (por sus siglas en inglés, Lowest-Observed-Effects Concentration)
LPS	Lipopolisaccarido bacteriano
LC_{50}	Concentración Letal en el 50% de los casos (por sus siglas en inglés, Letal Concentration)
MCIG	Concentración Minima que Inhibe el Crecimiento (por sus siglas en inglés, Minimum Concentration to Inhibit Growth)
$MnSO_4$	Manganeso II sulfato o Sulfato de manganeso
$NaSeO_3$	Sodio selenito o Selenito de sodio
NOEC	Concertación a la que no se observa ningún efecto (por sus siglas en inglés, No-Observed-Effects Concentration)
OECD	Organización para la Cooperación Económica y el Desarrollo (por sus siglas en inglés, Organization for Economic Cooperation and Development.
PAHs	Hidrocarburos Policíclicos Aromáticos (por sus siglas en inglés, Polycyclic Aromatic Hydrocarbons)
PCBs	Bifenilos Policlorados (por su nombre en inglés, polychlorinated biphenyls)
REACH	Registro, Evaluación, Autorización y restricción de sustancias Químicas (por sus siglas en inglés, Registration, Evaluation, Authorization and restriction of Chemical substances)
RSU	Residuos Sólidos Urbanos
SETAC	Sociedad de Química y Toxicología Ambiental (por sus siglas en inglés, Society of Environmental Toxicology and Chemistry)
SVL	Longitud hocico-base de la cola (por sus siglas en inglés, Snout to Vent Length)

TNF-α	Factor de Necrosis Tumoral α (por sus siglas en inglés, Tumor Necrosis Factor α). Escrita en cursivo hace referencia a el gen (*TNF-α*), si no a la proteína (TNF-α)
TSLP	Linfopoietina timica stromale (por sus siglas en inglés, Thymic Stromal Lymphopoietin)
TU	Unidades Tóxicas (por sus siglas en inglés, Toxic Units)
U.S. EPA	Agencia de Protección del medio Ambiente de los Estados Unidos (por sus siglas en inglés, Environmental Protection Agency of the United States)
UTR	Región 5'No Traducida de un gen (por sus siglas en inglés, Untraslated Region)
$ZnSO_4$	Zinc sulfato o Sulfato de zinc

RESUMEN

Introducción

El marco normativo ambiental vigente establece, para la evaluación ecotoxicológica de las sustancias químicas en los sistemas acuáticos de agua dulce, una serie de ensayos estandarizados que se desarrollan fundamentalmente sobre tres grupos taxonómicos básicos: los productores primarios (algas), los consumidores primarios (invertebrados acuáticos), y los consumidores secundarios (peces), dejando al margen un grupo de especial interés como son los anfibios. Hasta el momento, para los anfibios solo existen dos ensayos estandarizados: el ensayo de teratogénesis con embriones de *Xenopus* (ensayo FETAX), que estudia la teratogenicidad en embriones de *Xenopus laevis* y el ensayo de metamorfosis en anfibios (ensayo AMA), cuyo objetivo es el estudio de los efectos de perturbación de los xenobióticos sobre el eje hipotálamo-hipófisis-tiroides de los vertebrados.

Para poder identificar la mejor estrategia en la elección de los ensayos predictivos en ecotoxicología, recientemente se ha propuesto el uso de las que se definen como A*dverse Outcome Pathways* (AOP). Estas rutas suponen un nuevo concepto basado en identificar el vínculo entre un suceso que empieza a nivel molecular y el efecto adverso que se desencadena a un nivel superior de organización biológica, lo que permite establecer la relevancia del suceso inicial y considerarlo en la evaluación de riesgo ecotoxicológico.

Los efectos subletales tienen una importancia vital en ecotoxicología ya que, aunque no causan mortalidad directamente, afectan las posibilidades de supervivencia del individuo en el medio natural. El desarrollo de diferentes tipos de pruebas que utilizan concentraciones relevantes desde el punto de vista ambiental y seleccionan, mediante estudios más complejos, respuestas subletales más sensibles que la mortalidad, es esencial para determinar el impacto realmente producido por los contaminantes. En los últimos años ha aumentado la preocupación

acerca de los efectos que los xenobióticos pueden provocar sobre el sistema inmune, considerándose cada vez más relevante como sistema diana dentro de la evaluación de riesgo ambiental de las sustancias químicas. Tanto las citoquinas como las proteínas de choque térmico (HSPs) han demostrado ser buenos parámetros para predecir el potencial inmunotóxico de los contaminantes ambientales y pueden considerarse unos excelentes marcadores para el seguimiento de la exposición a contaminantes que afecten al sistema inmune.

El presente trabajo hace hincapié en las ventajas que aportaría la incorporación de los parámetros agudos y subletales en los anfibios en las directrices reguladoras para la protección de los organismos acuáticos y de la calidad de las agua; por lo que se recomienda su integración en los programas de evaluaciones de riesgo.

Objetivos

Objetivo general: Estudiar el potencial de integración de los ensayos con anfibios, tanto agudos como subletales, incluyendo efectos immunotoxicos, en los protocolos de Evaluación de Riesgo Ambiental (ERA) de sustancias químicas puras y de mezclas complejas.

Objetivos específicos:

1. Desarrollar un nuevo ensayo de toxicidad aguda con las larvas del anuro *X. laevis* para evaluar los efectos tóxicos de sustancias puras y mezclas complejas.
2. Comparar la sensibilidad del ensayo agudo sobre *X. laevis* con otros ensayos ya estandarizados o utilizados asiduamente en ecotoxicología como los ensayos agudos con peces, los ensayos *in vitro* con líneas celulares de peces (línea RTG-2 de trucha arcoíris) y el ensayo FETAX con embriones de *X. laevis*.

3. Evaluar la respuesta del nuevo ensayo dentro de una batería de ensayos de evaluación de riesgos ambientales de mezclas complejas (residuos tóxicos y peligrosos y lixiviados de vertederos).
4. Desarrollar un ensayo de toxicidad subletal sobre el anuro *Xenopus laevis* para evaluar los efectos inmunotóxicos potenciales a corto y medio plazo de los contaminantes mediante la expresión del acido ribonucleico mensajero (ARNm) para la proteína de choque térmico 70 (HSP70), la interleuquina 1 β (IL-1β) y el factor de necrosis tumoral α (TNF-α).
5. Evaluar la relación dosis-respuesta de dicho ensayo de inmunotoxicidad.

Resultados

Los valores de concentración letal en el 50% de los casos (LC_{50}) encontrados en la literatura para el ensayo FETAX fueron iguales o más bajos respecto a los obtenidos en el estudio con larvas de *X. laevis* expuestas a selenito de sodio (sodio selenito 5-hidrato: $NaSeO_3 * 5\ H_2O$), dicloruro de cobre (cobre II cloruro 2-hidrato: $CuCl_2 * 2\ H_2O$), sulfato de zinc (zinc sulfato 7-hidrato: $ZnSO_4*7\ H_2O$) e ivermectina. Por otro lado, los valores descritos en peces no siempre protegieron suficientemente a los anfibios. Por ejemplo, comparando la sensibilidad de las larvas de *X. laevis* expuestas en el presente estudio a $NaSeO_3$ (LC_{50} a 96 horas= 6,2 mg/L) respecto a los resultados del estudio de Buhl y Hamilton (1991) en trucha arcoíris, donde la LC_{50} a 96 horas (96h-LC_{50}) fue de 118 mg/L, se demostró una sensibilidad mayor para *X. laevis*.

Si bien para los antibióticos (oxitetraciclina, tetraciclina y sulfamida asociada a trimetoprim) y el sulfato de manganeso (manganeso II sulfato monohidrato- $MnSO_4 * H_2O$) no se mostraron efectos letales o subletales en larvas de *X. laevis*, la ivermectina, el cobre y el zinc produjeron unos efectos subletales que pueden considerarse como indicadores de alteraciones para distintos mecanismos fisiológicos. Las larvas expuestas al $CuCl_2$ eran

inmaduras y depigmentadas, las expuestas a la ivermectina presentaban alteraciones de locomoción y orientación (hiperactividad), y finalmente el $ZnSO_4$ provocó en los renacuajos edema cráneo-facial y abdominal.

El ensayo agudo con *X. laevis*, que fue incorporado a las baterías de ensayos que combinan pruebas *in vivo* e *in vitro* sobre diferentes grupos taxonómicos considerando diversos parámetros ecotoxicológicos, clasificó como toxicas el 60% de las muestras de residuos peligrosos ensayadas. Además, en comparación con las otras pruebas (ensayo de toxicidad en *Daphnia magna*, ensayo DR-Calux® y los ensayos con la línea celular RTG-2 de trucha arcoíris) el ensayo con *Xenopus* detectó el porcentaje más elevado (46,67%) de muestras clasificables como altamente toxicas. Los resultados obtenidos con las muestras de residuos sólidos urbanos (RSU) con el ensayo de toxicidad aguda en larvas de *Xenopus laevis* mostraron correlaciones muy altas entre propiedades fisicoquímicas y toxicidad (r=0,86), además de una caracterización biológica con un 0% de falsos positivos. Tanto con la muestras de residuos peligrosos y mezclas complejas potencialmente peligrosas como con los RSU se demostró y confirmó que dicho ensayo podía ser una prueba eficaz a la hora de valorar efectos tóxicos de mezclas complejas y que podría ser incluida dentro de cualquier batería de ensayos de monitorización ecotoxicológica.

Las respuestas inmunes de las larvas expuestas a tiabendazol, fluoxetina, cipermetrina y benzo[a]pireno (B[a]P) mostraron cómo la expresión del ARNm para la proteína HSP70 y la interleuquina IL-1β producidas por contaminantes a unos niveles ambientalmente relevantes podia ser empleada como un posible indicador temprano de los efectos tóxicos producidos por los contaminantes sobre el sistema inmune de *X. laevis*. Considerando todos los compuestos inmunotóxicos usados a lo largo de la presente tesis, el aumento de la expresión génica de ambas proteínas fue muy marcado en las exposiciones al tiabendazol y menos claro en el caso de la cipermetrina. Además, la inducción de ARNm para la HSP70 y la IL-1β se relacionó de una forma directa con la concentración del B[a]P, confirmando la posibilidad del uso de estos parámetros como biomarcadores

en la detección de compuestos inmunotóxicos. Por otro lado, la exposición a las diferentes concentraciones subletales de B[a]P no se asoció a una variación significativa en la expresión de ARNm para el TNF-α, con algunas excepciones que no se consideraron estadísticamente relevantes.

Conclusiones

1. Al comparar los efectos producidos por los contaminantes en dos ensayos con diferentes fases de desarrollo de *Xenopus laevis*, la larvaria y la embrionaria, se evidenció que frente a la mayoría de los compuestos estudiados la sensibilidad con el ensayo FETAX fue igual o superior que la detectada por las larvas. Sin embargo, estas últimas mostraron efectos subletales que en muchos casos podrían llegar a ser incompatibles con la vida. Por lo tanto, el ensayo agudo con larvas de *X. laevis* desarrollado en este trabajo no solo fue capaz de detectar mortalidad, sino también alteraciones subletales que derivan de las anormalidades asociadas a las exposiciones a corto plazo.
2. Al comparar los resultados de los ensayos agudos con larvas del género *Xenopus* con otros ensayos agudos con fases larvarias en peces, se comprobó que en algunos casos los valores obtenidos en peces pueden no proteger suficientemente a los anfibios, que representan una clase especialmente sensible al estrés químico. Por tanto, se destaca la utilidad de la inclusión del ensayo agudo con larvas de *X. laevis* en las baterías clásicas de ensayos de seguimiento ecotoxicológico, siguiendo además las directrices de los nuevos desarrollos de modelos ERA que requieren la consideración de diferentes organismos relevantes incluso cuando éstos no sean los más sensibles.
3. Los resultados de la caracterización biológica de los residuos tóxicos y peligrosos y los residuos sólidos urbanos corroboraron que el ensayo agudo con larvas del anfibio *X. laevis* es una prueba eficaz

a la hora de valorar los efectos tóxicos de mezclas complejas. Por tanto, podría ser incluido dentro de cualquier batería de ensayos de seguimiento ecotoxicológico para estos tipos de muestras. Además, este ensayo permitió detectar toxicidad de manera selectiva y favoreció la clasificación de la toxicidad de las muestras complejas en función de sus propiedades fisicoquímicas con el menor número de falsos positivos y negativos.

4. En los estudios de efectos subletales inmunotóxicos en los que se asoció la variación de expresión génica con la toxicidad de los contaminantes emergentes y persistentes, los resultados obtenidos con tiabendazol, fluoxetina, cipermetrina y B[a]P sugieren que la expresión de ARNm para la HSP70 y la IL-1β no depende del mecanismo de acción de los compuestos, lo que las hace más fiables en su uso como biomarcadores de inmunotoxicidad. Debido al hecho de que las proteínas TNF-α e IL-1β son mediadores de la respuesta inmune innata, en la exposición con B[a]P cabía esperar un mismo comportamiento para ambas proteínas. Sin embargo, la proteína TNF-α no se expresó de la misma manera que la IL-1β, lo que lleva a suponer que la TNF-α necesita un tipo de estimulo diferente, por ejemplo por naturaleza o intensidad, respecto a aquél que se logró con el B[a]P.
5. Demostrada la relación concentración-respuesta entre la exposición a concentraciones subletales de B[a]P y la expresión génica de *hsp70* e *IL-1β,* estos parámetros se ofrecen como indicadores precoces de riesgo que podrían ser incluidos en las baterías inmunotoxicológicas para el medio acuático y en futuros estudios de las rutas *Adverse Outcome Pathways* (AOP).
6. La conservación de la proteína HSP70 y la asociación de la misma y la IL-1β a la respuesta inmune no específica indican que HSP70 y IL-1β podrían ser útiles como indicadores de peligro subletales incluso para la salud humana.

ABSTRACT

Introduction

For ecotoxicological assessment of chemical substances in freshwater systems the current regulatory framework establishes standardized tests based upon three basic taxonomic groups: primary producers (algae), primary consumers (aquatic invertebrates) and secondary consumers (fish), leaving aside a group of interest represented by amphibians. At the moment, there are only two standardized tests in amphibians: the Frog Embryo Teratogenesis Assay Xenopus (FETAX) and the Amphibian Metamorphosis Assay (AMA).

Recently, the use of Adverse Outcome Pathways (AOP) has been proposed as the best tool for identifying useful predictive assays and testing strategies for regulatory endotoxicology. These pathways are conceptual construct that portray existing knowledge event and the adverse outcome at a biological level of organization relevant to ecological risk assessment.

Biological effects at a sublethal level are extremely important in ecotoxicology because, although they do not directly cause mortality, in the wildlife they may chance the individual survival rate. The development of multiples assays, with relevant environmental concentrations of contaminants that can detect sublethal responses more sensitive than mortality, is basic for the determination of the impact actually produced by pollutants. The consequences of exposure to xenobiotics should be considered as a priority for the proper interpretation of sublethal effects in the immune system. Thus, cytokines and heat shock proteins (HSPs) have proven to be good parameters for predicting immunotoxic potential environmental pollutants and they can be excellent biomarkers for monitoring exposure to contaminants which affect the immune system.

The current study emphasizes the benefits that would bring incorporating acute and sublethal parameters from amphibians to regulatory guidelines for the protection of aquatic organisms and water quality, and it

recommends the integration of amphibians into the risk assessment programs.

Objective

General objective: To explore the potential integration of acute and subacute assays, including immunotoxic effects, with amphibians to procedures of Environmental Risk Assessment (ERA) for pure toxic substances and complex mixtures.

Specific objectives:

1. To develop an acute toxicity test with larvae of anuran *X. laevis* for studying of pure toxic substances and complex mixtures.
2. To compare sensitivity of acute toxicity test with *X. laevis* larvae with other standardized tests, or well-known ecotoxicological assays such as acute toxicity fish assays, *in- vitro* test with the fish cell-line RTG-2 and FETAX assay with *X. laevis* embryos.
3. To evaluate its inclusion within a common battery of environmental risk assessment for complex mixtures (hazardous and toxic wastes and landfill leachates).
4. To develop a sublethal toxicity assay with *Xenopus laevis* for the assessment of the potential short- term or long-term immunotoxicity of pollutants. The immunotoxicity assay is based on the study of changing expression of ribonucleic acid messenger (mRNA) for heat shock protein 70 (HSP70), interleukin 1-β (IL-1β) and tumor necrosis factor α (TNF-α).
5. To evaluate the dose-response relationship of the immunotoxicity test.

Results

The lethal concentration into 50% of cases (LC_{50}) values available for FETAX assay were equal or lower than those calculated in the present study for later larvae of *X. laevis* when they were exposed to sodium selenite (sodium selenite 5-hydrate: $NaSeO_3$* 5 H_2O) copper dichloride copper II chloride 2-hydrate: $CuCl_2$* 2 H_2O), zinc sulfate (zinc sulfate 7-hydrate: $ZnSO_4$ *7 H_2O) and ivermectin. On the other hand, data from fish assays were not always protective enough for amphibians. For example, the current study showed a higher sensitivity for *X. laevis* when the results from larvae of *X. laevis* exposed to $NaSeO_3$ were compared to Buhl and Hamilton's results (1991) in rainbow trout; LC_{50} at 96-hours (96h-LC_{50}) were 6.2 mg/L and 118 mg/L, respectively.

In addition, although for antibiotics (oxitetracycline, tetracycline and sulfamethoxazole with trimethoprim) and manganese sulfate (manganese II sulfate monohydrate: $MnSO_4$ * H_2O) in larvae no letal or subletal effects were shown, ivermectin, copper and zinc showed sublethal effects that may cause same change in physiological mechanisms. Larvae exposed to $CuCl_2$ were underdeveloped and colourless, while larvae exposed to ivermectin were impaired locomotion and orientation (hyperactivity). Finally tadpole exposed to $ZnSO_4$ showed craniofacial and abdominal edema.

Through the incorporation of *X. laevis* assay to the battery of toxicity test combining *in vivo* and *in vitro* assays in different taxonomic groups and taking in consideration different ecotoxicological parameters, 60% of hazardous waste samples tested had been classified as toxic. Moreover, the *X. laevis* assay detected the highest percentage (46.67%) of samples classifiable as highly toxic in comparison with the other tests (toxicity test in *Daphnia magna*, DR-Calux® test and RTG-2 rainbow trout cell line tests). Results of *Xenopus laevis* larvae acute assay from samples of municipal solid waste (MSW) showed a very high correlation between physicochemical properties and toxicity (r=0.86), and a biological characterization with 0 % false positive. Both results, from hazardous waste

and MSW, proved and confirmed that the *X. laevis* larvae assay could be an effective test for assessing the toxic effects of complex mixtures. Moreover, it could be included within every battery of toxicity tests for the first screening of pollutants.

The immune responses of larvae exposed to thiabendazole fluoxetine, cypermethrin and benzo[a]pyrene (B[a]P) showed how mRNA expression of HSP70 and IL-1β produced by contaminants at low environmentally relevant concentrations, could be used as earlier indicator of possible risk for the immune system. Among all the immunotoxic substances used in the current thesis, the increasing gene expression for both proteins was remarkable for thiabendazole and less noticeably in the case of cypermethrin. Furthermore, evident concentration/response relationship between induction of mRNA expression for HSP70 and IL- 1β confirmed the possibility for using of these parameters as biomarkers for the detection of immunotoxic compounds. In addition, exposure to sublethal concentrations of B[a]P was not associated with a significant change in mRNA expression of tumor necrosis factor α (TNF-α), with certain exceptions that were not considered biologically relevant .

Conclusions

1. In the current study, with the most of the compounds used, when the effects of contaminants were compared between two assays using different stages of development of *Xenopus laevis*, embryo and larvae, it was demonstrated that sensitivity of FETAX assay was similar or higher than larvae sensitivity. However, larvae showed sublethal effects that could be incompatible with life. Therefore, the acute test with larvae of *X. laevis* was able to detect not only mortality but also sublethal abnormalities associated with short-term exposures.
2. When results of acute tests with larvae of the genus *Xenopus* were compared with acute tests with larvae of fish, it was found that in

some cases data obtained in fish could not be always enough protective for amphibians. In fact, amphibians are particularly sensitive to chemical stress. Therefore, the useful of including the acute assay with larvae of *X. laevis* into classical ecotoxicological test battery were highlighted. On the same way, it agreed with new models of guidelines of ERA that require taking into account different relevant organisms, even when these are not the most sensitive.

3. Results of biological characterization of toxic and hazardous waste and municipal solid waste confirmed that the acute test with larvae of the amphibian *X. laevis* is an effective test for the assessment of toxic effects of mixtures complex. Therefore, it could be included within any screening ecotoxicological test battery for these types of samples. The acute *X. laevis* larvae assay allowed a selectively classification of toxicity of complex samples with fewer false positives and negatives, based on their physicochemical properties.
4. In the immunotoxic studies, where the relationship between gene expression and toxicity of emerging and persistent pollutant were tested, the results obtained with thiabendazole, fluoxetine, cypermethrin and B [a] P suggest that expression of mRNA for HSP70 and IL-1β does not rely on the mechanism of action of the compounds. In this way, HSP70 and IL-1β are reliable for using as biomarkers of immunotoxicity. Hence, TNF-α and IL-1β are mediators of the innate immune response, in the exposure to B [a] P the same behavior for both proteins was expected. However, TNF-α protein was not expressed in the same way that the IL-1β, which leads to the assumption that the TNF-α requires a different type of stimulus, for example by nature or strength, compared to that which is achieved with B[a]P.
5. Once the concentration-response relationship between exposure to sublethal concentrations of B[a]P and *hsp70* and *IL-1β* gene expression has been shown, these parameters are given as early risk

indicators that could be included in inmunotoxicological battery tests for aquatic environmental and future studies of Adverse Outcome Pathways (AOP).

6. The conservation of HSP70 and the association of the same and IL-1β with non-specific immune response suggest that HSP70 and IL-1β could be used as sublethal risk indicators even to human health.

GUÍA PARA EL LECTOR

A lo largo de la presente tesis, desarrollada en 7 capítulos, se discute la posibilidad de incorporar los anfibios en los protocolos de evaluación de riesgo ambiental de las sustancias químicas para el compartimento acuático, además de estudiar la posible incorporación de nuevos parámetros subletales en los estudios de toxicidad de los contaminantes orgánicos persistentes y emergentes, tan ligados a residuos tóxicos y peligrosos. Este trabajo, permite ampliar la información ecotoxicológica de este tipo de compuestos con la generación de datos sobre otras especies diferentes a las habitualmente utilizadas, favoreciendo así la posterior gestión de dichos residuos.

El **capítulo 1** es una introducción general de los trabajos presentados en la presente tesis. Los capítulos que van a continuación son artículos publicados en revistas incluidas en el *Journal Citation report* (JCR). Los capítulos 2, 3 y 4 describen el desarrollo del ensayo de toxicidad aguda en el anfibio *X. laevis*, y la comparación de la sensibilidad de dicho ensayo con otras especies de referencia para el medio acuático y con los resultados obtenidos con metodologías *in vitro* (líneas celulares), con el objetivo de comprobar la posible integración de dicho ensayo en los protocolos de evaluación de riesgo ambiental de contaminantes puros y mezclas complejas.

El **capítulo 2** describe el desarrollo del ensayo de toxicidad aguda en larvas de *X. laevis*, no solo para comparar la sensibilidad de dicho ensayo con otras especies de referencia para el compartimiento acuático, como *D. magna* y peces, sino también con un ensayo ya estandarizado con *Xenopus laevis* (ensayo FETAX) pero desarrollado con otra fase de desarrollo, la fase embrionaria. Los compuestos empleados en el ensayo fueron sustancias biológicamente activas, como los medicamentos veterinarios y los elementos esenciales, que en pequeñas concentraciones pueden tener efectos potencialmente tóxicos en los organismos acuáticos. El capítulo discute los

resultados obtenidos con dicho ensayo frente a los datos disponibles en la literatura para *Daphnia*, peces y el ensayo FETAX, encontrando que, en general, suelen ser más protectores que los encontrados en este estudio, aunque existen algunas excepciones, como en el caso de la exposición al selenio, al cobre y al zinc.

En el **capítulo 3** se describe la incorporación de este nuevo ensayo en una batería de ensayos convencionales, que combinan pruebas *in vivo* e *in vitro*, sobre diferentes grupos taxonómicos y estudiando diversos parámetros ecotoxicológicos; esta batería se ha utilizado para la caracterización biológica de nueve residuos peligrosos, suministrados por empresas gestoras de residuos. Los resultados muestran que el ensayo agudo con larvas de *X. laevis* es una prueba eficaz a la hora de valorar efectos tóxicos de mezclas complejas que podría ser incluida dentro de cualquier batería de ensayos de seguimiento ecotoxicológico.

En el **capítulo 4** se presenta un estudio con muestras complejas potencialmente peligrosas procedentes de residuos sólidos de vertederos urbanos. Los resultados ratificaron la eficacia de la batería de ensayos propuesta anteriormente para la caracterización ecotoxicológica de dichos contaminantes. Al igual que en la prueba anterior, la sensibilidad de *X. laevis* fue de la misma magnitud, o incluso mayor para algunos compuestos que *Daphnia magna*. Los resultados de estos capítulos ponen en evidencia la relevancia del anfibio *X. laevis* en los ensayos ecotoxicológicos y la utilidad de su incorporación en las baterías de ensayos agudos de monitorización para el compartimiento acuático. Asimismo, en muchos casos se demostró que el ensayo de toxicidad en *X. laevis* es capaz de demostrar efectos subletales, pudiendo por tanto detectar efectos más específicos que en algunos casos se podrían relacionar con diferentes mecanismos fisiológicos.

Los capítulos 5 y 6 son los que describen los estudios de inmunotoxicidad desarrollados. Son ensayos subcrónicos de nueve días de duración, a lo largo de los cuales las larvas de *X. laevis* se exponen a diferentes contaminantes, y los efectos potencialmente inmunotóxicos de

dichas sustancias se miden a través de la expresión génica de citoquinas y proteínas específicas. En el ensayo descrito en el **capítulo 5**, frente a una exposición con cipermetrina, fluoxetina y tiabendazol, la proteína HSP70 y la interleuquina IL-1β se identificaron como parámetros capaces de detectar efectos inmunotóxicos potenciales. En el **capítulo 6**, los resultados del ensayo anterior fueron corroborados y ampliados mediante el desarrollo de un estudio de concentración-respuesta con benzo[a]pireno. Los efectos sobre la respuesta inmune de tipo innata producidos por el B[a]P en *X. laevis* fueron evidenciados a través del estudio de la expresión de ARNm para los genes *hsp70, IL-1β y TNF-α,* estableciéndose una clara concentración respuesta para dos de los tres parámetros estudiados. En el **capítulo 7** los resultados globales de la presente investigación han sido resumidos y discutidos coherentemente. Finalmente, se presentan las conclusiones generales de esta tesis.

CAPITULO 1

Introducción General

Objetivos

INTRODUCCIÓN GENERAL

La palabra ecotoxicología fue acuñada por Truhaut en 1975 para definir:

"*la rama de la toxicología que estudia los efectos tóxicos causados por los contaminantes de origen natural o antropogénico sobre los organismos vivos, ya sean animales o vegetales, terrestres o acuáticos, y las interacciones de estas sustancias con el medio ambiente*".

El objetivo primordial de la ecotoxicología es la identificación de los efectos que los contaminantes, de forma independiente o combinados con otros agentes de estrés, ejercen en el medio ambiente (Schirmer y cols., 2010), con el fin de predecir, y así disminuir o prevenir, los efectos prejudiciales sobre el mismo (Forbes y Forbes, 1994). La evaluación de los efectos tóxicos de los contaminantes se realiza fundamentalmente a través de los ensayos ecotoxicológicos; dichos ensayos pueden implicar diversos niveles de organización biológica, desde las alteraciones moleculares a nivel celular hasta la valoración de efectos sobre los ecosistemas (Calow, 1993). A medida que se desciende a lo largo de esta escala, los tiempos del ensayo se reducen y las condiciones del ensayo se vuelven más fácilmente controlables, por lo que las características que deben cumplir los ensayos (reproducibilidad, robustez, fiabilidad y repetibilidad) van aumentando. Por otro lado, si se aumenta la complejidad biológica de los ensayos empleando estudios de poblaciones o comunidades, es posible reproducir con mayor fidelidad la complejidad de la realidad ecológica, ganando así en relevancia, pero perdiendo en fiabilidad, particularmente en el sentido de la capacidad para interpretar los efectos observados y su relación con el agente estresante, debido a la complejidad del sistema (**figura 1**). Por lo tanto, es necesario llegar a un compromiso para la elección del ensayo más apropiado, y en general, combinar estudios realizados con diferentes niveles

de organización biológica. Debido a que, en un principio, todas las interacciones de las sustancias químicas tienen lugar a nivel molecular, en la actualidad se considera fundamental entender la ecotoxicología desde un punto de vista mecanicista, lo que permitirá obtener la información necesaria para predecir los efectos y por tanto salvaguardar el medio ambiente (Eggen y cols., 2004).

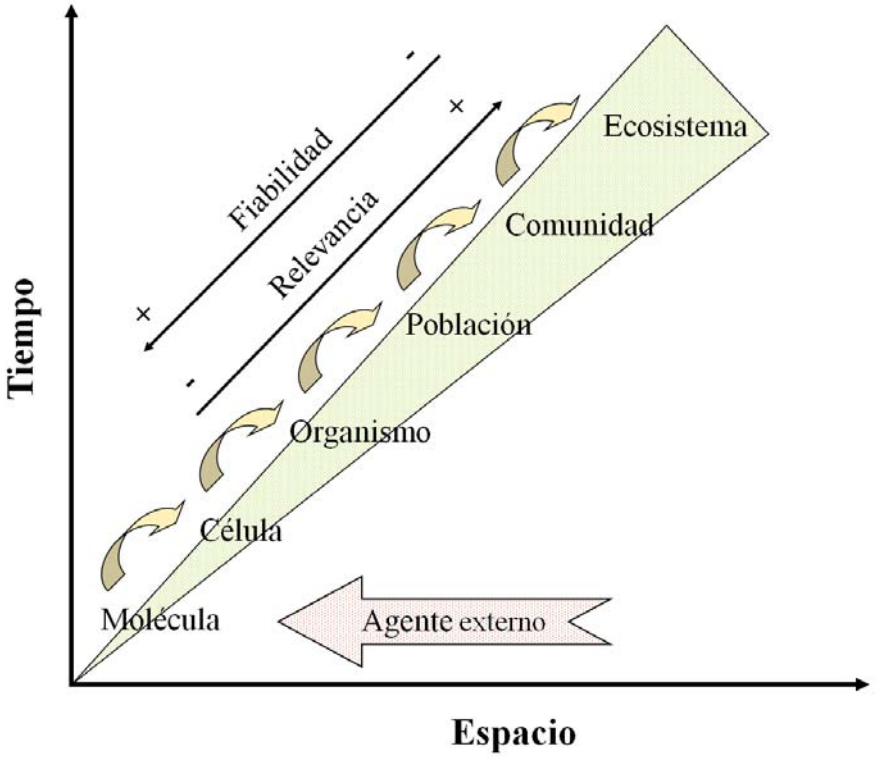

Figura 1. La ecotoxicología se basa en la integración de los conocimientos procedentes de los diferentes niveles de organización biológica. Cualquier respuesta al impacto de una sustancia química en un organismo se inicia a nivel celular, donde esa sustancia provoca una respuesta molecular a pequeña escala. A menos que los mecanismos celulares protectores no sean compensados, dichas respuestas celulares se trasladan a los niveles más altos de la escala espacial proporcionalmente al tiempo. Empleando las poblaciones o las comunidades, es posible reproducir con mayor fidelidad la realidad, ganando así en relevancia, pero perdiendo en fiabilidad, debido a la complejidad del sistema (adaptada de: Schirmer y cols., 2010).

Para poder identificar la mejor estrategia en la elección de los ensayos predictivos que sean más afines con las actuales necesidades del marco normativo en ecotoxicología, Ankley y colaboradores (2010) han propuesto recientemente el uso de las que definen como *Adverse Outcome Pathways* (AOP). Estas rutas suponen identificar el vínculo entre un suceso que empieza a nivel molecular y el efecto adverso que desencadena a un nivel superior de organización biológica, lo que permite establecer la relevancia del suceso inicial y considerarlo en la evaluación de riesgo ecotoxicológico.

Marco normativo

El marco normativo ambiental vigente establece, para la evaluación toxicológica de las sustancias químicas puras, de los efluentes, y de los residuos tóxicos y peligrosos en los sistemas acuáticos continentales, una serie de ensayos estandarizados desarrollados por diferentes organismos tanto internacionales como nacionales, tales como la Organización Internacional para la Estandarización (ISO), la Organización para la Cooperación Económica y el Desarrollo (OECD), la Agencia de Protección del medio Ambiente de los Estados Unidos (U.S. EPA), o la *American Society for Testing and Materials* (ASTM). Los ensayos establecidos están incluidos en marcos normativos tales como el Reglamento (CE) No 440/2008 de la Comisión Europea de 30 de mayo de 2008 por el que se establecen métodos de ensayo de acuerdo con el Reglamento (CE) No 1907/2006 del Parlamento Europeo y del Consejo relativo al registro, la evaluación, la autorización y la restricción de las sustancias y preparados químicos (REACH). Dichos ensayos se desarrollan sobre tres grupos taxonómicos básicos, que son comúnmente utilizados como referencia para la monitorización del medio acuático en general (Calow, 1993): los productores primarios (algas), los consumidores primarios (invertebrados acuáticos, como por ejemplo *Daphnia magna*), y los consumidores

secundarios (peces, como por ejemplo la trucha arcoíris, *Oncorhynchus mykiss*).

Las metodologías estandarizadas para los ensayos de ecotoxicidad en algas se basan en la determinación del efecto de la sustancia tóxica mediante un ensayo de inhibición del crecimiento de las poblaciones de microalgas en un medio enriquecido en nutrientes y en condiciones que permitan el crecimiento exponencial de la biomasa de la población control en un factor de al menos 16 veces durante un periodo de 3 días; un ejemplo de este ensayo es el ensayo estandarizado de la OECD 201 (2011). Por lo que respecta a los invertebrados, el microcrustaceo *Daphnia magna* se considera un taxón clave en las baterías de ensayos utilizadas en las evaluaciones del riesgo ambiental de primer nivel, por lo que existen ensayos estandarizados tanto para toxicidad aguda como para crónica. El ensayo de inmovilización aguda de *Daphnia magna* de la OECD 202 (2004), representa un ejemplo de ensayo de evaluación de la toxicidad aguda, mientras que para la evaluación de los efectos crónicos existen dos métodos generalmente aceptados por las agencias reguladoras: el ensayo a 7 días de supervivencia y reproducción en *Ceriodaphia* de la ASTM (ASTM E1295, 1989) y el ensayo de reproducción a 21 días en *Daphnia magna* de la OECD 211 recientemente revisado (2012).

Sin embargo, es dentro de la categoría de los consumidores secundarios donde se ha desarrollado el mayor número de ensayos ecotoxicológicos para la caracterización biológica de los contaminantes ambientales; los peces han sido tradicionalmente empleados como organismos "centinela" para comprobar la salud del compartimento acuático. Por lo tanto, los protocolos existentes cubren un amplio rango de métodos y de especies, incluyendo exposiciones agudas o crónicas (OECD 203, 1992; OECD 215, 2000); de tipo estático o en flujo continuo (OECD 203, 1992; OECD 210, 2013); con adultos o fases embrionarias (OECD 229, 2012; OECD 210, 2013; OECD 236, 2013) los que identifican alteraciones de crecimiento o de reproducción (OECD 215, 2000; OECD 229, 2012; OECD 230, 2009); y finalmente, los desarrollados para estudiar

efectos específicos de disrupción endocrina (OECD 234, 2011). La OCDE ha publicado recientemente un documento marco sobre los ensayos en peces (ENV/JM/MONO(2012)16 OECD, 2012) donde se analizan el conjunto de ensayos disponibles y se establecen las recomendaciones para su revisión. Entre todas las líneas directrices para los ensayos con organismos vivos de la OECD, destaca la ausencia de referencias a los anfibios; siendo la directriz OECD 231 (2009) la única actualmente existente. Esta guía describe el ensayo de metamorfosis en anfibios que permite seleccionar aquellas sustancias que pueden interferir con el funcionamiento normal del eje hipotálamo-hipófisis-tiroides. El ensayo se validó con la especie *Xenopus laevis*, que es la recomendada para su uso en esta guía.

Integración de los anfibios en las estrategias de evaluación de riesgo ambiental

La Evaluación de Riesgo Ambiental (ERA) se define como:

"*el procedimiento por el cual los efectos adversos, probables o reales de los contaminantes y otras actividades humanas, se estiman sobre los ecosistemas y sus componentes con un grado de certeza conocido mediante el uso de metodologías científic*as" (Depledge and Fossi, 1994).

Desde finales del siglo XX, los protocolos ERA constituyen la mejor herramienta disponible para dar soporte, con base científica, a la toma de decisiones por parte de los responsables de la gestión del medio ambiente (van der Oost y cols., 2002); si bien, el avance científico ha supuesto el desarrollo de nuevas necesidades y retos científicos y normativos (página web 1; Tarazona, 2013). Los programas de implementación normativa de procedimientos de ERA para los organismos acuáticos han ido emergiendo lentamente a lo largo de los últimos 20 ó 30 años, pero siempre considerando la evaluación de riesgo llevada a cabo en peces como

suficientemente protectora para cubrir a otros vertebrados acuáticos, incluidos los anfibios. La omisión por parte de la comunidad reguladora de los anfibios en las estrategias ERA fue probablemente el resultado de una menor familiaridad o interés en este clase menos estudiada, pero ecológicamente relevante entre los vertebrados.

La escasez de estudios ecotoxicológicos sobre anfibios y reptiles se demuestra en la **figura 2,** donde se puede ver la distribución de las referencias científicas entre las diferentes clases de vertebrados, para los años comprendidos entre el 2000 y el 2013. Basándonos en una búsqueda bibliográfica realizada en la base de datos del ScienceDirect, obtenida introduciendo como criterios de búsqueda las palabras "*pollution*" o "*contaminants*", junto con las diferentes categorías de vertebrados, se puede comprobar que la producción científica en los últimos 12 años ha originado un total de 50.961 citas, de las cuales el 53,7% (27.384) se refieren a peces, el 20,4% (10.409) a aves, el 19,1% (9.746) a mamíferos, y solamente el 4% (2.063) y el 2,7% (1.359) a anfibios y reptiles, respectivamente. Probablemente la diferencia en el volumen de las investigaciones ecotoxicológicas entre los anfibios (y reptiles) y el resto de los vertebrados se puede atribuir a diferentes factores; a pesar de que algunos anfibios tienen un valor económico (por ejemplo, la producción de ancas de rana), dicho valor es incomparable al de las otras clases de vertebrados. Otras razones de peso para explicar esta desigualdad en la generación de datos son: la mayor diversidad de especies y la relativa facilidad de cría en cautividad de los peces, además del mayor atractivo que estas especies tienen, comparadas con los anfibios, para el hombre.

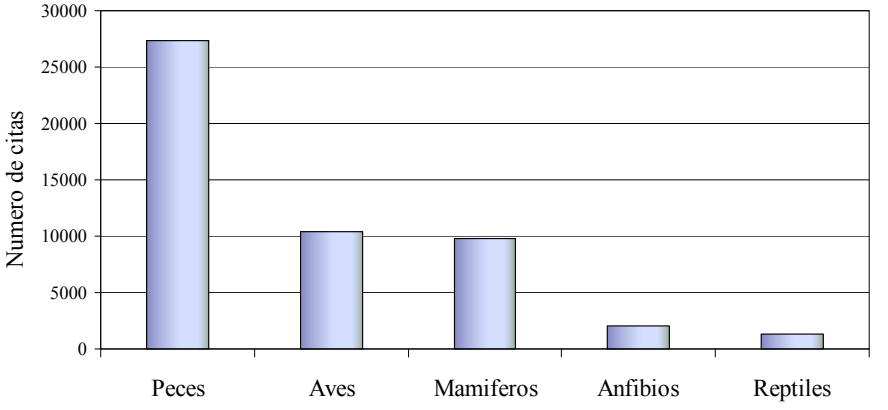

Figura 2. Número de citas obtenidas utilizando como criterios de búsqueda las palabras "*pollution*" o "c*ontaminants*" con las diferentes clases de vertebrados a partir de una búsqueda realizada en la base de datos del ScienceDirect (años: 2000-2013) (artículos, libros y trabajos científicos referenciados).

Desde nuestro punto de vista, es importante hacer hincapié en las características peculiares de los anfibios, así como en las ventajas que aportaría la incorporación de esta clase en las directrices reguladoras para la protección de los organismos acuáticos y de la calidad de las aguas. Además, sería recomendable que los anfibios se integren en los programas de evaluaciones de riesgo por las siguientes razones:

- El declive generalizado que están sufriendo las poblaciones de anfibios a nivel mundial Birge y cols., 2000; Venturino y cols., 2003; Ankley y cols., 2004; Marcogliese y cols., 2009) debido a varios fenómenos naturales (disminución de la capa de ozono, aumento a la exposición ultravioleta, infecciones fúngicas o bacterianas), y a las actividades antropogénicas directas (destrucción de los hábitats de los anfibios) o indirectas

(contaminación ambiental), hace evidente la sensibilidad de los anfibios frente a los contaminantes químicos en el medio acuático, por lo que los anfibios se pueden considerar un organismo diana crítico.

- Los estudios ecotoxicológicos muestran que muchas especies de anfibios, en comparación con los peces o los macroinvertebrados, son especialmente sensibles al estrés químico (Birge y cols., 2000; Sparling, 2000).

- Los anfibios experimentan una serie de cambios bioquímicos, morfológicos y fisiológicos durante todo el proceso de metamorfosis (Brown y Cai, 2007; Fort y cols., 2007), muchos de ellos regulados por hormonas tiroideas, lo que les hace ser un grupo particularmente importante para el estudio de los efectos de disrupción endocrina del eje tiroideo (OECD 231, 2009). La mayoría de los anfibios, por lo menos en su edad adulta, se alimentan de invertebrados, y a su vez son presas de otros vertebrados superiores, por lo que representan un eslabón importante de las cadenas tróficas acuáticas y terrestres (Sparling y cols., 2000).

- Desde el punto de vista de la evolución, los anfibios resultan ser una clase "conectora" entre mamíferos y vertebrados de orígenes más ancestrales (peces cartilaginosos y óseos), y representan un excelente modelo para aquellos estudios inmunológicos que no están filogenéticamente restringidos en un taxón específico (Robert y cols., 2004; Robert y Ohta, 2009).

- Ciertos anfibios han sido ampliamente utilizados como modelos experimentales para el estudio de alteraciones del desarrollo, estudios genéticos, moleculares, etc, lo que permite que exista

mucha información disponible para comprender la acción y los mecanismos biológicos que se producen por las agresiones químicas (ASTM E1439-91, 1998; Jelaso y cols., 2003; Ankley y cols., 2004; Robert y Ohta, 2009).

- Tanto por cuestiones coste/beneficio como éticas, la incorporación de los anfibios sería una posibilidad muy ventajosa, en las fases iniciales de los estudios de toxicidad *in vivo*, como alternativa a los ensayos *in vivo* en mamíferos.

- Finalmente, se dispone de una base de datos toxicológicos suficiente para evaluar y comparar los efectos químicos sobre especies de peces y anfibios, calculando los coeficientes de peligro agudo y crónico para la evaluación de las exposiciones en la columna de agua (Birge y cols., 2000).

A pesar de la reticencia por parte de los organismos reguladores para incluir de forma obligatoria los anfibios en los protocolos de ERA para la caracterización biológica de los contaminantes del compartimento acuático, los ensayos con anfibios tienen un uso cada vez más difundido. Aparte del ensayo agudo estandarizado de teratogenicidad en embriones de *Xenopus laevis*, ensayo FETAX (ASTM E1439-19, 1998), en los últimos años se están llevando a cabo iniciativas que facilitarán la implementación de los protocolos ERA con el uso de los anfibios. Cabe destacar, por ejemplo, los ensayos de metamorfosis y los estudios de toxicidad tiroidea con anfibios mencionados en las guías de la Agencia Europea para Sustancias Químicas (ECHA) (página web 2) que ponen los cimientos para la Directriz 231 (OECD 231, 2009) donde se desarrolla el Ensayo de Metamorfosis en Anfibios (AMA), fundamental para el estudio de disrupción endocrina de ciertas sustancias. Asimismo, los anfibios se han usado para la evaluación del riesgo asociado al cloroformo (página web 3) y en los anexos XV de identificación de sustancias de alta preocupación en

REACH (página web 4; página web 5; página web 6). Finalmente, es interesante hacer referencia a la formación, desde el año 2010, de un grupo de asesoramiento dentro de la Sociedad de Química y Toxicología Ambiental (SETAC) para temas ecotoxicológicos relacionados con los anfibios y los reptiles (AREAG).

Xenopus laevis* como modelo animal. Ensayos de toxicidad actuales con *X. laevis

Para poder llevar a cabo la parte experimental de la presente tesis, entre todos los anfibios cuya cría ha sido adaptada a las condiciones de laboratorio, se optó por el anuro *Xenopus laevis* por sus características biológicas adecuadas para la cría en cautividad (ciclo reproductivo corto y hábitat completamente acuático) y por ser el anfibio más estudiado y mejor conocido en investigación (Sparling y cols., 2000, Robert y Ohta, 2009). La clasificación sistemática de la especie *X. laevis* es la siguiente (Nieuwkoop y Faber, 1994):

CLASE Amphibia
SUB-CLASE Apsidospondyli
ORDEN Anura
SUB-ORDEN Opisthocoela
FAMILIA Pipidae
SUBFAMILIA Xenopodinae
GÉNERO *Xenopus*
ESPECIE *X. laevis*

La caracterización de la embriogénesis de *X. laevis* ha sido ampliamente documentada a nivel molecular y celular, proporcionando a los investigadores una descripción fiable de los diferentes estadios de su

desarrollo embrionario (**figura 3)**. De hecho, desde el año 1967, existe una tabla normalizada utilizada como guía para definir el desarrollo y el estadio larvario descrita por Nieuwkoop y Faber (1994). Además, el genoma del género *Xenopus* ha sido casi enteramente secuenciado (Ankley y cols., 2006) lo que hace de las especies de este género modelos animales de excelencia para investigaciones que requieren el uso de técnicas de biología molecular.

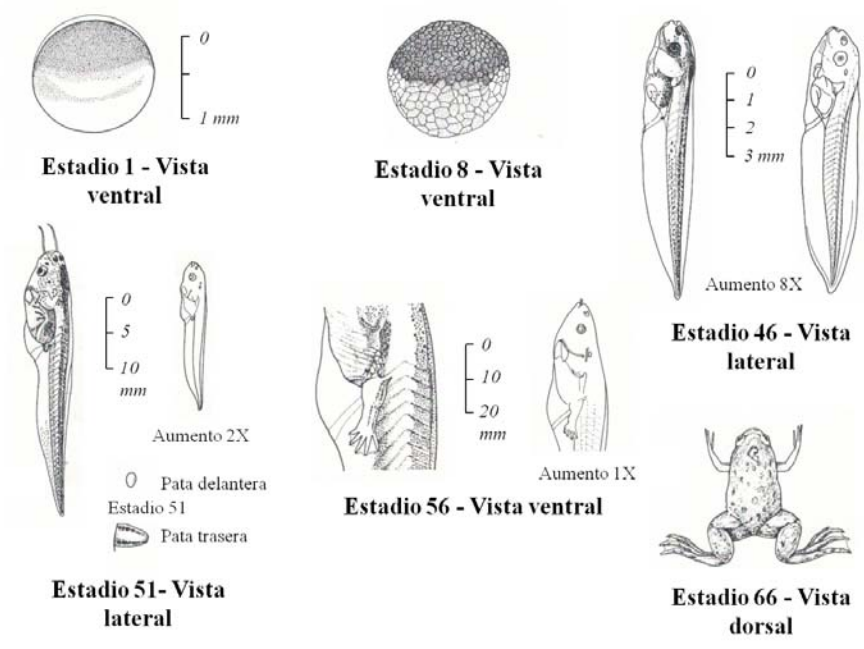

Figura 3. Representación de algunos de los estadios de desarrollo del anuro *Xenopus laevis* más significativos para la realización de los ensayos agudos y crónicos actualmente en uso (adaptada de Nieuwkoop y Faber, 1994).

Como se ha comentado con anterioridad, hasta el momento, los ensayos con *Xenopus laevis* que se pueden utilizar como estudios ecotoxicológicos se resumen en dos: el ensayo de teratogénesis con embriones de *Xenopus* (ensayo FETAX), como ejemplo de ensayo de toxicidad aguda y el ensayo de metamorfosis en anfibios (ensayo AMA)

como ensayo de evaluación de efectos de disrupción endocrina a nivel del eje tiroideo.

Ensayo de Teratogénesis con Embriones del anuro Xenopus laevis (FETAX)

Dumont y colaboradores (1983) desarrollaron el ensayo agudo de teratogenicidad en embriones de *Xenopus* (ensayo FETAX), definieron las bases del protocolo, fijaron los parámetros específicos que se medirían, y propusieron el concepto de índice teratogénico (IT) que permitía diferenciar las sustancias químicas que tenían un efecto sobre el desarrollo. Originariamente el ensayo FETAX se diseñó como una alternativa a los ensayos de evaluación de teratogénesis y alteración del desarrollo en mamíferos. Posteriormente, la ASTM definió un protocolo estandarizado (ASTM E1439-19, 1998), mediante una validación del ensayo realizada por diferentes laboratorios (Bantle y cols., 1994a; Bantle y cols., 1994b, Bantle y cols., 1996; Fort y cols., 1998; Bantle y cols., 1999) y que dio lugar a la elaboración de un Atlas de Anormalidades que tiene como intención facilitar la interpretación de los efectos obtenidos en dicho ensayo (Bantle y cols., 1990).

Este ensayo ha sido fundamentalmente concebido para identificar rápidamente tanto compuestos puros como mezclas complejas con efectos potenciales sobre el desarrollo. La guía propone, además, la posibilidad de que los resultados obtenidos con este estudio puedan ser extrapolados a otras especies, incluyendo los mamíferos, así como su posible utilización en estudios de relación estructura-actividad de compuestos y en estudios de biodisponibilidad (ASTM E1439-19, 1998; Hoke y Ankley, 2005). En los últimos años se ha discutido mucho acerca de la incorporación del ensayo FETAX en los protocolos ERA para la evaluación de los potenciales efectos ecológicos de las sustancias químicas en el compartimento acuático (Hoke y Ankley, 2005).

El ensayo FETAX es un ensayo agudo de 96 horas de duración en el que se determinan como parámetros finales la mortalidad, expresada como

la concentración letal en el 50% de los casos a las 96 horas (96h-LC_{50}), las alteraciones morfológicas, expresadas como la concentración efecto en el 50% de los casos a las 96 horas (96h-EC_{50}), y la mínima concentración capaz de inhibir el crecimiento (MCIG), que se determina midiendo la longitud del cuerpo entero de la larva, desde la cabeza hasta la cola (**figura 4**). El IT, que representa una medida del peligro de una sustancia para alterar el desarrollo, se calcula dividendo la 96h-LC_{50} entre la 96h-EC_{50}. Para valores de IT mayores de 1,5 se considera que existe una gran diferencia entre los rangos de concentraciones que producen mortalidad y los que producen malformaciones, por lo que aumenta la posibilidad de malformación para todos los embriones, aunque haya una ausencia significativa de la mortalidad. La utilización de otros parámetros, como por ejemplo, el tiempo de eclosión, las alteraciones en la pigmentación o en la locomoción, pueden ayudar a incrementar la utilidad del ensayo (ASTM E1439-19, 1998).

El uso rutinario de los bioensayos dentro del marco normativo requiere la optimización y estandardización del protocolo en el contexto de su uso previsto. En el caso del ensayo FETAX, varios aspectos metodológicos, como la calidad de los organismos (fuente, método de cría, alimentación, etc.), las condiciones del ensayo (temperatura, volumen de ensayo, etc.), los procedimientos de exposición (estático o con renovación del medio), y la recopilación e interpretación de los datos, pueden afectar a la validez de la prueba. En consecuencia, Hoke y Ankley (2005) presentaron una evaluación crítica de la incorporación del ensayo FETAX en los protocolos de la ERA, concluyendo que este ensayo presenta demasiadas incertidumbres, que sus parámetros finales deberían ser revisados, y que la evaluación de riesgo llevada a cabo usando los datos obtenidos con los ensayos con especies acuáticas tradicionales podría ser más protectora para los anfibios autóctonos que las evaluaciones de riesgos que usan datos del ensayo FETAX. Sin embargo, consideran valioso el anfibio *X. laevis* para el estudio de otros efectos, tales como la identificación de los compuestos

químicos con potenciales efectos de disrupción endocrina sobre el eje tiroideo o sobre la reproducción.

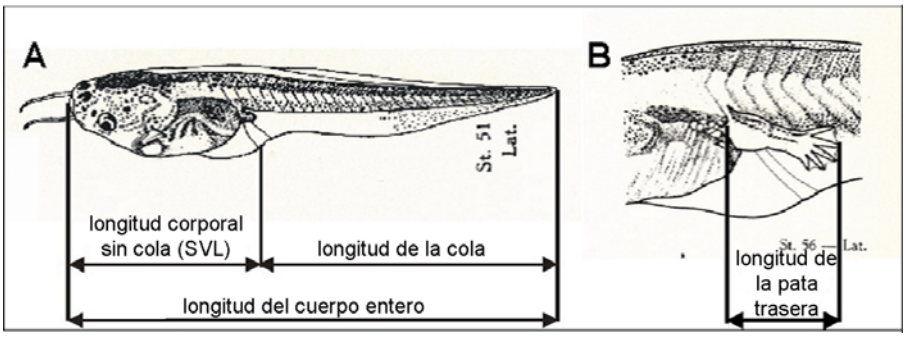

Figura 4. (A) Representación de los diferentes tipos de medidas de la longitud de la larva y (B) medida de la longitud de la pata trasera en renacuajos de *X. laevis* utilizadas en los protocolos estandarizados (Modificada de la Directriz OECD 231, 2009).

Ensayos crónicos: Ensayo de Metamorfosis en Anfibios (AMA)

En las últimas décadas la sociedad ha ido tomando conciencia de la existencia de determinados contaminantes ambientales que son capaces de interferir en el sistema endocrino del hombre y de los animales (Colborn y cols., 1993). Tales sustancias, llamadas disruptores endocrinos, tienen un origen fundamentalmente antropogénico y muchas de ellas presentan unas propiedades físico-químicas que les hacen ser persistentes en el medio ambiente, aumentando su potencial de bioacumulación en los organismos acuáticos. Esta creciente preocupación ha dado lugar a que diferentes organismos internacionales (Comisión Europea, Parlamento Europeo, U.S. EPA, OECD, Organización Mundial de la Salud, Agencia Europea para el Medio Ambiente) y la Industria Química trabajen en la elaboración de protocolos que permitan evaluar las alteraciones endocrinas. El Programa Internacional para la Seguridad de las Sustancias Químicas (IPCS) define un **disruptor endocrino** como:

"*una sustancia exógena, o una mezcla, que altera la(s) función(es) del sistema endocrino y consecuentemente causa efectos adversos en la salud de un organismo sano, o en su progenie, o a nivel sub-poblacional*" (página web 7).

Las alteraciones en la función endocrina, causadas por un disruptor endocrino, pueden ser debidas a la interferencia con la síntesis, la secreción, el transporte, la unión a un receptor, la actividad hormonal o la eliminación de las hormonas naturales presentes en el organismo; dichas hormonas son responsables de procesos fisiológicos tan importantes como la homeostasis, la reproducción, el desarrollo o el comportamiento. Por lo tanto, los productos químicos presentes en el medio ambiente, bien naturales o de síntesis, pueden actuar como perturbadores endocrinos que imitan, aumentando (agonistas) o inhibiendo (antagonistas), la acción de las hormonas (Vos y cols., 2000).

Aunque en un primer momento, la preocupación social por este tipo de compuestos se centró en sus efectos sobre el sistema endocrino reproductor, se ha comprobado en los últimos años que existen numerosos compuestos químicos que son capaces de alterar la función tiroidea (Brucker-Davis, 1998). Como modelo de evaluación de efectos adversos a nivel del eje tiroideo, se ha propuesto el ensayo de metamorfosis en anfibios (ensayo AMA) (Degitz y cols., 2005; Opitz y cols., 2005). Aunque los anfibios están muy separados filogenéticamente de otros vertebrados, son muchos los aspectos de la función tiroidea que están conservados entre los cordados, sobre todo a nivel morfológico y molecular (OECD 231, 2009); por tanto, son muchos los autores que proponen la extrapolación de los datos obtenidos con este ensayo a otras especies (Zoeller y Tan, 2007). Una de las causas por las que se ha elegido este ensayo, es el amplio conocimiento que existe de la funcionalidad del eje hipotálamo-hipófisis-tiroideo de los anfibios (Brown y Cai, 2007; Fort y cols., 2007); todos los cambios producidos durante el proceso de la metamorfosis son dependientes de las hormonas tiroideas, y por tanto cualquier alteración que se produzca

durante este proceso puede ser valorada como un efecto potencial de disrupción endocrina. Por todo ello, la OECD llevó a cabo un proceso de desarrollo y validación del ensayo AMA que ha sido publicado en el 2009 (OECD 231, 2009).

Inmunotoxicidad

Al igual que ciertos compuestos químicos pueden afectar a los sistemas reproductivo y tiroideo, otros mecanismos fisiológicos también pueden verse afectados, tales como la respuesta al estrés (Kloas y Lutz, 2006), o el sistema inmune. En los últimos años ha aumentado la preocupación acerca de los efectos que los xenobióticos pueden provocar sobre el sistema inmune, considerándose cada vez más relevante como sistema diana dentro de la evaluación de riesgo ambiental de las sustancias químicas. De ahí que haya surgido una nueva rama de la toxicología, la inmunotoxicología, que se dedica al estudio de la inmunotoxicidad, es decir, al estudio del efecto adverso de agentes o sustancias químicas sobre el sistema inmune. Estos agentes pueden provocar un incremento de la respuesta inmune (hipersensibilidad o autoinmunidad) o un efecto de inmunosupresión, disminuyendo la respuesta a agentes infecciosos e incrementando la incidencia del cáncer (Carfi y cols., 2007). La selección de los criterios de valoración de la inmunotoxicidad debe ser considerada como una prioridad para la adecuada interpretación de los efectos subletales a nivel inmunológico, que generalmente no son fácilmente cuantificables por los índices de salud de referencia (Kacmár y cols., 1999).

La cuantificación de citoquinas, en particular algunas interleuquinas, ha demostrado ser un buen parámetro para predecir el potencial inmunotóxico de los contaminantes ambientales (Pillet y Nicolas, 2005), debido a que las citoquinas se encuentran en todos los vertebrados y pueden constituir unos excelentes marcadores para el seguimiento de la exposición a contaminantes que afecten el sistema inmune. Las citoquinas son responsables de regular una gran variedad de procesos, incluyendo la inflamación, la apoptosis y la hematopoyesis, además de ser moléculas de

señalización que desempeñan un papel central en la iniciación, el desarrollo y la diferenciación de la respuesta inmune (Borish y Steinke, 2003; Haddad y cols., 2005) (**figura 5)**. Cuando las citoquinas se producen en respuesta a un daño en el sistema inmunológico, determinan el principio de la respuesta inmune, y posteriormente son las responsables de definir esa respuesta como humoral, mediada por células o de tipo alérgico. Las familias de citoquinas están resumidas en la **tabla 1**.

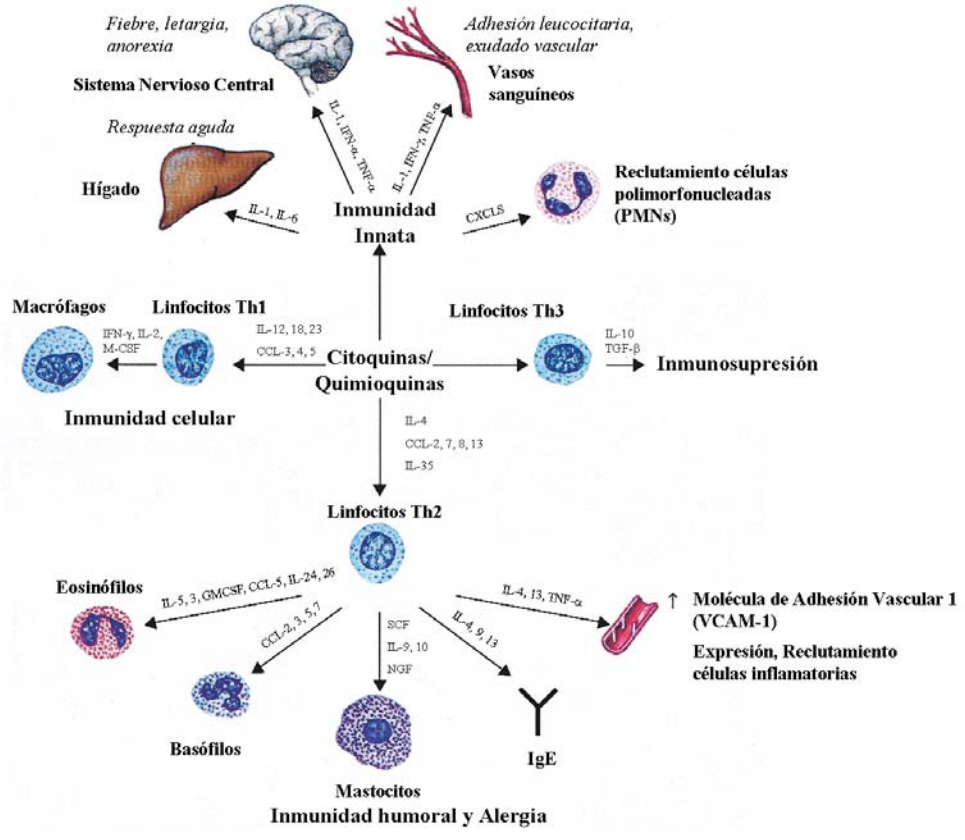

Figura 5. Resumen de las acciones de las citoquinas y quimioquinas en la respuesta inmune. Las citoquinas derivan principalmente de las células fagocitarias mononucleares. Son extremadamente importantes en la inmunidad innata y dan origen a los síntomas asociados a la respuesta inmune (adaptada de Borish y Steinke, 2003).

Tabla 1. Familias de citoquinas según Commins y colaboradores (2010).

Familia	**Miembros**
Hematopoyética	
Con cadena γ común	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
Con cadena β compartida (CD131)	IL-3, IL-5, GM-CSF
Compartidas	IL-2, IL-15
Con cadena IL-2β (CD122)	
Otras hematopoyéticas	IFN- γ, IL-7, IL-13, IL-21, IL-31, TSLP
Familia de IL-1	IL-1α, IL-1β, IL-1ra, IL-18, IL-33
Utilizadoras de gp130	IL-6, IL-11, IL-27, IL-31, CNTF, CT-1, LIF, oncostatina, osteopontina
IL-12	IL-12, IL-23, IL-35
Superfamilia de IL-10	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29
IL-17	IL-17A,-F, IL-25 (IL-17E)
Interferonas	
Interferonas tipo I	IFN-α, IFNβ, IFN-ω
Interferon tipo II	IFN-γ (también es una citoquina hematopoyetica)
Interferonas tipo III	IFN-λ1, (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B)
Superfamilia de TNF	TNF-α, TNF-β, BAFF, APRIL

APRIL:	Señal de inducción de proliferación (A proliferation-inducing signal)
BAFF:	Factor de activación de las células B desde la familia TNF (B-cell activation factor from the TNF family)
CNTF:	Factor neurotrópico ciliar (Ciliary neurotrophic factor)
CT-1:	Cardiotropina 1
GM-CSF:	Factor estimulante de colonias de granulocitos (Granulocyte colony-stimulating factor)
gp:	Glicoproteina
IFN:	Interferon
IL:	Interleuquina
LIF:	Factor inhibitorio de leucemia (Leukemia inhibitory factor)
TNF:	Factor de necrosis tumoral (Tumor necrosis factor)
TSLP	Linfopoietina timica stromale (Thymic stromal lymphopoietin)

Las moléculas estudiadas en este trabajo de investigación: proteína de choque térmico 70, interleuquina 1-β y factor de necrosis tumoral α

Como se ha dicho anteriormente, las citoquinas, incluyendo las interleuquinas, se están considerando como buenos parámetros para predecir el potencial inmunotóxico de los contaminantes ambientales (Pillet y Nicolas, 2005; Carfi y cols., 2007), y podrían también ser utilizadas como parámetros de toxicidad subletal de carácter inespecífico, debido a que nos brindan la capacidad de detectar cambios en la salud con bajos niveles de exposición, mucho antes de que aparezcan efectos evidentes en los organismos. También las proteínas de choque térmico (HSPs) han sido propuestas como indicadores de exposiciones subletales a contaminantes ambientales (Cruz-Rodríguez y Chu, 2002). En este sentido, en la presente tesis se estudia la expresión de tres genes proinflamatorios: la proteína de choque térmico 70 (*hsp70*), la interleuquina 1-β (*IL-1β*) y el factor de necrosis tumoral α (*TNF-α*).

Proteína de choque térmico 70 (HSP70)

Las proteínas de choque térmico (HSPs) fueron descubiertas por primera vez por Ritossa en 1962 en las glándulas salivares de *Drosophila melanogaster*. Son componentes celulares evolutivamente antiguos y altamente conservados, que se han encontrado en todos los organismos procariotas y eucariotas estudiados hasta hoy (Robert, 2003; Stromer y cols., 2003). Las HSPs son comúnmente nombradas y agrupadas según sus pesos moleculares, originando seis familias diferentes: HSP100, HSP90, HSP70, HSP60, HSP40, y las proteínas de choque térmico pequeñas (entre 15 y 30 KDa de peso molecular) (Katschinski, 2004). Aunque alguna HSP se expresa constitutivamente dentro de la célula en condiciones fisiológicas normales, ayudando en procesos vitales celulares tales como el plegamiento, ensamblaje y trasporte de proteínas, otras se expresan exclusivamente bajo la acción de perturbadores fisiológicos o estresantes, tales como: temperaturas elevadas, hipoxia, isquemia, exposición a metales, radiación,

aumento de calcio, privación de glucosa, cáncer o infecciones microbianas (Robert, 2003; Katschinski, 2004).

Las HSPs se definen como proteínas chaperonas que no se unen covalentemente a las superficies hidrofóbicas de otras proteínas exógenas (Robert, 2003), y que ejercen su función de mantener otras proteínas en un estado plegado, evitando así la agregación de proteínas desnaturalizadas o dañadas. (Agashe y Hartl, 2000; Heikkila y cols., 1997; Azzoni y cols., 2004) (**figura 6).** La respuesta al choque térmico es un mecanismo celular que asegura la supervivencia de las células después de sufrir cualquier tipo de estrés ambiental o endógeno. Numerosas investigaciones sobre la respuesta al choque térmico y los mediadores de dicha respuesta en diferentes modelos (Heikkila, 2003; Katschinski, 2004), han revelado una gran información sobre los mecanismos de acción que apuntan a una compleja cascada de eventos que rodean la respuesta al estrés (**figura 7**).

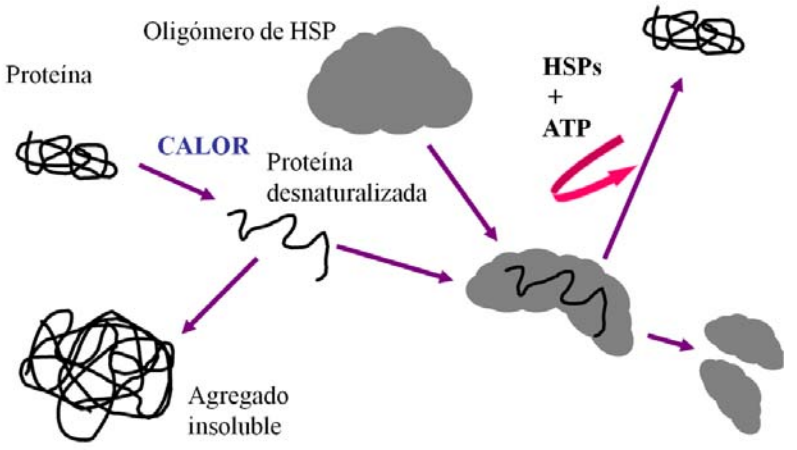

Figura 6. Modelo de la actividad chaperona de las proteínas de choque térmico (HSPs). Las proteínas presentes en las células pueden ser desnaturalizadas por la acción del calor o de otros agentes estresantes. En ausencia de las HSPs las proteínas desnaturalizadas se convierten en agregados insolubles que pueden provocar la muerte celular. Sin embargo, en presencia de las HSPs la proteína desnaturalizada se mantiene en un estado parcialmente plegado hasta que se elimine el agente estresante y se restablezcan las condiciones fisiológicas normales. En ese momento, las HSPs son fosforiladas para permitir la liberación de la proteína substrato. Finalmente, las HSPs pueden, en presencia de ATP, restablecer el estado competente plegado de la proteína (adaptada de van Montfort y cols., 2001).

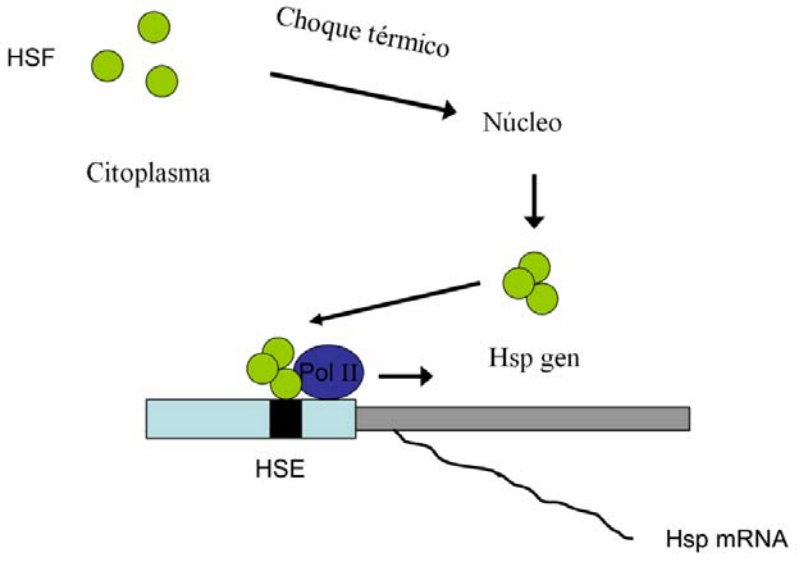

Figura 7. Modelo de síntesis *in vivo* del acido ribonucleico mensajero (ARNm) para las proteínas de choque térmico. El factor de choque térmico (HSF) se localiza en forma de monómero inactivo en el citoplasma de las células. En situación de estrés, tales como el choque térmico, el HSF migra hacia el núcleo donde se activa (trímero), y se une al elemento de choque térmico (HSE) localizado en la región 5' no traducida (UTR) del gen de la proteína de choque térmico. Dicha unión permite la asociación con la polimerasa II y la consecuente trascripción del gen en ARNm (adaptada de Heikkila, 2003).

Para cada familia de HSP se han definido numerosas funciones específicas, participando en muchos casos en diversos procesos inmunológicos. Un creciente número de datos sugiere que ciertas HSPs, como por ejemplo la HSP70, juegan un papel importante en la inmunidad innata y adaptativa (Robert, 2003). Las HSPs pueden provocar respuestas celulares específicas de la respuesta inmune (por ejemplo, la producción de los linfocitos T), debido a su función como proteínas chaperonas. Además, en varios estudios *in vitro* se ha visto que, independientemente de sus actividades como proteínas chaperonas, las proteínas de choque térmico gp96, la HSP90 y la HSP70 son capaces de estimular a los macrófagos para

que produzcan diferentes citoquinas pro-inflamatorias (IL-1β, TNF-α, interleuquina 12 (IL-12) y el factor de estimulación de colonias de los granulocitos y macrófagos (GM-CSF) (Basu y cols., 2000).

Interleuquina 1-β (IL-1-β)

La interleuquina 1-β es una de las principales citoquinas pro-inflamatorias, fundamental en los procesos de inmunidad innata que se induce *in vivo* por el lipopolisacarido bacteriano (LPS) (Zou y cols., 2000). Se considera un regulador principal de la respuesta inflamatoria e inmune en los mamíferos, pero a lo largo de los últimos años ha sido ampliamente estudiada también en los peces óseos y en los anfibios, lo que ha permitido la identificación de una serie de regiones bien conservadas entre los vertebrados (Bird y cols. 2002). Cuando ocurre una infección o una lesión en el organismo, la IL-1β estimula la respuesta inmune iniciando y promoviendo la producción de otras citoquinas, como las quimioquinas, y moléculas de adhesión celular (Engelsma y cols., 2002).

La IL-1β es segregada principalmente por las células del linaje fagocítico mononuclear, pero también se puede producir a partir de otras células. La producción de IL-1β es debida a una variedad de agentes, incluida, como se ha visto anteriormente, la endotoxina bacteriana, que estimula receptores específicos. Una de sus actividades biológicas más importantes es su capacidad de activar los linfocitos T mediante la inducción de la producción de interleuquina 2 (IL-2) y de la expresión de receptores de IL-2. En ausencia de IL-1β, es de esperar una disminución de la respuesta inmune o una tolerancia de la misma (Commins y cols., 2010).

Por otra parte, la IL-1β tiene una función anti-inflamatoria y es un factor importante en la inducción de la apoptosis *in vivo* en tejidos no linfoides (Haddad y cols., 2005; Islam y Pestka, 2003). Además, tiene la capacidad de inducir la producción de corticosterona, lo que provoca la apoptosis de los leucocitos y por consiguiente, un agotamiento del tejido linfoide (Islam y Pestka, 2003). Por estas razones, las alteraciones en la

síntesis de HSP70 y IL 1-β se han relacionado con el deterioro funcional de las células efectoras, lo que podría tener consecuencias en la respuesta inmune (Pillet y Nicolas, 2005; Robert, 2003). Finalmente, la alteración en la síntesis de estas moléculas podría aumentar la susceptibilidad a las infecciones bacterianas, víricas y parasitarias (Marcogliese y cols., 2009).

Factor de necrosis tumoral α (TNF-α)

El factor de necrosis tumoral α (TNF-α) es una citoquina altamente pleiotrópica, que participa en un amplio espectro de procesos fisiológicos que controlan la inflamación, las respuestas anti-tumorales y la homeostasis (Grayfer y Belosevic, 2009). Esta citoquina pro-inflamatoria puede estar unida a las membranas celulares, o bien estar producida por las diferentes clases celulares involucradas en la respuesta inmune: los macrófagos y los linfocitos activados, los neutrófilos, las células "natural killer", las células endoteliales y los mastocitos. Los efectos mediados por el TNF-α se deben a la trimerización de la proteína y su enlace con sus receptores específicos. El más poderoso inductor de TNF-α por parte de los monocitos es el LPS, que actúa a través de unos receptores específicos. El TNF-α se incluye en el grupo de las citoquinas que estimulan la reacción de fase aguda en los mamíferos y peces. Estudios recientes, llevados a cabo en mamíferos, sugieren que el TNF-α tiene un fuerte efecto antiviral, y que está involucrado también en la defensa antiviral en invertebrados (Morales y cols., 2010). Además, el TNF-α induce inmunidad antitumoral a través de efectos citotóxicos directos sobre las células cancerosas y estimulando la respuesta inmune antitumoral (Commins y cols., 2010). A pesar de que IL-1β y TNF-α comparten diversas actividades biológicas, la distinción más importante es que el TNF-α no tiene ningún efecto directo sobre la proliferación de linfocitos (Commins y cols., 2010).

OBJETIVOS

El objetivo general de este trabajo de investigación es estudiar el potencial de integración de los ensayos con anfibios, tanto agudos como subletales, incluyendo efectos immunotoxicos, en los protocolos de evaluación de riesgo ambiental de sustancias químicas puras y de mezclas complejas. Este objetivo se persigue a través de otros más concretos:

1. Desarrollar un nuevo ensayo de toxicidad aguda con larvas del anuro *X. laevis* para evaluar los efectos tóxicos de sustancias puras y mezclas complejas.

2. Comparar la sensibilidad del ensayo agudo sobre *X. laevis* con otros ensayos ya estandarizados o utilizados asiduamente en ecotoxicología, como los ensayos agudos con peces, los ensayos *in vitro* con líneas celulares de peces (línea RTG-2 de trucha arcoíris) y el ensayo FETAX con embriones de *X. laevis*.

3. Evaluar la respuesta del nuevo ensayo dentro de una batería de ensayos de evaluación de riesgos ambientales de mezclas complejas (residuos tóxicos y peligrosos, y lixiviados de vertederos).

4. Desarrollar un ensayo de toxicidad subletal sobre el anuro *Xenopus laevis* para evaluar los efectos inmunotóxicos potenciales a corto y medio plazo de los contaminantes mediante la expresión del ARNm para las proteínas HSP70, IL-1β y TNF-α.

5. Evaluar la relación dosis-respuesta de dicho ensayo de inmunotoxicidad.

CAPÍTULO 2

Are fish and standardized FETAX assays protective enough for amphibians? A case study on *Xenopus laevis* larvae assay with biologically active substances present in livestock wastes.

Martini F, Tarazona JV, Pablos MV.

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Índice de impacto: 1,730

Resumen

Cuando los residuos ganaderos se consideran para su uso como abono, las sustancias biológicamente activas contenidas en ellos pueden llegar al compartimento acuático causando alteraciones del mismo. Históricamente, en los estudios de ecotoxicología con vertebrados acuáticos siempre se han usado peces como organismos de referencia, o bien embriones del anfibio *Xenopus laevis* (ensayo FETAX). Recientemente, algunos investigadores han cuestionado tanto el uso del ensayo estandarizado FETAX, así como el uso de la evaluación de riesgo en peces para cubrir otras especies, como por ejemplo los anfibios, ya que la extrapolación podría no ser lo suficientemente protectora para estas. En el presente estudio se ha desarrollado un ensayo agudo con las larvas de *X. laevis* con el fin de comparar la sensibilidad de las larvas de dicha especie con respecto a la de los peces o del ensayo FETAX. Las larvas han sido expuestas a sustancias que se pueden encontrar en los residuos ganaderos, tales como medicamentos veterinarios (ivermectina, oxitetraciclina, tetraciclina, trimetoprim y sulfametoxazol) y elementos esenciales (zinc, cobre, manganeso y selenio). Los resultados se han obtenidos a través de la estimación de los efectos letales (LC_{50}) y no letales (identificación de parámetros subletales). En general, los datos disponibles para los peces y para el ensayo FETAX son equiparables o incluso más protectores que los valores encontrados en el presente estudio. No obstante, en el caso del selenio las larvas de *X. laevis* son más sensibles que la trucha arcoíris. Por otra parte, la presencia de efectos no letales causados por ivermectina, zinc y cobre, sugiere que varios mecanismos fisiológicos pueden verse afectados por los contaminantes del medio acuáticos. Es interesante investigar más detenidamente estos tipos de efectos. Los resultados obtenidos en el presente estudio podrían ampliar la información ecotoxicológica de los efectos sobre anfibios de microcontaminantes contenidos en los residuos ganaderos.

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Research Article

Are Fish and Standardized FETAX Assays Protective Enough for Amphibians? A Case Study on *Xenopus laevis* Larvae Assay with Biologically Active Substances Present in Livestock Wastes

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Biologically active substances could reach the aquatic compartment when livestock wastes are considered for recycling. Recently, the standardized FETAX assay has been questioned, and some researchers have considered that the risk assessment performed on fish could not be protective enough to cover amphibians. In the present study a *Xenopus laevis* acute assay was developed in order to compare the sensitivity of larvae relative to fish or FETAX assays; veterinary medicines (ivermectin, oxytetracycline, tetracycline, sulfamethoxazole, and trimethoprim) and essential metals (zinc, copper, manganese, and selenium) that may be found in livestock wastes were used for the larvae exposure. Lethal (LC_{50}) and sublethal effects were estimated. Available data in both, fish and FETAX studies, were in general more protective than values found out in the current study, but not in all cases. Moreover, the presence of nonlethal effects, caused by ivermectin, zinc, and copper, suggested that several physiological mechanisms could be affected. Thus, this kind of effects should be deeply investigated. The results obtained in the present study could expand the information about micropollutants from livestock wastes on amphibians.

1. Introduction

Veterinary medicines are widely used to treat disease and to protect animal's health [1]. Dietary growth-enhancing feed additives (growth promoters) are also incorporated into the feed of animals to improve their growth rates [2]. One of the most important problems that could occur when livestock wastes are considered for recovery, reuse, and recycling is the presence of biologically active substances in these wastes, such as veterinary medicines, biocides, and additives for animal feed, which in small concentrations could have potential toxic effects on aquatic organisms. In the present work, five veterinary medicines and four essential metals, used as mineral supplements or food additives in livestock, have been studied in acute static tests using *Xenopus laevis* as animal model.

The veterinary medicines selected to carry out the tests were ivermectin, oxytetracycline, tetracycline, sulfamethoxazole, and trimethoprim. The last two medicines were used maintaining the same proportions presented in the commercial chemotherapy Septrin (400 mg sulfamethoxazole and 80 mg trimethoprim). These drugs were selected because they are the most commonly used in animal husbandry within their respective categories [3–6]. The four studied essential metals were zinc (Zn), copper (Cu), manganese (Mn), and selenium (Se). Trace concentrations of essential metals are required in the diet for many biological processes, particularly enzyme functions, and they have a positive influence on livestock growth and reproduction [2]. Due to the low content of essential metals in some feeds compared to the recommendations, supplementation of these metals is necessary for most livestock species, and they are commonly added to daily rations as mineral supplements (e.g., Calfostonic, Bovis).

For the study of acute toxicity in amphibians, the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) [7] is currently used. The FETAX assay is a 4-day exposure standardized test with *Xenopus laevis* embryos from stage 8 to stage 46, according to Nieuwkoop and Faber table [8]. Over other

nonstandardized tests, the FETAX assay has the advantage to evaluate a large number of parameters in one study [9]. However, since it is unknown how the exposure to toxic substances in the embryonic stage may affect the sensitivity, the results obtained from FETAX test and their use for environmental risk assessment has been questioned by Hoke and Ankley [10].

Thus, one of the aims of the present study is to develop an acute assay to compare potential sensitivities to toxicants between larvae and embryo in *X. laevis*. Moreover, little is known concerning the relative sensitivity of amphibians to toxicants compared with other more traditional aquatic test species, such as fish. Although there has been a substantial amount of developmental biology research with *X. laevis*, there are few toxicology data for this species compared to fish [10]. Through the present acute larvae assay, we investigated the possibility that risk assessment carried out on fish could not be protective enough for other aquatic species, such as amphibians. The results obtained could expand the existing information on ecotoxicological effects of possible micropollutants present in livestock wastes on amphibians.

2. Materials and Methods

2.1. Chemicals. Sulfamethoxazole, trimethoprim, ivermectin, and triethylene glycol (99% pure) were purchased from Sigma (Steinheim, Germany). Tetracycline (tetracycline hydrate, 99% pure) was obtained from Aldrich (Milwaukee, WI, USA). Oxytetracycline (oxytetracycline hydrochloride ≥99% pure), zinc sulphate (zinc sulphate 7-hydrate ≥99% pure) and copper chloride (copper II chloride 2-hydrate) were provided by Panreac (Barcelona, Spain). Manganese sulfate (manganese II sulfate monohydrate ≥99% pure) and sodium selenite (sodium selenite 5-hydrate for analysis) were purchased from Merck (Germany). Ultrapure water was obtained by a Milli-Q Synthesis water purification system.

2.2. Test Organisms. *Xenopus laevis* tadpoles, stage 47 according to *Xenopus* table of development [8], were obtained from in-house breeding of adult animals. The adults were housed in plastic aquaria in group of 10, with 40 L of dechlorinated tap water. The room temperature was set at 22 ± 1°C under a 12 : 12 h light: dark photoperiod. Frogs were fed with trout feed chopped pellets (REPRODUCTORES, Dibaq, Spain) twice a week, 2-3 h before each water change. Animal manipulation was performed in accordance with the protocol of American Society for Testing Materials [7]. Spawning of adult *X. laevis* was induced by two injections of human chorionic gonadotropin (hCG-LEPORI 2500, Angelini, Italy) into the dorsal lymph sac, spaced 8 hours apart. Male received 400 International Units (IU) of hCG at each injections. Female received 250 IU on the first injection and 800 IU on the subsequent injection. Tadpoles were changed into fresh FETAX medium with a stainless steel strainer 5 d postfertilization and fed daily on commercially available fish powder dry food (SERA MICRON, Germany) *ad libitum*.

2.3. Toxicity Tests. All procedures were conducted under protocols approved by the Ethics Committee for animal research of the Spanish National Institute for Agricultural and Food Research and Technology. Preliminary range-finding experiments were performed to determine the appropriate concentration ranges for the tested chemicals (data not shown). Then, short-term tests (4 d) were carried out to establish the acute lethal toxicity of tested substances and to identify potential sublethal effects.

2.3.1. Veterinary Medicines. Tests were conducted in 52 glass jars located in a water bath maintained at 22 ± 1°C on a 12 : 12 h light: dark photoperiod. Jars were placed in one 4 × 13 blocks, and treatment and replicate positions were assigned randomly. Groups of 5 larvae were exposed in each glass jar containing 100 mL of medium solutions. All tests were conducted with four replicates. Exposures took place in a reconstituted water medium suitable for Frog Embryo Teratogenesis Assay-*Xenopus*, FETAX medium [11]. Tadpoles were exposed, in a static assay, during 4 days to serial dilutions of four different drugs: S + T, TC, and OTC with initial nominal concentrations of 50 and 100 mg/L, and IVE with initial nominal concentrations of 1.075, 2.15, 4.3, 8.6, and 17.2 μg/L. Because of limited aqueous solubility of tetracycline and ivermectin, triethylene glycol was used as carrier. In all experiments, the concentration of the solvent did not exceed the concentration of 1.6% (v/v), according to ASTM guidelines [7]. Larvae were checked every day for morphological abnormalities, developmental delay, abnormality swimming behaviours, and mortality, and all dead tadpoles were counted and removed.

2.3.2. Essential Metals. Exposure conditions were the same as described above. Jars ($n = 84$) were randomly placed in two 3 × 14 blocks. In this case, no SC was used. Tadpoles were exposed to five geometrical serial dilutions of four different compounds: zinc sulphate ($ZnSO_4 * 7\ H_2O$), copper chloride ($CuCl_2 * 2\ H_2O$), manganese sulphate ($MnSO_4 * H_2O$), and sodium selenite ($NaSeO_3 * 5\ H_2O$), with the aim to achieve the corresponding nominal concentrations of metals shown at Table 1.

2.4. Statistical Analyses. For each sample with visually distinguishable abnormalities, probit analysis (Statgraphics 5.1, StatPoint Technologies, INC., USA) was used to calculate effect concentrations in 50% of the cases (EC_{50}) with 95% confidence intervals. The same analysis was employed to calculate lethal concentrations (LC_{50}). The significance of the endpoints with respect to the control data was assessed by one way analysis of variance (ANOVA), with Fisher's least-significant difference procedure (LSD, $P < 0.05$), in the software Statgraphics 5.1.

3. Results

The embryo survival rate in the blank control (BC) and solvent control (SC) reached at least 90% throughout the duration of the tests, and SC did not show any significant effects

Table 1: Nominal concentrations (mg/L) of the four salts of metal, used as water-soluble forms, and the corresponding nominal concentrations (mg/L) of the four metals considered in the current study.

Water-soluble form	Concentration	Metal	Concentration
Zinc sulphate 7-hydrate	6	Zinc	1.36
	12		2.73
	24		5.46
	48		10.91
	96		21.83
Copper II chloride 2-hydrate	0.6	Copper	0.22
	1.2		0.45
	2.4		0.89
	4.8		1.79
	9.6		3.58
Manganese II sulfate monohydrate	3.75	Manganese	1.22
	7.5		2.44
	15		4.88
	30		9.75
	60		19.50
Sodium selenite 5-hydrate	1.56	Selenium	0.47
	3.12		0.94
	6.25		1.88
	12.5		3.75
	25		7.51

on normal *Xenopus* development. Thus, statistical analyses were related to BC. For tadpoles treated with OTC, TC, S + T, and Mn, no lethal or sublethal effects were found; therefore, LC_{50}s values were higher than the maximum exposure concentrations. For tadpoles exposed to IVE, Zn, Se, and Cu, the estimated LC_{50}s and EC_{50}s with their corresponding 95% confidence intervals, the sublethal effects, and the No Observed Effect Concentration (NOEC) based on sublethal effects are shown in Table 2. All tadpoles treated with IVE showed hyperactivity, rapid and uncontrollable swimming movements when a touch was given to the jar, at all tested concentrations except at the lowest one. In larvae exposed to Zn, edema (Figure 1) was detected at all exposure times and concentrations, except at 96 h at the lowest concentration. Furthermore, at 48 h the higher number of tadpoles with edema was found, while at the subsequent periods edema reabsorption in some individuals was observed (Table 2). Copper provoked two sublethal effects: developmental delay at 72 and 96 hours and abnormal pigmentation (whitish) in all tadpoles at all tested concentrations.

4. Discussion

The presence of xenobiotics in aquatic ecosystem does not, by itself, indicate injurious effects. Connections must be established between external levels of exposure, internal levels of tissue contamination, and early adverse effects [12]. Environmental Risk Assessment (ERA) is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainly using scientific methodologies [13]. Environmental Risk Assessment is currently considered the best available tool for environmental decision making [12]. The ERA of veterinary drugs for aquatic compartment uses the results of ecotoxicological tests of three basic taxa: algae (e.g., *Chlorella vulgaris*), aquatic invertebrates (e.g., *Daphnia magna*), and fish (e.g., *Oncorhynchus mykiss*). For years, the risk assessment carried out on fish was considered to be protective enough to cover other aquatic vertebrates, including amphibians. Since the mid-1990s, the significant decline suffered by amphibian populations has received the attention of both scientific community and popular media [14]. Several reasons have been put forward to explain such decline, some arising directly or indirectly from human activities, such as direct destruction of amphibian habitats by humans or chemical pollution, and others emerging from global and local climatic changes, for example, fungal and bacterial infection, which may be related to ozone depletion and an increase of ultraviolet exposure [14–16]. Due to the peculiarities of amphibians (combining aquatic and terrestrial phases in their life cycles, feeding and respiration rate, permeability of the skin), their susceptibility to contaminants in the aquatic environment could be considered greater than other aquatic organisms widely used in ecotoxicological tests, such as fish [17].

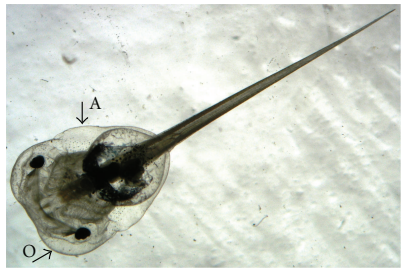

Figure 1: Edema in optic and abdominal areas of *X. laevis* tadpole caused by all nominal Zn exposure concentrations (2.73, 5.46, 10.91, and 21.83 mg/L), except for the lowest one (1.36 mg/L). O: optic and A: abdominal.

Environmental Risk Assessment protocols for pollutants or complex mixtures include ecotoxicological assays with fish to study effects of acute and chronic exposures on larval stages or adults. In the same way, in the case of amphibians, it would be particularly important to know the acute and chronic effects at different stages of development caused by biologically active substances such as biocides and veterinary medicines. Moreover, the lack of standardized toxicity tests with amphibians and the subsequent limitations in high-quality toxicology data for either prospective or diagnostic assessment continue to be a problem and often prevent the inclusion of amphibians in ERAs. The novel aspect of the current study lies in the use of an ecotoxicological assay with

Table 2: Endpoints studied at each exposure time for ivermectin (IVE), zinc (Zn), copper (Cu), selenium (Se). Lethal and effect concentrations in the 50% of the cases (EC_{50} and LC_{50}) were shown at 24, 48, 72 and 96 hours. At each exposure time, the EC_{50} values were related to the corresponding sublethal effect. For the water-soluble forms of the metals: zinc sulphate ($ZnSO_4 * 7\ H_2O$) and sodium selenite ($NaSeO_3 * 5\ H_2O$) the 96 h LC_{50} were reported, and for copper chloride ($CuCl_2 * 2\ H_2O$) both 48 h and 96 h LC_{50}s were showed.

Substance	Exposure time (hours)	LC_{50} (mg/L)	95% confidence intervals (mg/L)	EC_{50} (mg/L)	95% confidence intervals (mg/L)	NOEC[a] (mg/L)	Sublethal effect
IVE	24	$6.4 * 10^{-3}$		$>1.1 * 10^{-3}$ $<2.1 * 10^{-3}$			hyperactivity
	48	$5.6 * 10^{-3}$	$4.7 * 10^{-3}$–$6.6 * 10^{-3}$	$>1.1 * 10^{-3}$ $<2.1 * 10^{-3}$			hyperactivity
	72	$5.5 * 10^{-3}$	$4.6 * 10^{-3}$–$6.6 * 10^{-3}$	$>1.1 * 10^{-3}$ $<2.1 * 10^{-3}$			hyperactivity
	96	$5.5 * 10^{-3}$	$4.6 * 10^{-3}$–$6.6 * 10^{-3}$	$>1.1 * 10^{-3}$ $<2.1 * 10^{-3}$		$1.1 * 10^{-3}$	hyperactivity
Zn	24	14.0	12.0–16.8	7.4	3.6–12.3		edema
	48	13.4	11.3–16.1	4.5	2.6–6.3		edema
	72	13.4	11.3–16.1	7.6	5.1–11.2		edema
	96	12.8	10.8–15.6	8.5	6.4–12.0	1.4	edema
$ZnSO_4 * 7\ H_2O$	96	56.4	47.4–68.7				
Cu	24	1.3	0.6–2.0	>3.6			developmental delay[b]
	48	0.9	0.3–1.3	>3.6			developmental delay
	72	0.9	0.3–1.3	0.4	0.4-0.5		developmental delay
	96	0.9	0.3–1.3	0.4	0.4-0.5	<0.2	developmental delay
$CuCl_2 * 2\ H_2O$	48	2.4	1–3.7				
	96	2.3	0.8–3.6				
Se	24	4.1					no detectable effect
	48	4.1					no detectable effect
	72	2.2					no detectable effect
	96	1.9					no detectable effect
$NaSeO_3 * 5\ H_2O$	96	6.2					

[a] Based on sublethal effects observed at 96 hours.
[b] Apart from developmental delay at 72 and 96 hours, copper provoked and abnormal pigmentation (whitish) in all tadpoles at all tested concentrations ($EC_{50} < 0.22$ mg/L).

larvae stage of *X. laevis*, a not commonly used age stage, to compare the effects of acute exposures caused by biologically active substances with data obtained from FETAX or fish assays.

Concerning sensitivity to metals and organic contaminants to facilitate their use as bioindicators of pollution stress, early-life-stage toxicity tests were used by Birge [18] to classify 25 amphibian species as very sensitive, sensitive, moderately tolerant, or tolerant in comparison with the rainbow trout (*Oncorhynchus mykiss*), a sensitive benchmark species commonly used in toxicity criterion development. *Xenopus laevis* resulted to be one of the most tolerant species. Nevertheless, in the present study, *X. laevis* was selected as animal model for amphibians, since it is cultured and handled easily in laboratory setting, and there is a relatively wide knowledge in its developmental biology [10]. Combining data for all taxa studied by Birge [18] (on Table 3 were reported the most interesting LC_{50} values), based on 573 point-to-point comparisons between amphibian and fish LC_{50} values, amphibians were more sensitive than fishes in 386 (67%) of 573 cases. Table 3 shows the differences in metal sensitivity among selected amphibian species, as well as fish. Comparisons against Table 3 and the results of the current study (Table 2) demonstrate that *X. laevis* was not always the most tolerant species. For example, *X. laevis* was more sensitive than largemouth bass (*Micropterus salmoides*) to lethal effects of Se, but was in the same range of sensitivity than goldfish (*Carassius auratus*) and rainbow trout. In the same way,

Table 3: 96 h lethal concentration in the 50% of the cases (LC_{50}, mg/L) for early-life-stage amphibians and fish exposed to copper (Cu), zinc (Zn), selenium (Se) and manganese (Mn). (Source: [18]).

Species	LC_{50}			
	Cu	Zn	Se	Mn
Rana catesbeiana	0.02	0.08	0.07	
Gastrophryne carolinensis	0.02	0.01	0.09	1.42
Rana palustris	0.02	0.08	0.07	
Rana pipiens	0.05	0.05	0.14	318
Pseudacris crucifer	0.05			
Ambystoma barbouri	0.25	0.56		
Ambystoma jeffersonianum	0.37	1.00		
Ambystoma texanum	0.38	1.08		
Ambystoma maculatum	0.48	1.15		
Ambystoma tigrinum	0.50	2.00		
Ambystoma opacum	1.63	2.31		
Bufo fowleri	27.0	87.0		19.80
Oncorhynchus mykiss	0.09	1.06	5.17	2.91
Micropterus salmoides	6.68	5.18	114	25.60
Carassius auratus	5.20	2.52	8.91	10.40
Ictalurus punctatus	6.62	0.24	0.24	

X. laevis was more sensitive than fish (except for O. mykiss), and other amphibians (Ambystoma opacum and Bufo fowleri), to lethal effects of Cu. In addition, for Zn exposure, X. laevis was more sensitive than B. folweri. Moreover, considering the salt and taking into account the study of Buhl and Hamilton [19] where the 96 h LC_{50} on rainbow trout for $NaSeO_3$ was 118 mg/L, the species used in the current assay was an order of magnitude more sensitive than fish to lethal effects of $NaSeO_3$ (Table 2). On the contrary, for the same substance, there were no significant differences between the 96 h LC_{50} on gastrula stage of *X. laevis* (2.3 mg/L) [20] and the calculated value obtained in the present study for larvae (Table 2). In the same way, LC_{50}s for OTC (>100 mg/L) and $MnSO_4$ (60 mg/L) showed negligible toxicities, which were in the same range of the reported ones on rainbow trout by Office of Pesticide Programs (>116 mg/L) [21] and Davies (116 mg/L) [22], respectively. In the current study, the 48 h LC_{50} for $CuCl_2$ was 2.45, while, according to data from Office of Pesticide Programs [23], on rainbow trout it was 0.01 mg/L; thus, O. *mykiss* is clearly more sensitive than *X. laevis*. Nevertheless, in comparison with FETAX assay, the toxicity of $CuCl_2$ in *Xenopus* larva (Table 2) was close to the highest value of 96 h LC_{50} found by Buchwalter [24], which ranged between 0.042 and 1.180 mg/L. Published data for toxicity of $ZnSO_4$ exist for a variety of fish species and amphibians. For example, Alsop and Wood [25] reported a 96 h LC_{50} value of 2.615 mg/L on rainbow trout, while in *X. laevis* blastula the 96 h LC_{50} value was 3.6 mg/L [26]. Thus, the 96 h LC_{50} of 56.44 mg/L for $ZnSO_3$, obtained in the present study, showed that *X. laevis* sensitivity was lower than one order of magnitude compared with FETAX and fish assays. The ivermectin LC_{50} for *X. laevis* tadpoles was in the same range of fish and at least 100-fold less than are *Daphnia*. In fact, the 96 h LC_{50} for ivermectin on rainbow trout is 3.3 μg/L and the 48 h LC_{50} value for *D. magna* is 25 ng/L [27]. Due to ivermectin mechanism of action, *Daphnia* has been determined to be the most sensitive laboratory indicator organism [27].

The available data about acute effects in FETAX assay are generally more protective than the values found out in the current study for *X. laevis* 47 stage larvae, but previous data derived from fish assays could not be always enough protective. For example, *X. laevis* larvae exposed to $NaSeO_3$ showed a higher sensitivity than rainbow trout [19] (Table 2). In addition, the presence of no-lethal effects caused by IVE, Zn, and Cu suggested that these substances have been able to cause an organism response. For example, larvae affected by Cu were underdeveloped and colourless, while IVE impaired their locomotion and orientation. Similar effects could be problematic in natural environments by increasing the susceptibility of larvae to predation, as reported by Yuan [28] for the whitish caused by triphenyltin exposure, or reducing foraging success resulting in decreased grown and development. Changes in cognitive and psychomotor function, such as the hyperactivity induced by IVE, are commonly related to toxic neuropathy [29], while renal dysfunction, or more generally, an alter metabolism, could have caused the edema in the animals exposed to Zn (Figure 1).

Based on the studies, FETAX assay appears to be useful in ecotoxicological hazard assessment, but fish assays might be not always protective enough for amphibian. Moreover, data from several studies indicate that late-stage amphibian larvae may be more sensitive to some chemical than traditional aquatic bioindicators [30], as occurred in the present study for metals, and for those species of amphibians that spend their entire life cycle in water (e.g., Pipidae, Cryptobranchidae), larval exposure would be more accurate than FETAX assay [18]. It is necessary to highlight the need to study and prevent amphibian species. The presence of sublethal effects caused by different compounds should be investigated considering other endpoints that may affect several physiological mechanisms in a sublethal pattern, such as immunotoxicity, or a wider range of animal larvae stages.

Disclosure

The authors do not have any financial relation with the commercial identities mentioned in the paper.

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CAPÍTULO 3

Use of a novel battery of bioassays for the biological characterisation of hazardous wastes.

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Resumen

En el presente estudio, se utilizan cuatro bioensayos de toxicidad para la caracterización biológica de nueve residuos y extractos peligrosos. La evaluación de dichos residuos incluye ensayos biológicos convencionales y novedosos, y combina pruebas *in vivo* e *in vitro* con el fin de facilitar la caracterización de los efectos. Esta batería de pruebas incorpora diferentes grupos taxonómicos relevantes para el medio acuático y utiliza varios parámetros ecotoxicológicos. Los bioensayos de toxicidad que se han utilizado para esta caracterización son el ensayo de inmovilización aguda en *Daphnia*, y el ensayo de toxicidad aguda con larvas de *Xenopus laevis*, además de una prueba *in vitro* con la línea celular de peces RTG-2 para el estudio de la defensa y la viabilidad celular, y el ensayo DR-CALUXs para detectar compuestos similares a las dioxinas. El objetivo de este estudio es contribuir al desarrollo de una batería de costo-efectividad de las pruebas de toxicidad aguda para la selección de residuos tóxicos y peligrosos para el medio acuático.

Para lograr dicho objetivo se han estudiado las correlaciones entre los datos de toxicidad resultantes de todos los bioensayos utilizando un análisis multivariante, incluyendo el análisis de componentes principales. Los resultados han demostrado que los ensayos con *Daphnia* y *Xenopus* son eficaces para detectar la toxicidad y se podrían incorporar a una batería para las primeras fases de caracterización de los residuos y extractos peligrosos. Por otra parte, la prueba *in vitro* RTG-2 podría ser una buena alternativa a los ensayos *in vivo*, ya que demuestra una sensibilidad aceptable para la detección de toxicidad y aporta otras ventajas como la reducción de los costes y el uso de animales. Finalmente, el ensayo DR-CALUXs implementa las pruebas de las baterías para el rastreo de los residuos peligrosos cuando existe una sospecha de presencia de dioxinas en las muestras.

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Use of a novel battery of bioassays for the biological characterisation of hazardous wastes

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ABSTRACT

Four toxicity bioassays were used for the biological characterisation of nine hazardous wastes and extracts. This evaluation included conventional and novel bioassays, and combined *in vivo* and *in vitro* tests in order to facilitate the effect characterisation. This test battery incorporated different relevant taxonomic groups for the aquatic compartment and covered several ecotoxicological endpoints. The toxicity bioassays used for this characterisation were the acute immobilisation daphnia test, an acute toxicity test with larvae of *Xenopus laevis*, an *in vitro* test with the fish cell line RTG-2 comprising endpoints for cellular defence and viability, and finally the DR-CALUX® assay to detect dioxin-like compounds. The aim of this study is to contribute to the development of a cost-effective battery of toxicity tests for the acute screening of hazardous and toxic wastes for the aquatic compartment. For this objective, the correlations between toxicity data derived from all bioassay were studied using a multivariate analysis, including the Principal Component Analysis. The results showed that Daphnia and Xenopus were effective assays to detect toxicity and they could be incorporated to a screening test battery. On the other hand, the toxicity results with the *in vitro* test RTG-2 showed that this test could be a good alternative to *in vivo* tests, demonstrating an acceptable sensitivity for toxicity detection and contributing other advantages as reducing assays cost and animal testing. Finally DR-CALUX® test implemented the tests-batteries in the screening of hazardous wastes when there is a suspicious that dioxin-like compounds are presented in the samples.

1. Introduction

The management of toxic and hazardous wastes is considered an environmental matter of concern. The framework of the European Union, Directive 2006/12/EC (EC, 2006a), and more specifically the Hazardous Waste Council Directive (HWD) 91/689/EEC (EC, 1991) set the rules for the management, recovery and correct disposal of these wastes, considering, as an essential objective, the protection of the human health and the environment along the whole process.

The HWD has established, in its Annex I, different categories of wastes according to its nature or the activity which generated them. In order to characterise these wastes as hazardous, these residues must display any of the 14 properties established in Annex III of the aforementioned directive, that include, between others, physico-chemicals properties (i.e., if waste is considered explosive or highly flammable, etc.), toxicological (i.e., if wastes are carcinogenic, toxic, etc.) and, for an environmental point of view, "ecotoxic" property (substances and preparations which present or may present immediate or delayed risks for one or more sectors of the environment). This last category is defined in the HWD as Hazard 14 (H14) property.

For the attribution of the hazard 14 "ecotoxic" property, a chemical and biological approach can be used (Wilke et al., 2008); however, the chemical characterisation of wastes, in the majority of the cases, is not a practical approach, due to these residues are complex mixtures of chemicals or come from different waste management procedures; for that reason, ecotoxicological testing may be a complementary pragmatic approach to the chemical characterisation for the management of complex wastes (CEN, 2006; Ferrari et al., 2006; Leitgib et al., 2007; Wilke et al., 2008). Although ecotoxicological assessment of wastes is a regulatory requirement in Europe, unfortunately, for the evaluation of the H14 property, procedures have not been yet validated (Vaajasaari et al., 2004; Pandard et al., 2006).

In the literature, the examples of proposed tests-battery for the biological characterisation of different wastes are very varied (Rojíčková-Padrtová et al., 1998; Manusadžianas et al., 2003; Ren and Frymier, 2003; Pandard et al., 2006). The criteria for selecting bioassays that would be part of the test battery usually consider

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the incorporation of organisms that represent different trophic levels, the use of species that are easy to handle under laboratory conditions, tests that would involve a high cost efficiency and more recently, attempts for reducing the animal testing for ethical reasons (Devillers et al., 1988; Manusadžianas et al., 2003; Pandard et al., 2006).

The aim of this study was to contribute to the development of a cost-effective battery of toxicity tests for the first screening of hazardous and toxic wastes for the aquatic compartment, without taking into consideration the chemical characterisation of the samples. For this purpose, wastes from different nature and both organic and aqueous extracts of some of the samples were tested on four different bioassays that contributed at different levels to the biological characterisation of the hazardous wastes. The proposed battery in this work included, as reference, the acute *Daphnia magna* test, the most common toxicity test worldwide used for hazard and risk evaluation in aquatic ecotoxicology; but also comprised a novel acute toxicity test on the anuran *Xenopus laevis*, for generating ecotoxicological information in other taxonomic groups rarely considered up to now. In addition to these tests, the battery also incorporated an *in vitro* assay with the Rainbow trout cell line RTG-2, that evaluated cellular viability and cellular defence for exposure to hazardous wastes using different endpoints in the same test. Finally, a specific *in vitro* assay for Ah receptor active compounds (DR-CALUX®) was carried out in order to demonstrate the presence of Dioxin-like compounds in the wastes.

The incorporation of these very different assays in the test battery was carried out to demonstrate, among other reasons, the suitability of using *in vitro* versus *in vivo* test for the first screening of hazardous wastes. In addition, the ecotoxicological information obtained from the use of different bioassays also permitted to evaluate which assays could be incorporated in an efficiency test battery for the screening of the hazardous and toxic wastes in the waste management programmes.

The final recommendations of which tests should be considered were performed with a multivariate analysis using all the endpoints separately, trying to avoid the selection of assays with redundant ecotoxicological information.

2. Material and methods

2.1. Samples

For this work, six solid and three liquid toxic and hazardous residues were assayed with the novel battery. All wastes were supplied by representative waste management companies. A brief description of the samples used in this study is presented in Table 1. The samples were transported to the laboratory in glass containers (liquid samples) and plastic containers (solid samples), where they were stored at 4 °C in the dark for a maximum period of 2 weeks until they were used.

Considering their nature, the activity which generated them and/or their properties, these wastes should be classified as categories 5 (residue from substances employed as solvents), 14 (laboratory residues), 22 (ashes and/or cinder), 23 (soil, sand, clay including dredging spoils) following the criteria of Annex IA of the HWD (EC, 1991).

To assess the ecotoxic property (H14) of the samples, different methodologies were employed.

Due to the hydrophobic properties of the wastes, for Daphnia, Xenopus and the *in vitro* RTG-2 assay, liquid wastes were incorporated to the test mediums using a non-ionic emulsifier (Cremophor RH 40®, CAS no. 61788-85-0 BASF Aktiengesellschaft) at a maximum accepted concentration of 100 mg/L, as it was established by the former OECD protocol for Daphnia (OECD, 1984). For DR-CALUX® assay, no emulsifier was used.

In addition, liquid–liquid extraction of non-aqueous liquid samples was also performed in order to test the toxicity of the most polar components of the wastes. Fifty millilitres of liquid waste was extracted with 450 mL of the corresponding test medium; after phase separation, with the help of centrifugation (10 min, 2013 g in 50 mL vessels), several dilutions of these extracts were assayed in the test battery.

Solid wastes were extracted with the corresponding test medium (10 g/160 mL ratio) by mechanical stirring, at room temperature (20±2 °C) for 24 h. Then, extracts were centrifuged (5 min, 2013 g) and filtered through a 0.45 μm polyamide membrane filter. pH was determined before and after extraction. This procedure followed the leaching protocol established in the Spanish legislation (MOPU, 1989). After sample preparation, toxicity tests were performed within the following 24 h.

Organic extraction with dichloromethane was also carried out for solid wastes. One gram of waste was extracted with 25 mL of dichloromethane in an Accelerated Solvent Extractor (ASE 200, Dionex) and 5 mL of this extract was dried by vacuum using an evaporator (Genevac, 40 °C). With the help of 25 mL of tests medium (reconstituted water or Fetax) and sonication, dried residues were re-dissolved with the corresponding media until it reached the final test volume.

A summary of the applied methodology is presented in Fig. 1.

2.2. Ecotoxicity test

2.2.1. Daphnia magna toxicity test

Daphnia acute immobilisation test was conducted following the methodology described in ISO guidelines (ISO, 1996) with some modifications. For each sample (raw wastes or samples extractions) five serial dilutions were tested in duplicate, with *D. magna* Straus. Control medium and control with the maximum concentration of Cremophor RH 40® were carried out in parallel. Dahpnids unable to swim for 15 s after gentle stirring were considered as immobile. Toxicity tests were run in a thermostatised chamber (20±1 °C) with a 16-h light/8-h dark photoperiod. Waste toxicity was expressed as percent dilution able to produce an effect of 50% (ED_{50}). For all the samples, range finding tests were carried out prior to the ED_{50} determinations.

2.2.2. Xenopus laevis acute toxicity test

A 96-h acute toxicity test on Xenopus was developed in our laboratory. The endpoint of this test was mortality. Larvae at stage 47 (system of Nieuwkoop and Faber, 1994) were obtained from sexually mature animals reared in the laboratory. The assay was designed to test three replicates of each sample (raw waste or extracts), plus control medium (Fetax saline solution) (see ASTM, 1998 for composition) and, if necessary, a solvent or emulsifier control. Five serial dilutions of the test samples were assayed in a final volume of 100 mL of Fetax containing 10 larvae per replica. Test beakers were placed at 22±1 °C in a water bath with 12:12 h light:dark photoperiod. Waste toxicity was expressed as percent dilution able to produce a mortality of 50% (LD_{50}). For all samples, range finding tests were carried out previously to LD_{50} determinations. The acceptability criterion for the test was 90% survival in the control after 96 h.

2.2.3. Cytotoxicity test with fish cell lines RTG-2

The permanent fish cell line RTG-2 (ATCC CCL55) was grown at 20 °C in an incubator and propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum, L-glutamine, non-essential amino

Table 1
Description of the used hazardous wastes.

Sample code	Main matrix	Sample description
LR-1	Halogenated solvent	Liquid residue from industries and laboratories composed of a mixture of halogenated solvents
LR-2	Non-halogenated solvent	Liquid residue from industries and laboratories composed of a mixture of non-halogenated solvents
LR-3	Aqueous solution	Liquid residue from industries and laboratories composed of a mixture of aqueous solutions
SR-1	Sepiolite	Solid residue from industry and laboratories
SR-2	Mixture of solid wastes	Solid residue from the cosmetic industry
SR-3	Silica	Solid residue constitute of contaminated silica
SR-4	Sawdust	Solid residue formed of sawdust
SR-5	Ashes	Solid residue from an incinerator
SR-6	Soil	Contaminated soil

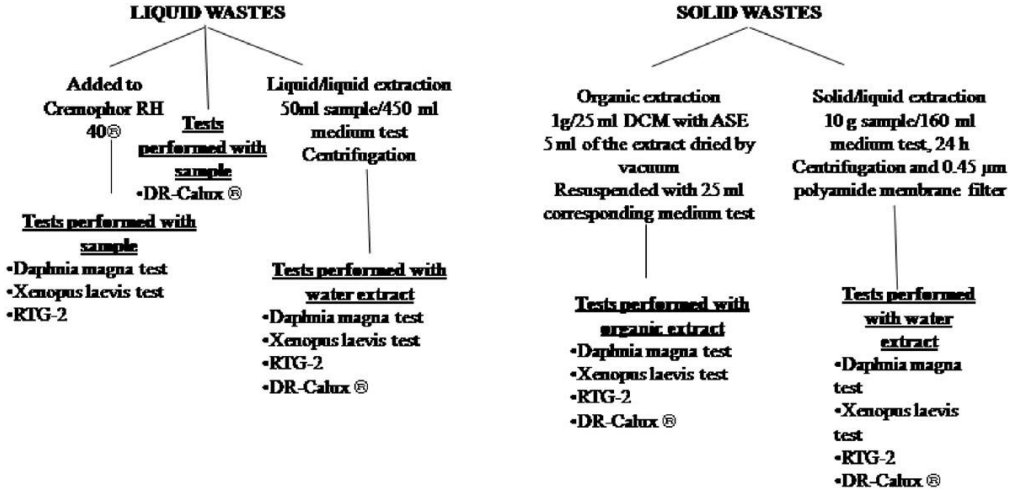

Fig. 1. Methodology applied for ecotoxicity estimation of wastes.

acids and penicillin–streptomycin. RTG-2 cells in exponential growth phase were plated at a density of 2.5×10^4 cells/well in 96-well tissue culture plates. After 24 h of attachment, culture medium was removed and samples and controls were incubated for a further 24 h.

The citotoxicity assay was conducted according to Babín and Tarazona (2005). Samples or their extracts were incorporated to the 10× concentrated EMEM media using serial dilutions up to 75% of the original sample. Untreated medium controls, consisting of water reconstituted 10× concentrated EMEM media, were included in the assay.

Cell viability was quantified using the neutral red assay (NR) (Borenfreund and Puerner, 1985). Presence of co-planar organic toxic chemicals was indicated by the stimulation of cytochrome CYP1A, which was measured as 7-ethoxyresorufin-O-deethylase (EROD) activity (Babín and Tarazona, 2005). Cellular defence was predicted by a beta-galactosidase (β-gal) assay (Babín and Tarazona, 2005). The TECAN-Genius spectrofluorometer was used to quantify fluorescence (kinetic way) and absorbance endpoints.

2.3. DR-CALUX® assay

The DR-CALUX® bioassay performed in this study is based on the use of a rat hepatoma (H4IIE) cell line transfected with a construct containing the luciferase reporter gene under direct control of dioxin responsive elements (DRE) (BioDetection Systems, Amsterdam, The Netherlands). Cell culture was performed as described (Boronat et al., 2007). After 24 h of exposure to the samples the luminescence emitted by the cells was quantified by means of the Steady-Lite system (Perkin Elmer, Shelton, CT, USA) in a MicroBeta luminescence counter (Perkin Elmer).

Liquid samples were added directly to the culture medium at a maximal dilution of 0.4%. Solid samples were extracted following the same procedure as the other assays. After that, medium was sterilised by filtration using 0.24 μm filters and maintained at −20 °C until use.

2.4. Data analysis

With the exception of the DR-CALUX® test, LC50s or EC50s and the 95% confidence intervals were calculated by log-probit regression, performed with Statgraphics version 5.1 (Statistical Graphics Corp.). For all the bioassays, toxicity endpoints were expressed as mL/100 mL medium.

In the DR-CALUX® system, exposure of the cells to each sample was performed in four independent experiments. Statistical analysis was performed using the Sigma Stat software (Jandel Scientific). This program automatically tests the normality of the data using Kolmogorov–Smirnoff test, and the equality of variance by checking the variability about the group means. Since data were not homoscedastic, with the exception of SR-6 sample, comparisons between means of exposed cells and those of control cells were carried out using Friedman's one-way repeated measurements analysis of variance on ranks followed by Dunnett's test. The effective concentration for which the 50% of the maximal response was detected (EC50) was calculated using Sigma Plot version 8.0 (Jandel Scientific) by fitting the DR-CALUX® data (one value for each concentration and assay, i.e. a total of four values for each concentration point) to a regression model equation for a four-parameter logistic curve as previously detailed (Casado et al., 2006).

For all bioassays, toxicity data expressed as 50% effect values (in % of dilution) were converted to toxic units (TU) based on the formula of Sprague and Ramsay (1965): $TU = [1/LC(E)_{50}] \times 100$. Based on the criteria of Manusadžianas et al. (2003), when no effect could be estimated or this effect was lower than 20%, toxicity units were considered as 0 values. Following the same work, toxicity data (expressed as toxic units) were classified in seven different categories corresponding to different toxic units intervals which come from 0 to >100, in the following ranges: category 1 (from 0 to ⩽0.4), category 2 (from >0.4 to ⩽1), category 3 (from >1 to ⩽3), category 4 (from >3 to ⩽10), category 5 (from >10 to ⩽30), category 6 (from >30 to ⩽100), and category 7 (>100).

In order to propose a pragmatic battery test for a rapid and cost-effective screening of hazardous and toxic wastes, a multivariate analysis, the Principal Component Analysis (PCA), with all the parameters obtained from the four bioassays was performed using Statgraphics version 5.1 (Statistical Graphics Corp.). The purpose of the analysis is to obtain a small number of linear combinations of the variables which account for most of the variability in the data, obtaining the principal components (PCs). PCA was carried out on the data expressed as toxic units and converted into log 10. Toxic values of 0 were arbitrarily considered as 0.2 for the log transformation, following the recommendation of Manusadžianas et al. (2003).

3. Results

The objective of this study was the biological characterisation of complex mixtures considered as hazardous and toxic wastes with different toxicity test without a previous chemical characterisation of the samples. A summary of the raw toxicity results is presented in Table 2. In general, these data show that the selected test battery had the capacity to detect toxic effects in complex samples in which a chemical characterisation has not been developed.

Compared to other tests, the standard assay *D. magna* showed toxicity in the majority of the samples (Table 3), confirming that this assay is a good indicator of general toxicity. Examining the distribution of the toxicity in different categories based on the ranges of toxic units (shown in Fig. 2), we can point out that Daphnia test was also capable to detect, to a greater or lesser extent, toxicity for almost all the categories in which the toxicity was classified.

Sixty percent of the samples tested with *X. laevis* assay showed toxicity. For some of these samples, Xenopus presented the capacity to identify the highest toxicity compared with other tests, as it is shown by the greatest percentage of samples

Table 2
Summary of the toxicity results for each bioassay (LC_{50} or EC_{50} expressed as ml sample100 ml^{-1} medium).

No.	Sample	Acute Daphnia test			Acute Xenopus test			RTG-2 cellular viability test			RTG-2 EROD activity			RTG-2 β-gal activity			DR-Calux® test[a]		
		EC50 (IC 95%)	TU	Tox. rank.[b]	LC50 (IC 95%)	TU	Tox. rank.[b]	EC50 (IC 95%)	TU	Tox. rank.[b]	EC50 (IC 95%)	TU	Tox. rank.[b]	EC50 (IC 95%)	TU	Tox. rank.[b]	EC50 (IC 95%)	TU	Tox. rank.[b]
1	LR-1	0.023 (0.021–0.025)	4348	7	0.028 (0.024–0.033)	3571	7	0.82 (0.59–1.31)	122	7	0.53[c] (0.36–5.1)	189	7	5.43[c] (3.3–34.3)	18.4	5	No effect	0	1
2	LR-1 (A)	7.52 (7.28–7.8)	13.29	5	4.11 (2.54–8.68)	24.33	5	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1
3	LR-2	0.024 (0.023–0.026)	4167	7	0.008 (0.006–0.01)	12,500	7	0.17 (0.072–0.719)	585	7	1.12	89.3	6	0.45	222	7	0.0092 (0.0072–0.012)	10,989	7
4	LR-2 (A)	4.11 (4.1–4.13)	24.33	5	1.27	78.74	6	40.99 (27.79–71.34)	2.43	3	No effect	0	1	No effect	0	1	0.0092 (0.0072–0.012)	10,989	7
5	LR-3	0.09 (0.07–0.107)	1111	7	0.045 (0.031–0.061)	2222	7	0.68 (0.42–1.45)	147	7	1.9	52.6	6	1.25	80	6	0.020 (0.014–0.029)	5050	7
6	LR-3 (A)	0.0258 (0.0257–0.0259)	3876	7	0.059 (0.049–0.079)	1695	7	4.55 (2.48–11.72)	22	5	No effect	0	1	No effect	0	1	0.020 (0.014–0.029)	5050	7
7	SR-1 (A)	10.82 (10.19–11.49)	9.24	4	0.55 (0.5–0.61)	182	7	3.30 (2.07–7.79)	30.3	6	No effect	0	1	No effect	0	1	No effect	0	1
8	SR-1 (O)	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1
9	SR-2 (A)	0.21 (0.18–0.24)	476.2	7	0.75 (0.55–1.1)	133	7	3.26 (2.43–4.92)	30.7	6	6.83[c] (4.43–15.57)	14.64	5	6.95[c] (4.35–19)	14.38	5	0.26 (0.13–0.56)	389	7
10	SR-2 (O)	50	2	3	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1
11	SR-3 (A)	4.9 (4.42–5.53)	20.40	5	>30	0	1	No effect	0	1	No effect	0	1	No effect	0	1	1.03 (0.61–1.75)	96.6	6
12	SR-3 (O)	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1
13	SR-4 (A)	0.048 (0.048–0.054)	2033	7	0.255 (0.23–0.3)	392	7	25.57	3.91	4	No effect	0	1	No effect	0	1	No effect	0	1
14	SR-5 (A)	1.65 (1.57–1.74)	60.6	6	>80	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1
15	SR-6 (A)	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	No effect	0	1	0.54 (0.30–0.96)	184	7

(A), aqueous extract.
(O), organic extract.
[a] For liquid samples, data of raw wastes and aqueous extracts are the same due to wastes which were added to the culture medium without an emulsifier.
[b] Toxicity ranking according to Manusadžianas et al. (2003).
[c] Inhibition of the activity.

classified as category 7 (highest toxicity) with this test; but unlike Daphnia, Xenopus test also presented a high percentage of samples with no toxicity. This might suggest that Xenopus could be very selective in their potential to detect toxicity for some compounds presented in the complex mixtures studied in this work.

The *in vitro* test with the fish cell line RTG-2, especially EROD activity and β-gal endpoints, did not show toxic effects for more than 70% of the tested samples; however, considering only neutral red (NR) as an endpoint for cytotoxicity, the capacity for detecting toxicity in the hazardous and toxic wastes was higher than the two other endpoints contemplated in the same assay (53.3% of positive responses versus 26.6% for EROD and β-gal activity).

DR-CALUX® assay detect toxicity in around 50% of the samples. This assay is determining the activation of the aryl hydrocarbon receptor (AhR) by dioxin-like compounds in mammal's cell line. Therefore, this assay is considered very specific for xenobiotics that bind to this ligand.

The Principal Component Analysis using the log 10 transformation of TUs separated all variables (endpoints) considered in the four assays in two different components; they account for 81.72% of the variability of the original data. The graphical representation (Fig. 3) reveals three different clusters which are Daphnia/Xenopus/NR, inhibition of the EROD activity/inhibition of the β-gal activity and finally stimulation of the EROD activity/stimulation of the β-gal activity/DR-CALUX®.

4. Discussion

In the literature, many attempts have been made in order to biologically characterise hazardous wastes (Rojíčková-Padrtová R., et al., 1998; Manusadžianas et al., 2003; Pandard et al., 2006; Wilke et al., 2008). These works include acute and chronic assays for both aquatic and terrestrial compartments. However, in our work, the aim is to improve the knowledge about how different assays could be used on the acute hazard identification of wastes for the aquatic compartment. This is why chronic assays or terrestrial organisms have not been contemplated. The aquatic compartment is usually considered the final receptor of contaminants in areas where contamination is a matter of concern (Antunes et al., 2007).

New alternative bioassays have been taken into consideration. For this reason, tests that are part of this battery are not the typical recommended assays (such as Microtox® assay, algal growth inhibition assay, etc.).

This study did not try either to develop a complete methodology for the ecological risk assessment of wastes such as other works did (Babut et al., 2002; Perrodin et al., 2006; Casado-Martínez et al., 2007; Irha and Blinova, 2007). In that case, the approach must include the description of exposure scenarios and a complete characterisation for both terrestrial and aquatic organisms (including sediment organisms). Therefore, our work would be considered as a first approach for the hazard characterisation of wastes in order to classify them as hazardous, within the waste management procedures.

Daphnia was selected as a standard test; it is a conventional assay being part of regular batteries of ecotoxicity tests, and its sensitivity to toxic compounds is well-known. The toxicity results with this test confirm these affirmations; this assay demonstrated the highest percentage of positive responses for toxicity.

The incorporation of Xenopus to the test battery contributed to create a better characterisation of samples with a mixture of unknown compounds, offering extra information about other

Table 3
Percentage of samples for which toxicity was detected, and % of samples showing toxicity for *D.magna* but not for the test.

Assays	% Positive responses[a]	% Toxic samples for Daphnia and not for other assays
Daphnia test	80	–
Xenopus test	60	20
Neutral red assay	53.3	26.6
EROD assay	26.6	53.3
β-gal assay	26.6	53.3
DR-CALUX®	46.6	40

[a] Percentage of samples that showed toxicity for this assay.

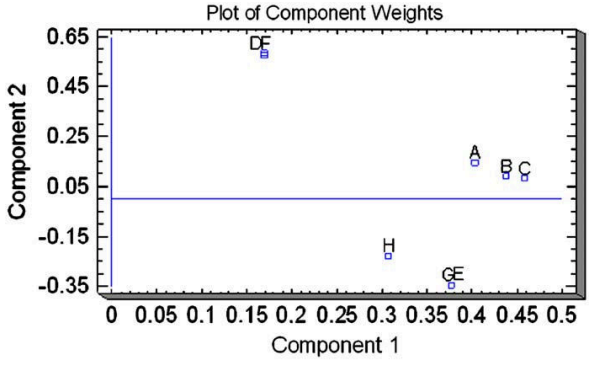

Fig. 3. Plot of component weights. A = Daphnia, B = Xenopus, C = neutral red, D = inhibition EROD activity, E = stimulation EROD activity, F = inhibition β-Gal activity, G = stimulation β-gal activity, H = induction DR-Calux®.

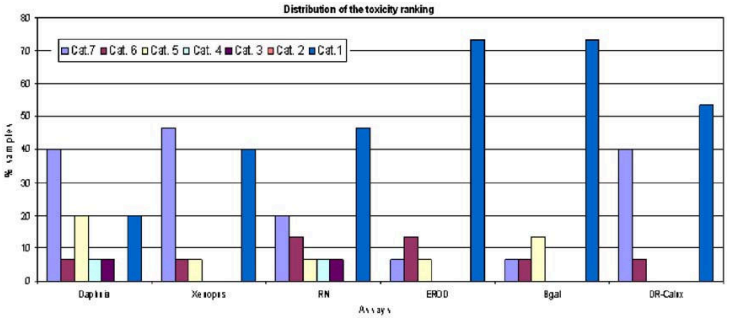

Fig. 2. Toxicity distribution of the samples based on the categories described by Manusadžianas et al. (2003). Cat 7: TU > 100; Cat 1: TU 0– < 0.4.

taxonomic group, such as amphibians, not well considered up to now in ecotoxicology. Xenopus test can also offer the possibility to detect not only mortality, but also abnormalities that could occur in short-term exposures (such as edema, axial malformations, etc.).

For the assessment of long-term effects, Xenopus has been previously incorporated to different test batteries for the detection of genotoxic effects (Perrodin et al., 2006). In addition, Xenopus could also be useful if specific mechanisms of action of the wastes want to be investigated (i.e. the identification of chemical disruption at hypothalamus–pituitary–thyroid axis level) (Opitz et al., 2005).

In this study Xenopus test has demonstrated the highest selectivity for certain compounds presented in these complex mixtures, in some cases up to one order of magnitude with respect to Daphnia test. The evidence of different responses to different types of toxicants justifies the inclusion of multiple assays. Although in some cases this information could seem redundant (i.e. Daphnia and Xenopus assays), the highest selectivity of the response exhibited in this work for the Xenopus assay could support the inclusion of another generic assay for a different taxonomic group.

The incorporation of an *in vitro* assay with RTG-2 tries to demonstrate the suitability of using alternative methods for the biological characterisation of hazardous wastes. The use of *in vitro* assays is currently promoted in the new EC policy on the registration, evaluation and authorisation of chemicals (REACH) following the intelligent testing strategies (EC, 2006b).

The origin of the samples tested in this work strongly suspected the existence of dioxin and dioxin-like compounds. This is the reason that DR-CALUX® assay was incorporated to the test battery. The existence of "specialised test" for the detection of specific families of pollutants, such is this case for dioxin-like compounds, offers new possibilities for the implementation of the screening methodologies for hazardous wastes. As new specialised assays are developed, they can be incorporated to the test batteries.

Comparing *in vivo* and *in vitro* assays, the conclusion is that in general, *in vivo* tests tend to offer a better performance in terms of capacity for detecting toxicity. The low sensitivity of fish cell line in relation with *in vivo* is widely known (Castaño et al., 1996); however in this case, the capacity of NR assay to detect toxic effects was reasonably similar to Daphnia and Xenopus test, but not EROD and β-gal activity. This conclusion was confirmed by the interpretation of the PCA that indicated that the *in vivo* assays and the NR endpoint were clearly related, denoting their capacity for detecting unspecific toxicity of the wastes. On the other hand, although a less sensitivity could be observed for RTG-2 cytotoxicity test comparing with generic viability endpoints (such as mortality), this kind of assays based on enzymatic activities offers an improved selectivity, particularly for assays that analyses simultaneously the potential for stimulation and for inhibition that is the case of the RTG-2 cytotoxicity test presented in this work.

PCA also revealed a cluster for stimulation of the EROD activity/stimulation of the β-gal activity/DR-CALUX®. The demonstrated relationship between the induction of the enzymatic activity EROD and DR-CALUX® assay is expected. DR-CALUX® is a test developed to detect dioxins and dioxin-like compounds by a binding at the AhR receptor (Murk et al., 1996). On the other hand, EROD activity is used as a biomarker for Cytochrome P450-1A1 activity (CYP1A1), whose induction is associated to exposures of polycyclic aromatic compounds (PAHs) (Billiard et al., 2004), polychlorinated binephyls (PCBs) (Eljarrat and Barceló, 2003), dioxins (Hahn, 2002), and so on. Many of these compounds also elicit relatively strong AhR activation in both mammalian and fish cellular models (Vondráček et al., 2004). The correlation between the two assays has been studied elsewhere (Behnisch et al., 2002). In our study, DR-CALUX® demonstrated a better capacity for toxicity detection than induction of EROD activity.

With regard to β-gal activity, this parameter has been considered as a sublethal stress indicator *in vitro* (Pablos et al., 1998; Babín et al., 2005). Its sensitivity has been proved for metals (Jung et al., 1996), complex mixtures (Babín et al., 2001), pesticides (Babín and Tarazona, 2005), and antimicrobials (Babín et al., 2005). In our work, the toxic response of this endpoint to wastes exposure was not very sensitive, and in all the cases was totally correlated to EROD activity, both for stimulation and inhibition. This could confirm that inclusion of EROD activity into the RTG-2 assay is not only used for addressing CYP1A1 activity, but also could be included just as a cost-effective and sensitive metabolic endpoint.

Among the enzymatic assays, DR-CALUX® offered the highest sensitivity but EROD and β-gal activity offered the added value of detecting both stimulation and inhibition, contributing to improve the biological characterisation of the wastes.

5. Conclusions

This approach has tried to explore new alternatives to be used on the biological characterisation of hazardous wastes, incorporating the ecotoxicological information of taxonomic groups not currently used up to now and using *in vitro* methodologies.

Both Daphnia and Xenopus assays were effective assays to detect toxicity in the samples (Daphnia for the great majority of the samples, Xenopus was the most sensitive assay in several occasions), demonstrated that Xenopus could be a good option to be part of any test battery. The incorporation of both assays with very similar toxic effects could create a dilemma, such as it has been established in other works, if it would be useful to increase the number of chosen assays for a test battery or on the contrary, whether it should be limited to the assays in the test batteries, reducing costs, which on the other hand, it will increase the uncertainty related to the ecotoxicological evaluation of these samples (Perrodin et al., 2006).

The other possibility, the cytotoxicity test with the fish cell line RTG-2, could be considered a good option for the assessment of the H14 property of hazardous and toxic wastes for the aquatic compartment. This assay that includes three different endpoints (cellular viability, cellular defence, and presence of co-planar compounds), will cover not only the unspecific toxicity of these complex mixtures, but also will allow detecting the effects of classes of compounds of concern as dioxins, furans and so on in a cost-effective way, and it will reduce the use of animal testing for the pre-screening of these samples. Comparing with other screening tests considered rapid and cost-effective (such as Microtox® test with *Vibrio fischeri*), this *in vitro* test would contribute more and very useful information for the hazard assessment of wastes.

Finally, the existence of "specialised assays" for specific families of pollutants (as DR-CALUX® is) open new possibilities in the screening of hazardous wastes when there is a suspicious that dioxin-like compounds are presented in the samples.

In any case, and in order to develop a risk characterisation of the management of these hazardous wastes, the two possibilities presented in this study (*in vivo* assays versus *in vitro* assays, both complemented with the specific assay DR-CALUX®) should be improved with the incorporation of other assays representing different taxonomic groups in the aquatic (i.e, primary producers and sediment organisms) and terrestrial compartment.

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CAPÍTULO 4

Correlation between physicochemical and ecotoxicological approaches to estimate landfill leachates toxicity.

Pablos MV, Martini F, Fernández C, Babín MM, Herraez I, Miranda J, Martínez J, Carbonell G, San-Segundo L, García-Hortigüela P, Tarazona JV.

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Resumen

Los lixiviados de los vertederos de residuos sólidos urbanos (RSU) pueden contener una gran variedad de contaminantes; estas aguas residuales deben ser consideradas como mezclas complejas potencialmente peligrosas y un potencial de riesgo ambiental para las aguas superficiales y subterráneas. La legislación actualmente vigente en tema de gestión de los RSU considera exclusivamente la caracterización físico-química sin contemplar la evaluación ecotoxicológica de los lixiviados de vertedero. Sin embargo, la presencia de sustancias altamente tóxicas en dichos residuos requiere la necesidad de reconsiderar sus posibles evaluaciones ecotoxicológicas. El objetivo principal de este estudio es evaluar la toxicidad de diferentes lixiviados que se originan de los RSU utilizando una batería de ensayos de toxicidad, incluyendo unas pruebas de toxicidad aguda con *Daphnia magna* y el anuro *Xenopus laevis*, y unas pruebas *in vitro* con la línea celular de peces RTG-2. El objetivo secundario es estudiar la posible correlación entre las propiedades físico-químicas y los resultados de toxicidad obtenidos para los lixiviados de vertederos sin tratar. Los resultados muestran que la batería de ensayos propuesta es eficaz para la caracterización ecotoxicológica de los lixiviados de vertedero de RSU. En todos los ensayos de toxicidad se detecta una correlación variable, entre moderada y elevada, entre las propiedades fisicoquímicas y las unidades de toxicidad calculadas. Se han encontrado factores de 0,85, 0,86 y 0,55 respectivamente para *Daphnia*, *Xenopus* y el ensayo RTG-2. El análisis discriminante muestra que ciertos parámetros físico-químicos se podrían utilizar para una clasificación inicial de la toxicidad aguda potencial de los lixiviados para el medio acuático; este hallazgo puede facilitar la gestión de los lixiviados ya que la caracterización físico-química es actualmente el método más común de vigilancia empleado en la gran mayoría de los vertederos. El amoníaco, la alcalinidad y la demanda química de oxígeno (DQO), junto con cloruro, permiten una clasificación adecuada de la toxicidad de los lixiviados en hasta un 75% de las muestras analizadas, con un pequeño porcentaje de falsos negativos.

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Correlation between physicochemical and ecotoxicological approaches to estimate landfill leachates toxicity

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ABSTRACT

Leachates from municipal solid waste (MSW) landfills may contain a huge diversity of contaminants; these wastewaters should be considered as potentially hazardous complex mixtures, representing a potential environmental risk for surface and groundwater. Current MSW landfill wastes regulatory approaches deem exclusively on the physicochemical characterization and does not contemplate the ecotoxicological assessment of landfill leachates. However, the presence of highly toxic substances in consumer products requires reconsideration on the need of more specific ecotoxicological assessments. The main aim of this study was to evaluate the toxicity of different MSW landfill leachates using a battery of toxicity tests including acute toxicity tests with *Daphnia magna* and the anuran *Xenopus laevis* and the *in vitro* toxicity test with the fish cell line RTG-2. The additional objective was to study the possible correlation between physicochemical properties and the toxicity results obtained for untreated landfill leachates. The results showed that the proposed test battery was effective for the ecotoxicological characterization of MSW landfill leachates. A moderate to strong correlation between the measured physicochemical parameters and the calculated toxicity units was detected for all toxicity assays. Correlation factors of 0.85, 0.86 and 0.55 for *Daphnia*, *Xenopus* and RTG-2 tests, respectively, were found. The discriminant analysis showed that certain physicochemical parameters could be used for an initial categorization of the potential aquatic acute toxicity of leachates; this finding may facilitate leachates management as the physicochemical characterization is currently the most common or even only monitoring method employed in a large majority of landfills. Ammonia, alkalinity and chemical oxygen demand (COD), together with chloride, allowed a proper categorization of leachates toxicity for up to 75% of tested samples, with a small percentage of false negatives.

1. Introduction

Landfills are commonly used for municipal solid waste (MSW) disposal. Spain, with 350 kg of municipal solid wastes per inhabitant managed through landfills in 2007, is well above the EU average of 214 kg per inhabitant (MMA, 2008). The European Directive 1999/31/EC (EC, 1999) defines different kind of landfills (landfills for hazardous waste, landfills for non-hazardous waste and landfills for inert waste), specifies the wastes that are accepted in each landfill, establishes the control and monitoring procedures in the operational and closure phases and includes the general requirements for all classes of landfills. Regulatory requirements include the measures intended to prevent surface and groundwater contamination by landfills. The presence of hazardous substances (metals, organic and inorganic compounds) in these leachates is well documented (Clément et al., 1997; Slomczyńska and Slomczyński, 2004; Bortolotto et al., 2009; Li et al., 2009). Leachates are complex wastewaters and their release represents a risk for aquatic systems and groundwater. The toxic effects of the leachates have been evaluated using different batteries of bioassays covering different trophic levels (Clément et al., 1996; Ward et al., 2002; Bortolotto et al., 2009); all studies concluded on the high toxicity of municipal landfill leachates, deeming it equivalent to leachates from industrial landfills.

Although hazardous wastes legislation contemplates the direct ecotoxicological assessment as an option for waste characterization (CEN, 2006; Ferrari et al., 2006; Leitbig et al., 2007; Wilke et al., 2008), the EU landfill legislation (EC, 1999) does not consider this option when setting leachates sampling conditions and parameters to be measured. Potential relationships between leachate physicochemical properties and their ecotoxicity have been proposed by some authors (Clément and Merlin, 1995; Clément

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et al., 1997; Olivero-Verbel et al., 2008); in particular, the role of ammonia, heavy metals and alkalinity on the toxicity of landfill leachates were studied.

The aim of this work was to study the correlation between the physicochemical and ecotoxicological characterization in order to facilitate the eco-management of the leachates. Landfills management practices are exclusively based on physicochemical characterizations; therefore, a reliable correlation between these parameters would avoid the ecotoxicological characterization of samples with high levels of some physicochemical parameters (i.e., ammonia, alkalinity, COD and chloride), considering them as "potentially toxic", and thus following an appropriate management practices of these leachates. On the other hand, samples that do not have high levels of these physicochemical parameters should be ecotoxicologically characterize in order to demonstrate the presence or absence of toxicity.

2. Materials and methods

2.1. Samples

Twenty-one samples, untreated leachates or compost leachates, from MSW landfills were used for this study. Samples were collected from four landfill sites in the Madrid region (central Spain), from June 2007 to October 2008. Leachate samples were collected using a pump system or immersing a swing sampler in the leachate collection tanks; samples were collected in 1 L amber glass bottles and maintained at 4 °C.

After a first broad sampling campaign including five landfills, three landfills were selected based on the toxicity results. Landfill leachates or compost leachates from these sample points were regularly collected. The samples' description is presented in Table 1.

Table 1
Description of the studied MSW landfills.

Sample code	Landfill	Sample description
1	A (Active landfill)	Compost leachate
2	A (Active landfill)	Landfill cell leachate
3a	B (Closed landfill)	West Landfill cell leachate
4a	B (Closed landfill)	East Landfill cell leachate
5	C (Active solid waste treatment plant)	Compost leachate
6	D (Closed landfill)	Landfill cell leachate
7a	D (Closed landfill)	Landfill cell leachate
8	E (Active solid waste treatment plant)	Effluent from treatment plant leachate
9a	E (Active landfill)	Landfill cell leachate
10a	E (Active solid waste treatment plant)	Compost leachate
10b	E (Active solid waste treatment plant)	Compost leachate
9b	E (Active landfill)	Landfill cell leachate
7b	D (Closed landfill)	Landfill cell leachate
3b	B (Closed landfill)	West Landfill cell leachate
4b	B (Closed landfill)	East Landfill cell leachate
10c	E (Active solid waste treatment plant)	Compost leachate
3c	B (Closed landfill)	West Landfill cell leachate
4c	B (Closed landfill)	East Landfill cell leachate
10d	E (Active solid waste treatment plant)	Compost leachate
3d	B (Closed landfill)	West Landfill cell leachate
4d	B (Closed landfill)	East Landfill cell leachate

Samples with the same number are leachates from the same sampling point; subscripts a, b, c and d identify the different periods that the samples were collected throughout the year.

2.2. Physicochemical analysis

Leachate parameters (ammonium, nitrite, nitrate, phosphate, sulphate, chemical oxygen demand (COD), chlorides, electrical conductivity (EC) and alkalinity) were analysed using the standard methods for the examination of water and wastewater (Eaton et al., 1995). Levels of un-ionized ammonia were estimated from measured ionized concentrations of ammonium, considering the equilibrium between ammonia and ammonium and taking into account a Kb of 1.8×10^{-5} (Kotz et al., 2009). The used formula was:

$$NH_3 = NH_4^+ \times 10^{(pH-14)}/1.8 \times 10^{-5}.$$

2.3. Ecotoxicity tests

Samples were incorporated into the test media at serial dilutions. For *in vivo* assays, the pre-screening tests consisted of three different dilutions up to a maximum of 10% in the media; the results of these pre-screening tests were used for the establishment of the corresponding LC_{50} or EC_{50} in a screening test. In the *in vitro* assay, eight dilutions with a factor of 2 were assayed, with a maximum dilution of 74% in the medium.

2.3.1. Daphnia magna acute toxicity test

This assay was conducted in compliance with the ISO Standards (ISO, 1996) with some modifications. Toxicity tests were run in a thermostatised chamber (20 ± 1 °C) with a 16 h-light/8-h dark photoperiod, with five serial dilutions in duplicate, 20 animals per concentration. Control medium was carried out in duplicated. Waste toxicity was expressed as percent dilution able to produce an effect of 50% (Effect concentration 50 or EC_{50}).

2.3.2. Xenopus laevis acute toxicity test

The *Xenopus* acute toxicity test was developed as described in Pablos et al. (2009). Briefly, early larvae of *Xenopus* were exposed to five serial dilutions of the samples in a saline medium known as FETAX (ASTM, 1998). The endpoint of this test was mortality in a 96-h period. Four replicates (five animals per replicate) plus a control medium were incorporated. Waste toxicity was expressed as percent dilution able to produce lethality of 50% (Lethal concentration 50 or LC_{50}).

2.3.3. Cytotoxicity test with fish cell lines RTG-2

The cytotoxicity test with fish cell lines RTG-2 was conducted following the methodology proposed by Babín and Tarazona (2005). Three different endpoints were measured in the same test: neutral red assay (Borenfreund and Puerner, 1985) for cellular viability, the 7-ethoxyresorufin-*O*-deethylase (EROD) activity for presence of co-planar organic toxic chemicals (Babín and Tarazona, 2005) and cellular defence by beta-galactosidase (β-gal) assay (Babín and Tarazona, 2005). Samples were incorporated to a 10× concentrated Eagle's minimum essential medium, EMEM, (Lonza, Spain) at eight different serial dilutions during an exposure period of 24 h. Waste toxicity was expressed as percent dilution able to produce effect of 50% (Effect concentration 50 or EC_{50}).

2.4. Data analysis

Toxicity data for all bioassays were expressed as ml/100 ml medium. Both, LC_{50} and EC_{50} were estimated using a log-probit regression, with the Statgraphic Plus version 5.0 (Statistical Graphics Corp., USA). Results of the three assays were converted to toxic units (TU) following the formula of Sprague and Ramsay (1965): $TU = [1/L(E)C_{50}] \times 100$. In the case of the *in vitro* assay, the three

different endpoints measured in the test were considered independently.

The relationship between the nine physicochemical measured parameters was investigated by a principal component analysis (PCA) using the Statgraphics Plus version 5.0 (Statistical Graphics Corp., USA). The relationship between TU for each bioassay and the extracted components from PCA was studied using a simple regression analysis with the same statistical software package.

On the other hand, a discriminant analysis was also developed; with this procedure we tried to develop a model which could help to predict a toxicity classification based on physicochemical parameters. The parameters selected for this analysis were those that had a larger weight for the first component of the PCA. For the toxicological classification, four categories, based on the toxic unit ranges, were established as proposed by Lapa et al. (2002) and Wilke et al. (2008): highly toxic (TU > 100), very toxic (10 < TU < 100), toxic (1 < TU < 10) and no toxic (TU < 1).

3. Results and discussion

The toxicity results and the corresponding toxic units are shown in Table 2. *Daphnia* and *Xenopus* tests showed toxicity for the same samples, with *Xenopus* been equally or more sensitive than *Daphnia*. These results are in line with those from a previous related work on hazardous wastes (Pablos et al., 2009). Atwater et al. (1983) found significant correlation between the leachate toxicity on *D. magna* and fish.

Daphnia test was selected for the test battery as it is a standard assay currently used in monitoring programs of different kind of samples; this assay has demonstrated a good sensitivity for hazardous wastes (Pablos et al., 2009) and for municipal solid leachates (Clément et al., 1996; Isidori et al., 2003; Bortolotto et al., 2009). However, there is a recognized need for including organisms representing different trophic levels in the test batteries (Slomczyńska and Slomczyński, 2004; Thomas et al., 2009); this reason justifies the inclusion of an assay with amphibian larvae, which incorporates new ecotoxicological information on an insufficiently considered taxonomic group.

Based on previous results, the *in vitro* test, neutral red assay, was selected as a valid method for detecting toxicity (Schulz et al., 1995; Castaño et al., 1996). This test was a suitable alternative for the ecotoxicological characterization of hazardous wastes (Pablos et al., 2009); but for MSW landfill leachates, this assay showed less sensitivity compared with the *in vivo* assays. It should be noted that the β-gal endpoint could not be measured due to the background fluorescence of the samples. Nine samples showed cytotoxicity in the neutral red assay and the EROD activity showed a dose–response inhibition in only three of those samples (10a, 3b and 4b) at sublethal concentrations; a possible explanation for this could be that complex samples can cause toxicity through hundreds of undefined interactions with cellular components and processes, and this could be better detected by a non-specific assay like the neutral red. No induction of CYP1A (EROD activity) was observed in the tested samples suggesting a low content of dioxin-like planar compounds.

In general, leachates composition is highly variable, depending on several factors such as waste composition and age, precipitation rate, landfill management (Kjeldsen et al., 2002; Ward et al., 2005; Žaltauskaitė and Čypaitė, 2008; Alkassasbeh et al., 2009). As a consequence, leachates toxicity may vary for each sample collection, as observed by Olivero-Verbel et al. (2008). However, in this study samples collected at different seasons from the same sampling points showed similar toxicity levels with narrow confidence intervals, suggesting that the toxicity of leachate is maintained in the same landfill.

Although the toxicity of leachates from MSW landfill have been related to the high content of heavy metals in these samples (Clément et al., 1997; Olivero-Verbel et al., 2008; Bortolotto et al., 2009), the present study aimed to assess the leachates overall toxicity using direct measurements. It is recognized that chemical characterization may underestimate the toxicity of complex mixtures like municipal landfill leachates (Clément et al., 1996; Thomas et al., 2009); as a consequence, bioassays have been incorporated in the hazard evaluation of different wastes (Isidori et al., 2003; Slomczyńska and Slomczyński, 2004; Wilke et al., 2008; Žaltauskaitė and Čypaitė, 2008). This approach, named direct toxicity assessment (DTA), tests dilutions of complex anthropogenic or environmental samples in a set of bioassays in order to quantify the toxicity of the original sample (Wharfe, 2004; Environmental Agency, 2006). DTA offers a combine measurement for

Table 2
Summary of the toxicity results for each bioassay (LC_{50} or EC_{50} expressed as ml sample100 ml^{-1} medium) and the corresponding toxic units.

Sample	Acute *Daphnia* test			Acute *Xenopus* test			RTG-2 neutral red			RTG-2 EROD activity		
	48 h EC_{50}	CI (95%)	TU[a]	96 h LC_{50}	CI (95%)	TU[a]	24 h EC_{50}	CI (95%)	TU[a]	24 h EC_{50}	CI (95%)	TU[a]
1	>10	–	0	N.E.	–	0	>74	–	0	N.E.	–	
2	>10	–	0	N.E.	–	0	70.1	(44.4–202.4)	1.4	N.E.	–	
3a	>10	–	0	2.4	2.2–2.7	40.8	>74	–	0	N.E.	–	
4a	2.6	(2.3–11.7)	37.9	0.6	(0.5–0.7)	166.6	52.4	(35.9–91.5)	1.9	N.E.	–	
5	4.1	(3.4–5.1)	24.3	3.4	(3.1–3.6)	29.5	>74	–	0	N.E.	–	
6	>10	–	0	>10	–	0	>74	–	0	N.E.	–	
7a	>10	–	0	>10	–	0	>74	–	0	N.E.	–	
8	>10	–	0	>10	–	0	>74	–	0	N.E.	–	
9a	9.4	(7.1–13.5)	10.6	4.5	(4.2–4.9)	22.1	>74	–	0	N.E.	–	
10a	3	(2.6–6.5)	33.1	3.8	–	26.6	62.4	(44.2–115.3)	1.6	4.6	(0–10.4)	
10b	3.7	–	26.6	1.5	(1.3–1.7)	68	23.2	(18.7–30.2)	4.3	N.E.	–	
9b	10	–	10	3	(2.7–3.4)	33	>74	–	0	N.E.	–	
7b	>10	–	0	N.E.	–	0	>74	–	0	N.E.	–	
3b	9	(9.9–10)	11.9	1.2	–	79.4	88.6	(68.6–136.9)	1.1	6.5	(5.2–8.5)	
4b	2.6	(2.34–10)	33.9	0.4	(0.3–0.6)	222.2	27.7	(21.4–39.9)	3.6	2.8	(2.1–3.8)	
10c	3.7	–	27	0.99	(0.9–1.1)	101	29.9	(21.8–49.5)	3.3	N.E.	–	
3c	7.5	–	13.3	1.2	(1.1–1.3)	83	>74	–	0	N.E.	–	
4c	1.8	–	55.5	0.6	(0.59–0.63)	163.9	>74	–	0	N.E.	–	
10d	5–10	–	13.3	1.3	(0.9–1.6)	78.7	>74	–	0	26.9	(17.4–61)	
3d	5–10	–	13.3	1.6	(1.4–1.8)	63.3	50.6	(25.9–286.6)	1.97	N.E.	–	
4d	1.2–2.5	–	39.8	0.5	(0.5–0.6)	196	25.8	(17.9–45.9)	3.8	N.E.	–	

N.E., No effect.
[a] Toxic units based on the formula of Sprague and Ramsay (1965).

all compounds present in the leachate assessing synergism/antagonism and their bioavailability (Pivato and Gaspari, 2006).

There is a clear tendency in DTA approaches for using test batteries, covering different taxonomic groups (Tinsley et al., 2004). This approach has been used for evaluating toxicity of MSW landfill leachates (Clément et al., 1996; Ward et al., 2002; Isidori et al., 2003; Slomczyńska and Slomczyński, 2004; Thomas et al., 2009). However, current landfills management practices are exclusively based on physicochemical characterizations; thus, a reliable correlation between physicochemical and ecotoxicological characterization would facilitate the management of landfill leachates, allowing the prioritisation of leachates that should be ecotoxicologically characterized.

The physicochemical characterization of the samples considered in this study is summarized in the Table 3; in general, the results obtained in this work are in line with those published by several authors (Sisinno et al., 2000; Slomczyńska and Slomczyński, 2004; Ward et al., 2005; Alkassasbeh et al., 2009) with high levels of chemical oxygen demand (COD), conductivity and nitrogen among others. The pH values ranged from 6.5 to 8.96; the alkalinity of the majority of the leachates could indicate landfills exploited from a long time (Slomczyńska and Slomczyński, 2004; Alkassasbeh et al., 2009). The higher values for conductivity corresponded to leachates from landfill "B" and compost leachates from landfill "E"; on the contrary, compost leachate from landfill "A" did not show high levels of conductivity; differences between values of conductivity from distinct compost leachates could be explained due to the different procedures of separation of the organic matter chosen for compost preparation between the two landfills. In addition to this, the original residue could also influence the conductivity values of the compost leachates. Many of the leachates or compost leachates had high concentrations of nitrogen compounds (ammonium, nitrate).

Previously to assess the potential relationship between the physicochemical parameters and the ecotoxicological characterization, a PCA was developed; this analysis searched for possible relationships between physicochemical parameters that allowed reducing of the number of variables used for later correlations. PCA allowed extracting three components, which they account for 72.55% of the variability of the original data. Fig. 1 shows the three-dimensional graphic for the principal component analysis

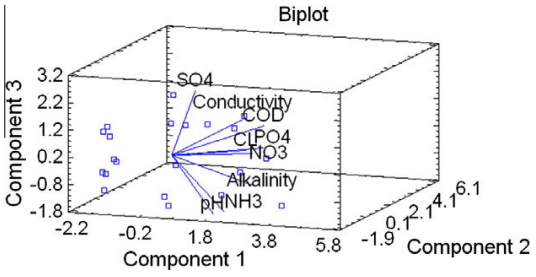


Fig. 1. 3D plot of component weights for physicochemical parameters. A 3D biplot graphic for a principal component analysis display a three dimensional biplot with rays that represent the magnitude and direction for each variable.

that represents the magnitude and direction of each variable. The results of this analysis did not demonstrate clear groups between parameters. The parameters with larger weight for the first component were chlorides (Cl^-), alkalinity (HCO_3^-), conductivity, ammonia (NH_3) and COD.

The correlation between physicochemical properties of leachates and their toxicity was studied through a regression analysis; this test correlated the physicochemical parameters, considering their weight in the component 1 of the PCA and the derived toxic units obtained for *D. magna*, *X. laevis* and neutral red assays. The EROD assay covers a very specific group of chemicals is not intended for these general toxicity assessments. The results (Fig. 2) showed a moderate to strong relationship between physicochemical variables and the toxic units from the three bioassays. This correlation was higher for *Daphnia* ($r^2 = 0.85$) and *Xenopus* tests ($r^2 = 0.86$) than for the *in vitro* assay ($r^2 = 0.55$ for neutral red assay).

Taking into account the moderate (*in vitro* assay) to strong (*in vivo* assays) correlation between physicochemical parameters and toxicity units, a discriminant analysis was applied, obtaining a model that offers a categorization of the leachate, predicting toxicity based on the physicochemical properties. Discriminant analysis is designed to develop a set of discriminating functions which can predict classification based on the values of other quantitative variables. The method allows predict additional observations by

Table 3
Summary of the physicochemical characterization of landfill leachates or compost leachates.

Sample code	pH	EC (μS/cm)	COD (mg O_2/L)	SO_4^{2-} (mg/L)	NH_4^+ (mg/L)	NH_3[a] (mg/L)	NO_3^- (mg/L)	Alkalinity HCO_3^- (mg/L)	Cl^- (mg/L)	PO_4^{3-} (mg/L)
1	7.3	2525	872	140	27	0.3	35.2	458	170	4.5
2	7.4	4447	951	101	47.8	0.7	35.2	793	227	5.4
3a	7.4	40,319	2798	4070	0.8	0.01	26.4	2044	5524	7.4
4a	8.1	64,487	9529	1573	1.6	0.1	136	8937	7086	42.4
5	7.6	16,751	19,244	641	12	0.3	968	5368	966	319
6	6.8	5836	422	2700	27.4	0.09	61.6	610	142	3.2
7a	6.5	5750	118	1845	4.8	0.008	96.8	610	227	2.4
8	8	1119	12	23	0.9	0.05	4	250	40	0.7
9a	7	10,398	1087	263	11.5	0.06	79.2	1769	852	5.2
10a	7.6	25,676	37,845	2540	5.6	0.12	132	8662	2556	143
10b	6.5	21,488	27,780	773	1715	3.01	378	9028	3919	47
9b	7	11,207	1374	165	726	4.03	44	3111	1022	5.4
7b	7.1	4760	65	2485	0.1	0.0007	88	305	196	2.6
3b	7.5	11,050	4518	3920	1020	17.9	334	3416	4757	13.8
4b	7.8	18,438	9668	1840	3045	106.7	898	8388	5481	51
10c	8.96	15,122	2857	910	5	2.4	158	8540	1599	5.6
3c	7.7	14,441	4054	910	1081	30.1	312	3050	6836	21.9
4c	8.3	19,315	9030	1728	1729	191.6	15	7717	7725	50.2
10d	8.2	15,255	3005	430	1187	104.5	47	9516	2272	11.4
3d	7.4	18,044	4264	3725	1535	21.4	95	10,736	6532	16.9
4d	8	24,253	9101	2140	3870	215	204	29,890	8307	50.2

[a] Concentrations estimated from NH_4 and pH values.

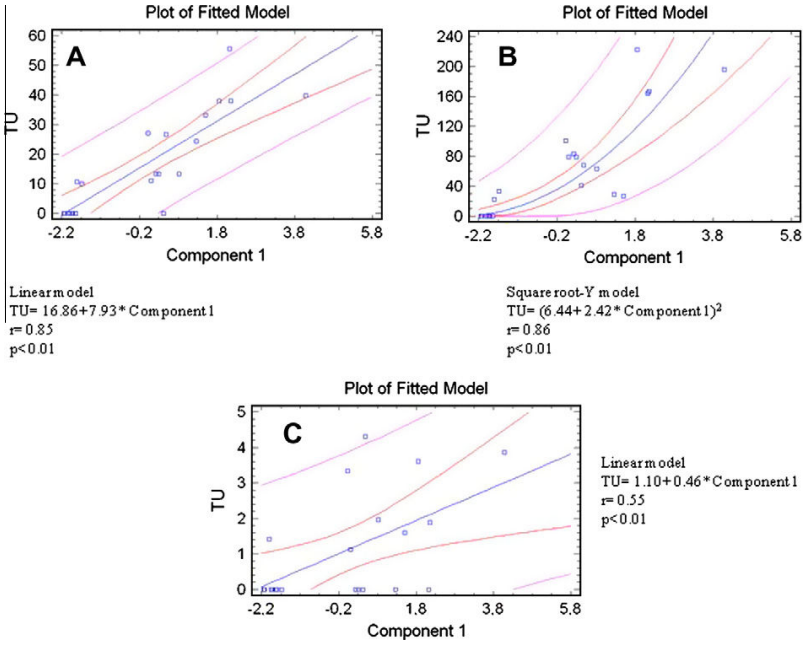
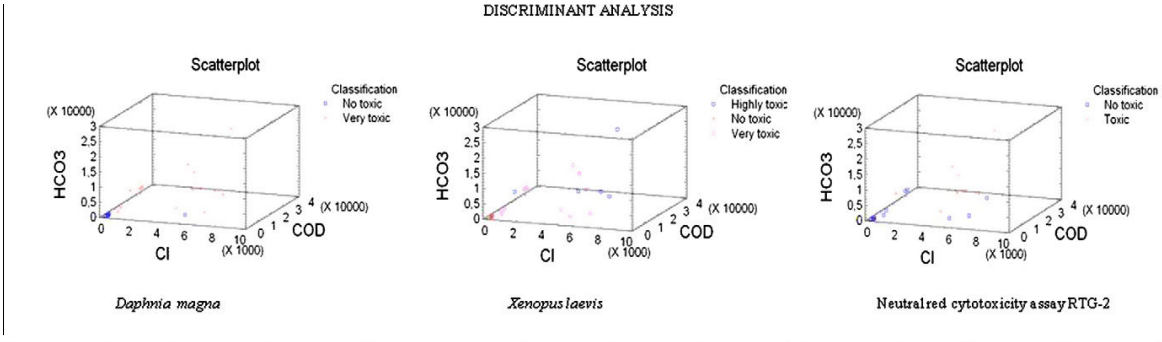


Fig. 2. Regression analysis between values of the component 1 (from PCA) obtained for each sample leachates and the toxic units achieved for each sample from three different bioassays: (A) *Daphnia magna* assay; (B) *Xenopus laevis* test; (C) neutral red RTG-2 cytotoxicity assay.



Actual Classific.	Group size	Predicted Classific. Very Toxic	Predicted Classific. No Toxic
Very Toxic	14	11 (78.6%)	3 (21.4%)
No Toxic	7	1 (14.3%)	6 (85.7%)

80.95% cases correctly classified
False negatives: 3 (14.3%)

Actual Classific	Group size	Predicted Classific. Highly Toxic	Predicted Classific. Very Toxic	Predicted Classific. No Toxic
Highly Toxic	5	3 (60%)	2 (40%)	0 (0%)
Very Toxic	10	2 (20%)	6 (60%)	2 (20%)
No Toxic	6	0 (0%)	0 (0%)	6 (0%)

71.43% cases correctly classified
False negatives: 2 (9.53%)

Actual Classific.	Group size	Predicted Classific. Toxic	Predicted Classific. No Toxic
Toxic	9	6 (66.7%)	3 (33.3%)
No Toxic	12	1 (8.3%)	11 (91.7%)

80.95% cases correctly classified
False negatives: 3 (14.3%)

Fig. 3. Discriminant analysis for toxicity categorization based on physicochemical parameters (Cl^-, COD, NH_3 and HCO_3^-). The toxicity categorization was established using toxic unit ranges (highly toxic (TU > 100); very toxic (10 < TU < 100); toxic (1 < TU < 10); and no toxic (TU < 1).

adding the independent variables. The model used the four physicochemical parameters that had a larger weight for the first component of the PCA (Cl^-, COD, HCO_3^- and NH_3). Due to the narrow relationship between chlorides and conductivity, this last parameter was excluded of the correlation. In Fig. 3, boxes represent the three-dimensional scatterplots of the relationship between physi-

cochemical variables and the assigned category in the discriminant analysis, whilst tables show the summary of the results using the derived discriminant functions to classify observations. Actual classification is the result of the ecotoxicological characterization, and the predicted classification is the predicted value obtained from the discriminant analysis using the physicochemical parameters.

The results showed that the physicochemical parameters may offer a good initial categorization of the expected toxicity for all bioassays. A similar toxicity categorization based on physicochemical parameters and bioassay results was observed for a large majority of the samples (81% for both *Daphnia* and neutral red assay and 71% cases for *Xenopus*). It should be noted that the lower percentage observed for *Xenopus* was mostly related to differences in the specific toxic category (i.e., samples classified as highly toxic by the model and very toxic by the *Xenopus* data or vice versa).

The percentages of false negatives (toxic samples classified as "no toxic") were small for all the bioassays (less than 10% considering *Xenopus* test and less than 15% in the other assays). It should be noted that the correlation is not based on a real cause-effect relationship but a simple association between the toxicity and a set of parameters which describe the leachate characteristics, in terms of waste characteristics, potential for extracting different classes of toxic chemicals, dilution, ageing, etc. Therefore, the percentage of false negatives should be considered relatively low. The results of these discriminant analyses would indicate that certain parameters could be used as a first screening of the potential acute toxicity of leachates on aquatic organisms. Previous studies have correlated toxicity and physicochemical characteristics of leachates; Clément et al. (1997) suggested that ammonia, alkalinity and COD were related with increasing toxicity. Ward et al. (2002) found a relationship between the ammonia concentration in the leachates and the toxicity in *Cerodaphnia dubia* and *Kirschneriella subcapitata* assays. Similar results were found in the study of Pivato and Gaspari (2006), correlating the ammonia content of the leachate with the toxicity on *Vibrio fisheri* test.

Although some studies concluded that it is difficult to predict the toxicity of the leachates by their chemical parameters (Thomas et al., 2009), some physicochemical parameters may be used as markers of the acute toxicity. Ours results are in agreement with the study of Clément and co-workers (1997), confirming that ammonia, alkalinity and COD, together with chloride, allowed a correct categorization of the leachates toxicity for more than 75% of the samples.

4. Conclusions

The proposed test battery presented in this work (*D. magna*, *X. laevis* and RTG-2 cytotoxicity assay) was effective for the ecotoxicological characterization of MSW landfill leachates. The discriminant analysis showed that certain physicochemical parameters could be used as an initial categorization method for acute leachates toxicity in aquatic organisms. The correlation should not be considered a cause–effect relationship but an association between the physicochemical parameters and the overall content of toxic substances in the leachate. Samples with high levels of certain physicochemical parameters (i.e., ammonia, alkalinity, COD and chloride) should be directly classified as potentially toxic, and managed accordingly, e.g., treatment before emission. False positives (no toxic samples but based on their physicochemical characterization could be considered as toxic) represent an economic problem for the management of the leachates, because they will be treated as toxic; however the results of this work highlight that the percentage of these samples is very low (0% for *Xenopus* characterization and 4.8% for both *Daphnia* and RTG-2 assays). The other samples should be considered as candidates for the ecotoxicological characterization. New studies to corroborate these results should be necessary.

Acknowledgments

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CAPÍTULO 5

Assessment of potential immunotoxic effects caused by cypermethrin, fluoxetine, and thiabendazole using heat shock protein 70 and interleukin-1β mRNA expression in the anuran *Xenopus laevis*.

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Resumen

En general, las citoquinas, y más concretamente las interleuquinas, son consideradas buenos indicadores del potencial inmunotóxico de los xenobióticos. En el presente estudio se describen los efectos de la cipermetrina, la fluoxetina y el tiabendazolo, a unas concentraciones ambientales relevantes, en la expresión de la proteína de choque térmico 70 (HSP70) y de la interleuquina 1β (IL-1β). Como modelo animal se han usado las larvas del anuro *Xenopus laevis*. Los renacuajos, en un estadio de desarrollo 47 según la tabla de desarrollo de Nieuwkoop y Faber (1994), han sido expuestos bajo condiciones estáticas a 0,3 y 30 µg/L de fluoxetina, 0,7 µg/L de tiabendazol y 0,24 µg/L de cipermetrina. Los efectos se han evaluado a las 7, 24, y 72 horas, y a los 6 y 9 días. Una parte de los renacuajos, elegidos al azar, han sido utilizados como material genético para la determinación de la inducción del ARNm para el gen *hsp70* y el gen *IL-1β* a través de la técnica de RT-PCR. Los renacuajos expuestos a 30 g/L de fluoxetina muestran una expresión de ARNm de ambos genes en todos los tiempos de exposición, mientras que a 0,3 g/L se observa un pico del ARNm para *hsp70* después de 24 horas y un aumento estadísticamente significativo respecto al control del ARNm para *IL-1β* a 72 horas después de la exposición. El tiabendazolo induce una alta expresión del ARNm tanto para el *hsp70* como para el *IL-1β* en todos los tiempos de exposición. La cipermetrina aumenta los niveles del ARNm del *hsp70*, con un pico a las 24 horas, y provoca una alta expresión del ARNm del *IL-1β* en todos los tiempos de exposición. Teniendo en cuenta la relación entre la proteína HSP70 y la interleuquina IL1-1β y sus participaciones (principalmente de la IL-1β) en la respuesta inmune, ciertos cambios observados en sus expresiones podrían considerarse como unos indicadores de advertencia de posibles efectos inmunotóxicos en las larvas de *Xenopus*.

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ASSESSMENT OF POTENTIAL IMMUNOTOXIC EFFECTS CAUSED BY CYPERMETHRIN, FLUOXETINE, AND THIABENDAZOLE USING HEAT SHOCK PROTEIN 70 AND INTERLEUKIN-1β mRNA EXPRESSION IN THE ANURAN *XENOPUS LAEVIS*

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Abstract—The current study describes the effect of cypermethrin, fluoxetine, and thiabendazole, at environmentally relevant concentrations, on the expression of heat shock protein 70 (HSP70) and interleukin 1β (IL-1β), using *Xenopus laevis* larvae as animal model. Cytokines and interleukins are considered good predictors of the immunotoxic potential of xenobiotics. Tadpoles at stage 47 (normal tables of *X. laevis*) were exposed under static conditions to: 0.3 and 30 μg/L fluoxetine, 0.7 μg/L thiabendazole, and 0.24 μg/L cypermethrin. The effects were evaluated at 7, 24, and 72 h, and 6 and 9 d. Randomly chosen tadpoles were used as genetic material for detection of *hsp70* and *IL-1β* mRNA induction through reverse transcription PCR. Tadpoles exposed to 30 μg/L fluoxetine showed mRNA expression of both genes at all exposure times, whereas at 0.3 μg/L a peak response for *hsp70* was observed after 24 h, and the increase in *IL-1β* mRNA was statistically significant with respect to the control 72 h after exposure. Thiabendazole induced a high expression of mRNA for both *hsp70* and *IL-1β* at all exposure times. Cypermethrin increased the *hsp70* mRNA levels, with a peak at 24 h, and provoked high expression of *IL-1β* mRNA at all exposure times. Considering the relationship between HSP70 and IL-1β and their involvement (mainly of IL-1β) in immune responses, certain changes observed in their expression could be considered warning indicators of potential immunotoxic effects of these substances on *Xenopus*. Environ. Toxicol. Chem. 2010;29:2536–2543.

Keywords—*Xenopus laevis* Heat shock protein 70 Interleukin-1β Immunotoxicity

Environmental releases of pesticides and pharmaceuticals are associated with agricultural activities and discharges from urban wastewater treatment plants. Pyrethroid insecticides are widely used because of their general low toxicity to birds and mammals. Among these pyrethroids, cypermethrin is one of the most used insecticides. During the application period, cypermethrin may reach water bodies by spray drift and runoff. Cypermethrin is highly toxic to aquatic macroinvertebrates and fish [1], and it is strongly bound to sediment and organic matter [2]. Cypermethrin concentrations within the 0.1 to 0.71 μg/L range have been measured in runoff and water bodies [1].

The effects of pharmaceuticals on nontarget aquatic organisms are still poorly understood. Fluoxetine is among the most prescribed antidepressants and acts on the central nervous system as a selective serotonin reuptake inhibitor. Fluoxetine has been detected in municipal effluents and receiving surface waters in the 0 to 1 μg/L range [3,4]. Limited information is available on the potential hazard of sublethal fluoxetine exposures to aquatic organisms [5].

Thiabendazole is a systemic fungicide that forms a protective deposit on the treated surfaces of fruit and tubers. Because of its moderate water solubility (0.16 g/L, pH 4, and 0.03 g/L, pH 7 and 10) and hydrolytic stability [6], thiabendazole may reach water bodies. The European Scientific Committee on Plants calculated a predicted environmental concentration in surface water of 53.6 μg/L ([7]; http://ec.europa.eu/food/fs/sc/scp/out74_ppp_en.pdf).

Amphibians are increasingly used as bioindicators of environmental health, so it is important to develop methods for evaluating the effects of environmental contaminants on these species. Amphibian immunotoxicology is an important area of research within the field of amphibian ecotoxicology [8]. The evaluation of the immune system is increasingly recognized as a critical target in the process of xenobiotic risk assessment [9]. Ecoimmunotoxicological methods can provide useful data that may be leveraged to evaluate immunotoxicity potential in wildlife species. The value of such research is the identification of potential, unintended harm to the environment expressed through subtle immunological endpoints [9]. In particular, the ecotoxicological relevance of the amphibian *Xenopus laevis* has already been well established in a large array of studies [10]. The selection of immunotoxicity endpoints is a priority for the adequate interpretation of sublethal immune effects not easily quantified by standard health indices [11]. Risk assessment predictions employ a variety of toxicological and community structure measurements to predict risk [12]. Ideally, molecular biomarkers should offer insight into the molecular nature of adverse effects, such as the progression of environmentally induced disease or the organism's response to the exposure [13].

Biomarkers of exposure, effect, and susceptibility are needed for understanding the relationship between the levels of environmental pollutants and the organism's response. Biomarkers range from those that are highly specific (such as the inhibition of the aminolevulinic acid dehydratase by Pb) to those that are nonspecific (i.e., the effects of the immune system) [14]. In this context, biomarkers facilitate the health status assessment of amphibian populations by serving as sublethal toxicity endpoints [15]. Recent advances in identifying new and better biomarkers have depended on understanding the inter- and intraindividual variability of these markers, how their responses relate to ecological disturbances, and whether increasing incidence of disease is associated with exposure to the pollutant [13]. Quantifications of cytokines and interleukins

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were shown to be a good predictor of the immunotoxic potential of environmental chemicals [12]; cytokines are found in all vertebrates and represent excellent markers for following the course of exposure and disease. The proinflammatory cytokine interleukin-1β has been used as an exposure biomarker in frogs ([16]; http://www.osti.gov/bridge/servlets/purl/469639-Z9ePCD/webviewable/469639.pdf). Heat shock proteins (HSPs) have also been proposed as sensitive indicators of sublethal exposure to environmental pollutants [17].

This study assesses the potential immunotoxic effects of fluoxetine, thiabendazole, and cypermethrin in tadpoles. The response was measured as changes in gene expression of interleukin-1β (IL-1β) and HSP70. Changes in gene expression are being explored by industry and regulatory agencies worldwide as screening and prioritization tools for functional evaluation of environmental pollutants [18]; thus, the current study offers easy endpoints and novel techniques for the screening assessment of immunotoxicological effects in amphibians.

MATERIALS AND METHODS

Xenopus *breeding and maintenance*

Sexually mature *Xenopus laevis* were obtained from our laboratory stocks. Adults were kept in dechlorinated tap water on a 12:12 h light:dark photoperiod at 21 to 23°C, with a density of six animals per 40 L in 125 L plastic aquaria. Frogs were fed with trout feed chopped pellets (Reproductores, Dibaq) twice a week, 2 to 3 h before each water change. Animals were treated as described by American Society for Testing Materials [19]. *Xenopus laevis* tadpoles were obtained by injecting the dorsal lymph sac of adult breeder frogs with human corionic gonadotropin (HCG-LEPORI 2500, Angelini) as described by Schultz and Dawson [20]. Tadpoles were changed into fresh frog embryo teratogenesis assay—*Xenopus* (FETAX) medium with a stainless steel strainer 5 d postfertilization, and fed daily with commercially available fish powder dry food ad libitum (Sera Micron).

Exposure conditions

After prescreening assays to select substances, exposure concentrations, assay duration, and the sampling regimen (data not shown), we developed the definitive experiments. The tests began with larvae at stage 47, according to the *Xenopus* table of development [21]. All exposures took place in a reconstituted water medium suitable for FETAX [22]. Tadpoles were exposed, in a static assay, for 9 d to serial dilutions of three different substances: fluoxetine (fluoxetine hydrochloride, Interchim; product number 09674, batch number TT0821), with initial nominal concentrations of 0.3 and 30 μg/L; thiabendazole (99% pure, Sigma), with an initial nominal concentration of 0.7 μg/L; and cypermethrin (α-cypermethrin Pestanal, 99.8% pure, Sigma-Aldrich), with an initial nominal concentration of 0.24 μg/L. Because of limited aqueous solubility of cypermethrin, triethylene glycol (99% pure, Sigma-Aldrich) was used as a solvent. Thus, stock solutions were adjusted to obtain final dilutions in FETAX solution with a concentration of 1% (v/v) of triethylene glycol. Groups of 20 larvae were exposed in 2-L glass beakers containing 1 L medium solutions; the assay used different beakers for each exposure time, and all tests (including FETAX and solvent control) were performed twice in parallel (replicates A and B). The beakers ($n = 60$) were placed in a 22 ± 1°C controlled environmental chamber on a 12:12 h light:dark photoperiod. During exposure, larvae were fed with Sera Micron fish food four times (at 1, 3, 6, and 8 d) at a rate of 20 mg/beaker. Larvae were checked every day for deformity, abnormality swimming behaviors, and mortality, and all dead tadpoles were counted and removed. Samples for genetic analysis were collected at 7, 24, and 72 h, and 6 and 9 d. At each sampling time, 10 randomly chosen tadpoles from each treatment were killed by immersion in an overdose of MS-222 (ethyl 3-aminobenzoate methanesulfonate; Fluka) and, after having been preserved in RNAlater Solution (Ambion), were used as genetic material for detection of *hsp70* and *IL-1β* mRNA induction. Snout–vent length was measured, and developmental stage was determined according to a *Xenopus* table of development [21]. At the end of the test, the remaining tadpoles ($n = 10$) were fixed in Carnoy's fixative (60% absolute ethanol, 30% chloroform, 10%, acetic acid) to record their snout–vent length and developmental stage. To measure snout–vent length, a photograph of each tadpole was taken and analyzed with the image analysis program Image Pro-Plus 6.0 (Infaimon S.L.).

Contaminant analyses

Water samples were collected immediately after each toxic substance addition (time-zero samples) and subsequently at 7 h, 24 h, 72 h, and 6 and 9 d. One sample was taken from each beaker.

Determination of fluoxetine. Chemical analysis of fluoxetine was performed by solid phase extraction and high-performance liquid chromatography (HPLC) with mass spectrometry detection. The analytical procedure is based on methods described by Sánchez-Argüello et al. [23] and Vlase et al. [24] with some modifications (i.e., volume of sample and cartridge supplier).

Briefly, the analytical procedure was the following: a solid phase extraction procedure was applied to 500-ml water samples, filtered through a glass fiber filter (Millipore APFF04700), using Oasis HLB (divinylbenzene/N-vinylpyrrolidone copolymer) cartridges (60 mg, 3 ml) from Waters. The cartridges were preconditioned with 6 ml methanol and 6 ml MilliQ water. After the precondition step, a 500-ml sample was passed through the cartridge and then rinsed with 6 ml MilliQ water before the elution. After that, cartridges were dried by vacuum (30 min) and eluted three times with 3 ml methanol. The extracts were evaporated until dry using a Genevac EZ-2 evaporator (Ipswich, IPI, 5AP) at 40°C. The dried residues were then reconstituted with 1.5 ml methanol/water (10/90 v/v). The analysis of fluoxetine was performed by injecting 20 μl of the extract in an HPLC system (HP 1200 series, Agilent Technologies) equipped with a triple quadruple mass detector G6410A QQQ (Agilent Technologies); separation was performed with a Zorbax Eclipse XDB (4.4×150 mm, 5 μm; Agilent Technologies) column at 40°C, and a linear gradient (0.8 ml/min) in 20 min from 100% of 0.1% formic acid pH 5.5 to 100% acetonitrile.

Fluoxetine was detected by multiple reactions monitoring using electrospray ionization mass spectrometry. The recovery studies were carried out by quadruplicate with spiked samples containing 0.4 μg/L fluoxetine. The method detection limit (3 ng/L) and the method quantification limit (9 ng/L) were determined by injection of fortified blank water and calculated as the concentration that supplies a peak with signal-to-noise ratio of 3 and 10, respectively.

Determination of thiabendazole. The concentration of thiabendazole was quantified by solid phase extraction followed by HPLC determination with fluorescence detection. The procedure used is based in the method described by Young et al. [25], with several modifications. Briefly, 500-ml water samples, filtered through a glass fiber filter (Millipore APFF04700), were extracted by solid phase extraction (Strata-X 33 μm

200 mg/6 ml, Phenomenex 8B-S029-FBJ) previously conditioned with 6 ml methanol and 6 ml 0.15 M ammonium hydroxide ($HONH_4$) solution. After sample absorption, the cartridge was washed with 6 ml 0.15 M $HONH_4$ and dried by vacuum for 30 min; then thiabendazole was desorbed with 9 ml methanol containing 0.3 M $HONH_4$. The methanolic extract was dried at 40°C (Genevac EZ-2) and redissolved in 1.5 ml HPLC mobile phase. Thiabendazole was analyzed by liquid chromatography (Waters Alliance model 2690 with a Waters 776 fluorescence detector, 315 nm excitation, 385 nm emission), injecting 100 μl extracted sample in the column (Luna 5 μm C18, 250 × 4.60 mm, Phenomenex) at 40°C with buffer phosphate pH 6.8/Acetonitrile 65/35 at 1 ml/min.

Quantification was performed using external standards (fortified water samples 0.05–0.8 ng/ml). The limits of quantification and detection were established in 0.05 ng/ml and 0.03 ng/ml, respectively, and in recoveries greater than 90%.

Determination of cypermethrin. Quantification of cypermethrin concentration in water samples was performed by solid phase microextraction followed by gas chromatography with electron capture detection. The analytical procedure is based on previously described methods [26,27]. Briefly, extraction was performed (100 μm polydimethylsiloxane, Supelco) by immersion (constant magnetic stirring, 60°C, 30 min) in a vial containing 3 ml sample and 0.1 ml of a 30 ng/ml methanolic solution of λ-cyhalothrin (Pestanal, Rielel-de-Häen), used as an internal standard for quantitative purposes. After extraction, analysis was performed in an HP 6890 gas chromatograph coupled to an HP 5973N selective mass detector working in electron impact single ion monitoring mode (ions 163, 181, 209 *m/z*). Calibration curves were performed with standard fortified solutions ranging from 0.06 ng/ml to 0.48 ng/ml. This method showed a detection limit of 0.01 ng/ml (limit of quantification, 0.03 ng/ml).

RNA extraction and RT-PCR

The genetic material was obtained from 10 tadpoles per beaker (one beaker for each exposure time), pooled two by two, thus achieving five samples per beaker. The mRNAs were extracted from a pool of tissues of the abdominal region. According to the literature, *IL-1β* mRNA expression is high in kidney, liver, and spleen [28], whereas *hsp70* mRNA is expressed in liver and skin [29]. Thus, samples of tissues from the abdominal region were considered the best samples for detecting mRNA expression of both proteins. The mRNA isolation procedure was carried out with 0.5 ml TRI Reagent (Ambion), according to the manufacturer's instructions. Before reverse transcription polymerase chain reaction, mRNA samples were treated with DNase (DNA-free, Ambion). The quality and integrity of all mRNAs produced was assessed by measuring the absorbance of the RNA solutions and calculating the ratio A260/A280, and then confirmed by electrophoresis on a 2% agarose-formaldehyde gel.

The concentrations of mRNAs were determined with the Ribogreen RNA-Specific Quantitation Kit with DNase I (Molecular Probes).

For the reverse transcriptase (RT) the first strand of cDNA was synthesized by using 0.1 μg mRNA according to Bioscript (Bioline) indications. The PCR was performed with Biotaq DNA Polymerase (Bioline). Amplification of cDNA specific for the protein HSP70 was performed using primers resulting in a PCR product of 480 bp, and the PCR reaction method described by Gornati et al. [30]. The sequences of *hsp70* primers (Ecogen, Spain) were forward: 5′-AGTCGGCATTGACCTCGGC-3′ and reverse: 5′-TGATTCTCAAAATATTCAGTCC -3′. Amplification of cDNA specific for the protein IL-1β was performed using primers resulting in a PCR product of 152 bp [28] and the PCR reaction method described by Metz et al. [31]. The sequences of primers (Ecogen) were forward: 5′-AACAGAAGATGGCCAAGACTC-3′ and reverse: 5′-ATGCAACCGATTCAAAGCTG-3′. The housekeeping gene elongation factor 1 α (*ef1α*) of *X. laevis* was used as internal standard. The PCR reaction was performed using the primer sequences (Ecogen) forward: 5′ TGCCAATTGTTGACATGATCCC-3′ and reverse: 5′ TACTATTAAACTCTGATGGCC-3′, resulting in a PCR product of 285 bp, and amplification was developed according to the method described by Bögi et al. [32]. Amplification was carried out in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research) using the specific thermal cycling condition for each case.

The positive control (c +) for *hsp70* mRNA expression was obtained by the exposure of three tadpoles (stage 47) to 33°C for 1 h, whereas for *IL-1β*, one *X. laevis* adult was intraperitoneally injected with 200 μg lipopolysaccharide (*E. coli* 0111:B4, Sigma) dissolved in 0.2 ml phosphate buffered saline and was killed 24 h later, following the method described by Zou et al. [28].

The PCR products were loaded into 2% agarose gel and run in tris-borate-EDTA buffer 0.5X at 110 mV for 60 min. Staining of cDNA was achieved by adding 0.1 μl SYBR Safe DNA Gel Stain (Molecular Probes) per 10 ml liquid agarose gel. The PCR products were visible under ultraviolet light. The fluorescent bands of cDNA were quantified by densitometric measurements using the image analyzer Gel-Pro Analyzer 3.1 (Media Cybernetics). A linear relationship exists between quantity and optical density, which allows semiquantitative measurements compared with a base value. The densitometric values of the cDNA for the housekeeping protein EF1α were used as internal standard. Therefore, the amounts of amplified *hsp70* and *IL-1β* were normalized for the differences obtained by the PCR products of *ef1α*.

Statistical analyses

The significance of the relative expression with respect to the negative controls data was assessed by ANOVA, with Fisher's least-significant difference procedure ($p < 0.05$), in the software Statgraphics 5.1 (Statpoint Technologies). The values were expressed as means ± standard deviation. To evaluate statistical significance of snout–vent lengths observed in the treatments relative to controls, the same statistic method described previously was used, whereas for the statistical analysis of developmental stage, the nonparametric Kruskal-Wallis test was used. In this case the values were expressed as median ± standard deviation. A statistical probability of $p < 0.05$ was considered significant.

RESULTS

The embryos' survival rate in the FETAX and solvent control reached 90%, and thus the assay was considered acceptable. When a solvent control was necessary, no statistical differences between FETAX control and solvent control were observed. In these cases, statistical analyses were related to solvent control.

Contaminant analyses

Fluoxetine waterborne concentrations ranged from 73% to 53% of nominal concentrations from day 0 to day 9 (Fig. 1a). Thiabendazole was measured at concentrations near nominal

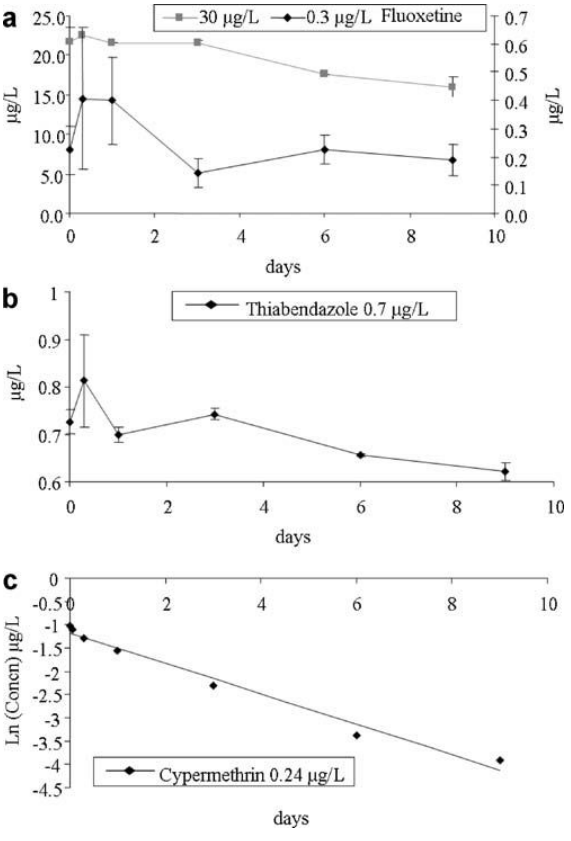

Fig. 1. Dissipation in water of fluoxetine at the two tested concentrations (0.3 and 30 µg/L) (**a**), thiabendazole at 0.7 µg/L (**b**), and cypermethrin at 0.24 µg/L (**c**). Water samples were analyzed immediately after each toxic substance addition (time-zero samples) and subsequently at: 7, 24, and 72 h, and 6 and 9 d. One sample for each beaker was taken.

levels at all sample times (Fig. 1b). Initial cypermethrin concentrations in water samples ranged from 0.26 to 0.41 µg/L; a first-order decay was observed with dissipation of the substance at the end of experiment (Fig. 1c). The half dissipation time of cypermethrin was 33 h.

hsp70 *and* IL-1β *mRNA expression*

Results of reverse transcription PCR for the samples exposed to fluoxetine showed mRNA expression of both proteins at the two tested concentrations (Fig. 2). Concerning *hsp70* mRNA induction, the lower concentration of fluoxetine (0.3 µg/L) caused the peak response at 24 h but drastically declined at 72 h (Fig. 2a). However, the expression of *hsp70* mRNA was high for the maximum concentration (30 µg/L), reaching the same levels as the positive control after 6 d. Although at 9 d *hsp70* mRNA expression decreased, there were still significant differences with respect to the control (Fig. 2b). A similar tendency was observed for the expression of *IL-1β* at the exposure concentration of 30 µg/L, showing high levels of mRNA expression (higher than the positive control) during all the assay period (Fig. 2d). The expression of *IL-1β* mRNA at the lowest concentration was statistically significant with respect to the control at 72 h, increasing its value until the last sampling day, when it doubled the positive control value (Fig. 2c). Thiabendazole induced a statistically significant expression with respect to the control for both *hsp70* and *IL-1β* mRNA during all exposure times (Fig. 3). For *IL-1β*, an mRNA induction of approximately twofold increase with respect to positive control was detected at 7, 24, and 72 h (Fig. 3b). In animals exposed to cypermethrin, the level of mRNA expression for *hsp70* reached the highest peak at 24 h but drastically decreased at 72 h (Fig. 4a). As in the previous cases, cypermethrin caused an *IL-1β* mRNA expression higher than the control until the end of the assay (Fig. 4b). Unexpectedly, mRNA for both *hsp70* and *IL-1β* increased its expression abnormally 9 d after exposure.

Other effects

When compared with the control animals at the end of the bioassay, all tadpoles exposed to the three different contaminants were not significantly different in length or in developmental stage ($p > 0.05$; data not shown). No deformities or abnormal swimming behaviors were observed.

DISCUSSION

This study presents nonspecific immunity (HSP70) or innate immune (IL-1β) responses at low environmentally relevant concentrations of the three immunotoxic chemicals [1,3,4,7]. Obviously, the structural and functional integrity of the immune system are crucial in performing its protective role against pathogenic agents; any chemically induced perturbation of the host's immune system may compromise its capacity, leading to adverse health consequences. Considering the link of IL-1β and HSP70 with the immune system response [28,33], the increase or decrease of their expression should be considered sublethal alterations, which may progress through evident damage of the immune system.

To our knowledge, this is the first report in which the correlation between *hsp70* and *IL-1β* mRNA expression caused by contaminants at relevant environmental levels has been studied as a possible early warning indicator in *X. laevis* for screening hazard identification of immune effects associated with chemical exposures.

Taking into account that *X. laevis* is considered an excellent model for studying those immunological effects that are not phylogenetically restricted to a given taxon [34], as well as the high degree of structural conservation of HSPs among vertebrates and the involvement of HSPs and interleukins in nonspecific immune response, the endpoints selected for this study could be proposed as indicators of potential sublethal immunotoxicity hazards in human health assessment as well. Although most quantitative and functional immune assays were developed and validated on mouse and rat, the increased use of other species in immunotoxicity studies has prompted the development and validation of a number of immune assays in species other than rodents [9,35].

Heat shock proteins are evolutionarily ancient and highly conserved cellular components that have been found in all prokaryotic and eukaryotic organisms that have been studied [33,36]. Although some HSPs are expressed constitutively within the cell under normal physiological conditions, aiding in vital cellular processes such as protein folding, assembly, and transport, others are only expressed on heat shock or under other stress conditions [37]. Many other more specific functions have been characterized for particular HSP types, including a role in immunological processes. An increasing body of data suggests that certain HSPs (such as HSP70) play a role in both innate and adaptive immunity [33]. The HSPs can elicit potent specific cellular adaptive immune responses based on their ability to

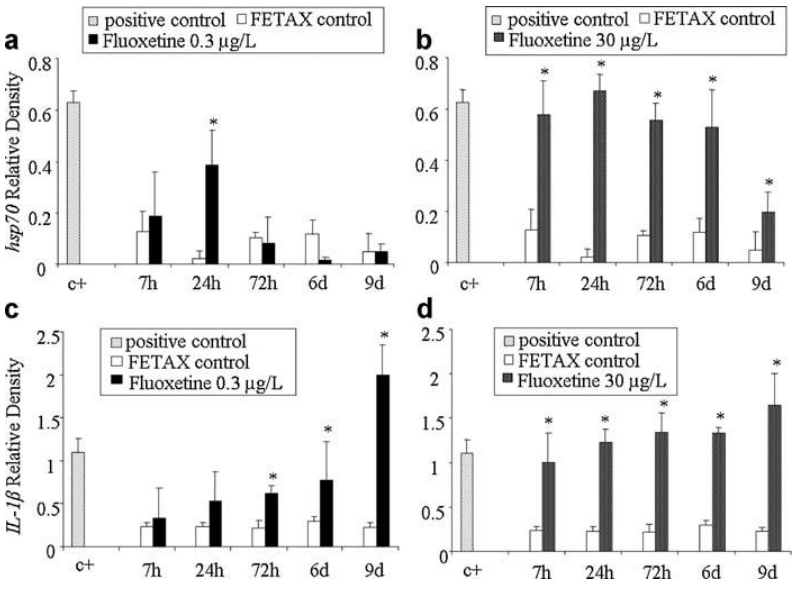

Fig. 2. Induction of heat shock protein 70 (*hsp70*) mRNA caused by 0.3 (**a**) and 30 µg/L (**b**) fluoxetine exposure. Induction of interleukin-1β (*IL-1β*) mRNA caused by 0.3 (**c**) and 30 µg/L (**d**) fluoxetine exposure. Data are shown as mean ± standard deviation of gel densitometric evaluation obtained from genetic material samples ($n = 10$). The letter c+ symbolizes the corresponding positive control, which for the expression of *hsp70* was obtained by the exposure of three tadpoles (stage 47) to 33°C temperature for 1 h; for *IL-1β* it was obtained by intraperitoneal injection of a *Xenopus laevis* adult with 200 µg lipopolysaccharide dissolved in 0.2 ml phosphate buffered saline. Asterisks indicate significant differences with respect to the corresponding FETAX (frog embryo teratogenesis assay-*Xenopus*) control ($p < 0.05$).

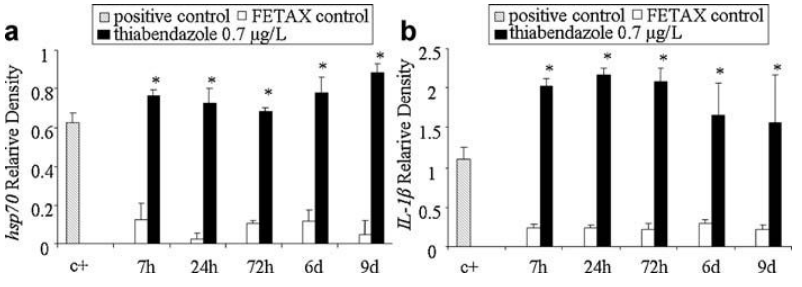

Fig. 3. Effects of exposure to 0.7 µg/L thiabendazole on the mRNA expression of heat shock protein 70 (*hsp70*) (**a**) and interleukin-1β (*IL-1β*) (**b**). Data are shown as mean ± standard deviation of gel densitometric evaluation obtained from genetic material samples ($n = 10$). The letter c+ symbolizes the corresponding positive control, which, for the expression of *hsp70,* was obtained by the exposure of three tadpoles (stage 47) to 33°C temperature for 1 h; for *IL-1β,* it was obtained by intraperitoneal injection of an *Xenopus laevis* adult with 200 µg lipopolysaccharide dissolved in 0.2 ml phosphate buffered saline. Asterisks indicate a significant increase in band densities relative to the corresponding FETAX (frog embryo teratogenesis assay-*Xenopus*) control ($p < 0.05$).

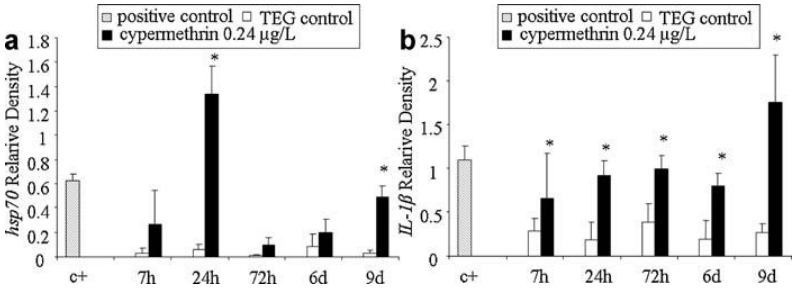

Fig. 4. Effects of 0.24 µg/L cypermethrin exposure on heat shock protein 70 (*hsp70*) (**a**) and interleukin-1β (*IL-1β*) (**b**) mRNA expression level. Data are shown as mean ± standard deviation of gel densitometric evaluation obtained from genetic material samples ($n = 10$). The letter c+ symbolizes the corresponding positive control, which, for the expression of *hsp70,* was obtained by the exposure of three tadpoles (stage 47) to 33°C temperature for 1 h; for *IL-1β,* it was obtained by intraperitoneal injection of an *Xenopus laevis* adult with 200 µg lipopolysaccharide dissolved in 0.2 ml phosphate buffered saline. Asterisks indicate significant differences with respect to the corresponding triethylene glycol (TEG) control ($p < 0.05$).

chaperone antigenic peptides. Heat shock proteins can also act independently of chaperoned peptides to directly stimulate innate immune response [33]. Several in vitro studies have shown that, independent of their bound peptides, gp96, HSP90 and HSP70 are able to induce macrophages to produce the proinflammatory cytokines IL-1β, tumor necrosis factor alpha, IL-12, and granulocyte-macrophage colony-stimulating factor [38].

Cytokines are responsible for regulating a variety of processes, including immunity, inflammation, apoptosis, and hematopoiesis [39]. Cytokines are signaling molecules that play a central role during the initiation of the immune response. In innate immunity, the effector cytokines are produced primarily by mononuclear phagocytes. These molecules elicit neutrophil-rich inflammatory reactions that serve to contain and, when possible, eradicate microbial infections [39].

Interleukin-1β is a principal pro-inflammatory cytokine that is induced in vivo by lipopolysaccharide [28] and is critical for innate immunity. Interleukin-1β modulates immune and inflammatory responses, and it is an important factor in inducing apoptosis in vivo in nonlymphoid tissues [39,40]. Interleukin-1β has a capacity to induce corticosterone that results in leukocyte apoptosis and resultant lymphoid tissue depletion [40].

For these reasons, alteration of HSP70 and IL-1β synthesis has been linked to the functional impairment of effector cells that could have consequences on the entire immune response [12,33]. Alteration of their synthesis could increase the susceptibility to infections and parasitism [41]. Immunocompetence is controlled through many compensatory mechanisms, and disruption of one parameter may not necessarily impair the overall immune response. However, any consistent suppression of a measurable immune function may represent a potential risk to an organism. Hence, for hazard identification purposes, any statistically significant and consistent alteration of an immune function should be considered an indicator of potential concern [12].

Previous studies have demonstrated the capacity of different contaminants to produce a stress protein response (HSP70) that in some cases was able to induce the production of proinflammatory cytokines, such as IL-1β [17,30,33]. The results of the exposure to the three xenobiotics used in the current study (Figs. 2, 3, and 4) suggest an evident (thiabendazole) or a less marked (cypermethrin) relationship between the expression of *hsp70* and *IL-1β* mRNA, because both mRNA expressions underwent an increase after the exposure. Results of the current study were in agreement with previous works [33,38], with the exception of the study by Schmidt and Abdulla [42], in which the induction of the human acute monocytic leukemia cell line with lipopolysaccharide at 39 to 41°C for 2 to 4 h resulted in the expected increased synthesis of heat shock proteins HSP70 and HSP90 but decreased synthesis of IL-1β precursor protein p35 (and its mRNA). A study conducted by Blake et al. [29] on tissues of rats exposed to elevated ambient temperature explained how in liver the expression of *hsp70* mRNA, after the removal of the animals from the stress, gradually rose until the last time detected.

The maximal induction did not occur until 6 h after heat stress, and it showed a tendency to increase. Although in the current study the peak of expression of *hsp70* mRNA was reached 24 h after exposure initiation, at 7 h mRNA had already been detected for each compound. Looking at Figure 3 in the present study, thiabendazole evidently was the most remarkable immunostimulating compound. The induced mRNA response for both proteins was maintained during the whole assayed period, which could be explained by the high persistence of thiabendazole in water. The concentration of fluoxetine in water was not maintained constant during the 9 d, but it ranged from 73 and 53% of nominal concentrations from day 0 to day 9 (Fig. 1a). The fate observed for fluoxetine in our experiment is consistent with that expected. When the compound is added to an aqueous medium, a first dissipation is produced because of adsorption to particulate organic matter; thus, the initial measured concentration is lower than nominal values. Then, further degradation or dissipation processes are responsible for subsequent concentration decay. The use of measured concentrations is essential for a proper quantification of exposure levels. Results of the current study are in agreement with those of Cruz-Rodríguez and Chu [17], who observed, for stress proteins in eastern oyster (*Crassostrea virginica*), how mRNA expression for *hsp70* and *IL-1β* exhibited a fluctuating response with time, dose, or both. In the current study, at the lowest concentration of fluoxetine (0.3 μg/L), *hsp70* mRNA expression showed a peak at 24 h, and *IL-1β* slowly increased to reach the maximum value at day 9, whereas at 30 μg/L, both mRNA expressions were strongly induced. Considering that analytical determination for cypermethrin did not detect any metabolites, this compound may have dissipated because of its absorption by glass and organic material, rather than through a real degradation. The calculated half dissipation time for cypermethrin in this assay was similar to the half dissipation time of 24 h reported by González-Doncel et al. [26]. Despite the rapid dissipation from water, cypermethrin induced and maintained mRNA expression of *hsp70* and I*L-1β*. The expression of *hsp70* mRNA reached its peak at 24 h, and *IL-1β* mRNA was highly expressed during all test periods. This fact might confirm the high sensitivity of *hsp70* and *IL-1β* mRNA expression to peak stimuli. The unexpected increases of mRNA expression of both *hsp70* and *IL-1β* at day 9 had to be attributed to unknown causes, independent from the compound exposure.

The low level of *hsp70* expression observed in control animals was also observed in the study by Blake et al. [29]. Because toxicity is not responsible for this basal *hsp70* induction, other factors, possibly physiological, neural, or humoral, may be influencing its expression. Nevertheless, the information reported by Washburn et al. [43] that no effects of handling stress, MS-222, or both were observed in the tissues most frequently sampled in ecotoxicology research and environmental monitoring for HSP60 and HSP70 in rainbow trout, together with the fact that dietary restriction did not affect production of HSP70 [44], provide a further validation for the use of HSP70 as biomarker. Interestingly, we found a basal expression of the *IL-1β* mRNA in nontreated larvae, also observed by Pelegrín et al. [45] but not by Zou et al. [28]. The role of IL-1β as mediator of both physiological and pathological processes is well known [46].

As suggested by Krzystyniak et al. [35], a variety of structural changes in the immune system, such as metabolic/biochemical interactions observed during subchronic and chronic toxicity studies, are useful indicators of a potential immunotoxic effect of chemicals and can be used as a base in designing further immunotoxicity studies.

CONCLUSION

Because of the sensitivity of the immune system to chemicals at low levels and its importance for maintaining host resistance against disease, emphasis has been placed on the

development of immune endpoints that can predict exposure to and effects from environmental chemicals. Through the efforts of a number of laboratories, a battery of well-characterized immune assays, originally developed in rodents and organized into tiers to test for functional and histopathological parameters, are currently available [35,47]. Tier 1 is usually a screen for potentially immunotoxic chemicals, focusing on the hazard identification, and tier 2 tests consider functional parameters and a more comprehensive evaluation of the mechanisms involved, specific immune function tests, and host resistance challenge assays [47]. No one immune endpoint is going to be the *magic bullet* for determining xenobiotics-induced immunotoxicity in the amphibian. The greatest strength for predicting immunotoxicity in wildlife populations lies in the use of a suite of selected immune assays. Using these together with data from those indicators measuring biochemical, physiological, and histopathological processes should prove most successful for predicting toxicological risks to species residing in contaminated aquatic environments. Thanks to biomolecular technology, the results of our study offer new early warning endpoints that may be included in an aquatic immunotoxicological battery. Further research about the expression of these parameters in dose–response assays should be considered. Based on the correlation between cytokine production and phagocytosis [8,35,45,48], the possibility of correlating these endpoints with phagocytosis capacity also should be explored.

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CAPÍTULO 6

Gene expression of heat shock protein 70, interleukin-1β and tumor necrosis factor α as tools to identify immunotoxic effects on *Xenopus laevis:* a dose-response study with benzo[a]pyrene and its degradation products.

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Resumen

Entre todos los hidrocarburos policíclicos aromáticos (PAHs) presentes en el medio ambiente, el benzo[a]pireno (B[a]P) es el más representado, por lo que es frecuentemente usado como modelo para los estudios de exposición y efectos de estos contaminantes. La exposición a B[a]P puede dar lugar a una alteración de la función inmune en mamíferos y peces y, por otro lado, el análisis de los niveles de ARNm de citoquinas ha sido sugerido para la predicción del potencial inmunomodulador de los productos químicos. Para obtener evidencia de cómo la respuesta inmune innata puede ser alterada por los efectos del B[a]P, el presente estudio evalúa la expresión del ARNm para la interleuquina 1-β (IL-1β), el factor de necrosis tumoral α (TNF-α) y la proteína de choque térmico 70 (HSP70), en una exposición controlada en laboratorio en el anuro *Xenopus laevis*. Los renacuajos expuestos a un única dosis de las siguientes diluciones seriadas de B[a]P: 8,36; 14,64; 89,06 y 309,47 µg/L, se utilizan para la detección de la inducción de ARNm de los genes *hsp70*, *IL-1β* y *TNF-α*. Los resultados muestran un incremento en la expresión génica del *hsp70* y del *IL-1β* relacionado con la concentración del B[a]P. Los resultados de este estudio confirman el uso de la proteína HSP70 y de la IL-1β, pero no del TNF-α, como indicadores para los efectos inmunotóxicos del B[a]P en *X. laevis*.

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Gene expression of heat shock protein 70, interleukin-1β and tumor necrosis factor α as tools to identify immunotoxic effects on *Xenopus laevis*: A dose–response study with benzo[a]pyrene and its degradation products

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ABSTRACT

The exposure to benzo[a]pyrene (B[a]P) results in an alteration of immune function in mammals and fish, and the analysis of cytokine mRNA levels has been suggested for predicting the immunomodulatory potential of chemicals. To obtain evidence of the innate immune responses to B[a]P in *Xenopus laevis*, the present study monitored the mRNA expression of interleukin 1-β (IL-1β), tumor necrosis factor α (TNF-α) and heat shock protein 70 (HSP70) in a laboratorial exposure. Tadpoles exposed to 8.36, 14.64, 89.06 and 309.47 μg/L of B[a]P,were used for detecting *hsp70*, *IL-1β* and *TNF-α* mRNA induction. A dose–response increase in the expression of *hsp70* and *IL-1β* mRNA was found. The results of this study confirmed the use of *hsp70* and *IL-1β*, but not *TNF-α*, as sensitive indicators of immunotoxic effect of B[a]P in *X. laevis*. Further research would be required for the validation of these endpoints.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants derived primarily from incomplete combustion of organic matter. Since the onset of industrialization, the environmental load of PAHs, which may be found in air, soil, water, and food, has increased several orders of magnitude due to the increasing use of fossil fuels. While some PAHs have a relatively low toxicity, other, such as benzo[a]pyrene (B[a]P), dibenzanthracene, and certain dibenzopyrenes and nitropyrenes, are compounds with cytotoxic, genotoxic, teratogenic, and carcinogenic effects (Salo et al., 2005).

Benzo[a]pyrene is presented as a major component of the total content of polycyclic aromatic compounds in the environment; thus it is frequently used as a model for exposure and effects of PAHs (Schellenberger et al., 2009). After ingestion, inhalation or dermal absorption, B[a]P acquires its mutagenic and carcinogenic properties in a multi-step process (De Buck et al., 2005). In addition, B[a]P has been found to be a potent immunosuppressant; effects on cell-mediated immunity, humoral immunity, and host resistance have been documented in a study reported on human, rat and murine cells by Carfi' et al. (2007), in which both, cell proliferation and cytokine production, were used to assess the immunotoxic potential of two chemicals classified as not immunotoxic (urethane and furosemide), and four compounds with different effect on the immune system (tributyltin chloride, verapamil, cyclosporine and B[a]P).

The immune system is an important target organ for chemical exposure. Immunotoxicity can be defined as the adverse effect of chemicals or agents on the immune system. The effect may increase immune activity, manifested as either hypersensitivity or autoimmunity, or decrease immune activity, with reduced ability to fight against infectious agents and increased incidence of cancer (Snodin, 2004).

Cytokine release is considered to be very relevant to investigate the toxicity towards the immune system (Carfi' et al., 2007). In mammals, it has been well established that cytokines are required to initiate and regulate immune response, both innate and adaptive. Some cytokines that have a role in innate response, such as the pro-inflammatory cytokines (e. g. interleukin 1-β (IL-1β), IL-6, IL-8 and tumor necrosis factor α (TNF-α)) may be universal, or have functional equivalents in vertebrates and invertebrates (Kaiser et al., 2004). In this regard, the present study monitored the expression profile of three typical proinflamatory genes (*IL-1β*, *TNF-α* and heat shock protein 70 (*hsp70*)) after a single exposure to sublethal concentrations of B[a]P on the anuran *Xenopus laevis*.

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Interleukin 1-β is a primary regulator of inflammatory and immune responses in mammals, but over the lasts years, it has been extensively studied on bony fish and amphibian, allowing the identification of a number of regions conserved throughout vertebrates (Bird et al., 2002). After infection or injury, IL-1β stimulates the host immune response by initiating and promoting production of other cytokines, chemokines, and adhesion molecules (Engelsma et al., 2002). In the same way, TNF-α is highly pleiotropic cytokine involved in a spectrum of physiological processes that control inflammation, anti-tumor responses and homeostasis (Grayfer and Belosevic, 2009). Increasing evidence suggests that HSP70 is a key player in immune responses, and the ability of HSPs to evoke potent immune responses relies on their capacity to produce pro-inflammatory cytokines, as IL-1β and TNF-α, and chemokines, as well as to up-regulate co-stimulatory molecules (Robert et al., 2009). Furthermore, HSPs have been proposed as sensitive indicators of sublethal exposure to contaminants in the environment (Cruz-Rodríguez and Chu, 2002).

We have recently published a study (Martini et al., 2010) in which the expressions of *hsp70* and *IL-1β* mRNA have been suggested as warning indicators of potential immunotoxic effects of different xenobiotics (thiabendazol, fluoxetine and cypermethrin) for the pre-screening assessment of immunotoxicological effects in amphibians. In the current study we focused on the establishment of dose–response relationship between the exposure and the increase of the expression of these genes. Thus, the aim of this study was to explore the validity of analyzing cytokine mRNA levels as an earlier tool for predicting immunotoxicological effects caused by B[a]P on *X. laevis*, in a dose–response pattern, and to improve the knowledge of B[a]P immunotoxic effects in other taxonomic groups rarely considered up to now, such as amphibians. *Xenopus laevis* was selected as model for amphibians, since is a well-known quality indicator of aquatic ecosystems (Colombo et al., 2003; Mouchet et al., 2006), as well as an increasing laboratory model for immunological studies (Morales et al., 2010). Sublethal concentrations of B[a]P (Saka, 2004), which is a well-studied environmental pollutant with a potential immunotoxic effect, were used in a dose–response assay at different times.

2. Materials and methods

2.1. Animals and husbandry

Xenopus laevis tadpoles were obtained from in-house breeding of adult animals housed in plastic aquaria (10 animals in 40 L of dechlorinated tap water). The room temperature was set at 22 +/− 1 °C under a 12:12 h light: dark photoperiod. Frogs were fed with trout feed chopped pellets (REPRODUCTORES®, Dibaq, Spain) twice a week, 2–3 h before each water change. Animal manipulation was performed in accordance with the protocol of American Society for Testing Materials (ASTM, 1998). Spawning of adult *X. laevis* was induced by two injections of human corionic gonadotropin (hCG-LEPORI 2500®, Angelini, Italy) into the dorsal lymph sac, spaced 8 h apart. Male received 400 International Units (IU) of hCG at each injections, while female received 250 IU on the first injection and 800 IU on the subsequent injection. Tadpoles were changed into a reconstituted water medium suitable for Frog Embryo Teratogenesis Assay-*Xenopus*, FETAX (Dawson and Bantle, 1987) with a stainless steel strainer 5 d post-fertilization, and fed daily on commercially available fish powder dry food (SERA MICRON®, Germany) *ad libitum*.

2.2. Exposure conditions

Xenopus laevis larvae at stage 48, according to the *Xenopus* table of development (Nieuwkoop and Faber, 1994), were used to perform the experiment. All experimental aspects were conducted in compliance with the institutional guidelines for the care and use of animals detailed for the Ethics Committee for animal research of the Spanish National Institute for Agricultural and Food Research and Technology.

All exposures took place in FETAX medium. Tadpoles were exposed to B[a]P (97% pure, Sigma–Aldrich, Germany) in a dose–response static assay during nine days; the measured concentrations were 8.36, 14.64, 89.06 and 309.47 μg/L. Because of limited aqueous solubility of B[a]P (0.0038 mg/L; Juhasz and Naidu, 2000), a solvent other than FETAX solution was needed. Because of its low toxicity, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol (TEG), in a concentration lower than 1.6% *v/v*, is considered a good and safety organic solvent to prepare stock solutions (Rayburn et al., 1991; ASTM, 1998). Thus, TEG (99% pure, Sigma–Aldrich, Germany) was used as carrier solvent with a final concentration of 0.1% *v/v* in FETAX solution, in all treatments.

Groups of 20 larvae were exposed in 2 L glass beakers containing 1 L of medium solutions. All tests, including FETAX control (blank control (BC)) and solvent control (SC), were performed twice in parallel (replicates A and B). The beakers ($n = 12$) were placed in 22 +/− 1 °C controlled environmental chamber on a 12:12 h light: dark photoperiod. During exposure, larvae were fed with SERA MICRON® fish food four times at 1, 3, 6 and 8 day at a rate of 30 mg/beaker. Larvae were checked every day for mortality, and all dead tadpoles were counted and removed. Samples for genetic and water analyses were obtained from replicate A and B, and were collected at 1, 6 and 9 days. At each sampling time 6 random chosen tadpoles from each treatment were used as genetic samples. The tadpoles were euthanized by immersion in an overdose of MS-222 (Fluka, Germany), preserved in RNAlater SOLUTION® (AMBION®, USA) and used as genetic material for detection of *hsp70*, *IL-1β*, *TNF-α* mRNA induction.

2.3. Determination of B[a]P in aqueous samples

Water samples were collected immediately after B[a]P addition (time-zero samples) and subsequently at 1, 6 and 9 days. Two measurements in the same beaker (two beakers per concentration) were taken. The concentration of B[a]P (but not its degradation products) was quantified by HPLC with fluorescence detection. The procedure used is based in the 550 EPA method (1990), but injecting directly the water sample after filtration. Briefly, 10 ml of water samples filtered through a glass fiber filter (Millipore, USA) and transferred to the injection vial was analyzed by liquid chromatography (Waters Alliance, USA) with a Waters 776 fluorescence detector (380 nm excitation, 430 nm emission); 50 μl were injected in the column (Luna 5 μm C18 (2) 250 × 4.6 mm, Phenomenex, USA) at 40 °C with MilliQ water/Acetonitrile 30/70 with a flow of 1.2 ml/min.

Quantification was performed using external standards in the following way: calibration curves were prepared daily using spiked FETAX solution by adding B[a]P acetonitrile solution to obtain standard solutions with B[a]P concentrations ranged from 0.20 to 0.3 ng/ml. Standard B[a]P solutions were injected in parallel with samples, and B[a]P sample concentrations were calculated using the calibration curve built (peak area vs concentration). Following the above described method, quantification limit was established at the lowest calibration point (0.3 ng/ml) and detection limit was established at 2.5 times below the quantification limit (0.1 ng/ml), with a signal/noise ratio higher than 10.

2.4. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The genetic material was obtained from 6 tadpoles per beaker of replicate A and B, pooled two by two, achieving thus 3 samples per beaker. The mRNAs were extracted from a pool of tissues from the abdominal region obtained by cutting off head, tail, forequarter and hind quarter, and ensuring that liver, kidney, spleen and muscle were collected. The mRNA was isolated using 0.5 ml TRI REAGENT® (AMBION®, USA) per sample, with an additional DNase (DNA-free™, AMBION®, USA) treatment according to manufacturer's instructions. The concentrations of mRNAs were determined spectrophotometrically (NanoDrop-2000c™, ThermoScientific, USA). The quality and integrity of mRNA were also assessed by calculating the ratio A260/A280 and then confirmed by electrophoresis on a 2% agarose-formaldehyde gel. For the reverse transcriptase (RT) the first strand of cDNA was synthesized by using 0.5 μg of total RNA according to BIOSCRIPT™ (Bioline GmbH, Germany) indications. The PCR was performed with BIOTAQ™ DNA Polymerase (Bioline GmbH, Germany). The cDNA products were amplified in the presence of primers (Ecogen, Spain) for HSP70, IL-1β, TNF-α and β-actin (Table 1). Amplification of cDNA was performed in a thermocycler (MJ Research, USA) using the specific PCR reaction method described by Gornati et al. (2002), Metz et al. (2006), and Wang et al. (1996) for HSP70, IL-1β and β-actin,respectively. For TNF-α protein, the amplification protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 40 s, with a final extension at 72 °C for 5 min.

The positive control for *hsp70* mRNA expression was obtained by the exposure of 3 tadpoles (stage 47) to 33 °C for 1 h; for *IL-1β* and *TNF-α*, one *X. laevis* adult was intraperitoneally injected with 200 μg lipopolysaccharide (LPS; Sigma, Germany) dissolved in 0.2 ml phosphate buffered saline (PBS) and euthanized 24 h later following the method described by Zou et al. (2000).

The PCR products were loaded into 2% agarose gel and run in TBE 0.5X buffer at 110 mV for 60 min. Staining of cDNA was achieved by adding 0.1 μl SYBR Safe™ DNA Gel Stain (Molecular Probes™) per 10 ml liquid agarose gel. The PCR products were visible under UV-light. The fluorescent bands of cDNA were quantified by densitometric measurements using the image analyzer Gel-Pro Analyzer 3.1 (Media Cybernetics, L.P). The densitometric values of the cDNA for the house-keeping protein β-actin were used as internal standard. Therefore, the amounts of amplified *hsp70*, *IL-1β* and *TNF-α* were normalized for the differences obtained by the PCR products of *β-actin*.

Table 1
Primers used in the study.

Name	Sequence (from 5′ to 3′)	Product length (pb)	Reference
HSP70-frw[a]	AGTCGGCATTGACCT CGGC	480	Gornati et al. (2002)
HSP70-rev[b]	TGATTCTCAAAATAT TCAGTCC		
IL-1β-frw	AACAGAAGATGGCC AAGACTC	152	Zou et al. (2000)
IL-1β-rev	ATGCAACCGATTCAA AGCTG		
TNF-α-frw	GCTCAAGGATAACTC CATCG	210	Morales et al. (2010)
TNF-α -rev	AACCAAGTGGCACCT GAATG		
β-actin-frw	GGTGTCATGGTTGG AATGG	359	http://www.urmc.rochester.edu/mbi/resources/Xenopus/available-res.cfm#PrimerPairs
β-actin-rev	TGGGTTACACCATC ACCTGAG		

[a] Frw: forward.
[b] Rev: reverse.

2.5. Statistical analyses

The significance of the relative expression with respect to the negative control was analyzed using a multifactor ANOVA with type III sum of squares, considering the effects of the following factors: concentration, time and replicate nested within each concentration. Mean values were compared using multiple range tests with Fisher's least significant difference (LSD) intervals ($\alpha = 0.05$). The statistical analysis was performed with Statgraphics 5.1 software (Statpoint Technologies, INC., USA).

3. Results

3.1. Contaminant analyses

The initial measured B[a]P waterborne concentrations were: 8.36, 14.64, 89.06 and 309.47 μg/L. Table 2 shows the concentration of B[a]P in water measured at different times. The decrease of B[a]P concentration in water samples was in relation to both exposure concentration and time; the lower the initial B[a]P exposure concentration was, much less persistent was the compound into water. The estimated dissipation half time (DT_{50}) of B[a]P was 73 h (Fig. 1), but it should be noted that solubility was facilitated by a solvent.

3.2. hsp70, IL-1β and TNF-α mRNA expression

The larvae' survival rate in the FETAX control (blank control (BC)) and solvent control (SC) reached 90%, consequently the assay was considered acceptable. No statistical differences between BC and SC were observed; thus statistical analyses were related to SC. Benzo[a]pyrene induced a statistically significant expression of both *hsp70* and *IL-1β* mRNA at all exposure concentrations in comparison to SC ($P < 0.05$; Figs. 2 and 3). The B[a]P related *hsp70* mRNA induction showed a clear concentration/response relationship (Table 3; Fig. 2), despite the fact that a solvent carrier has been used. At the two highest concentrations, a significant gene expression increase was detected between day 1 and 6 ($P < 0.05$; Fig. 2). The induction of *IL-1β* mRNA also showed a concentration/response relationship (Table 3; Fig. 3) but the levels of induction did not show significant changes from day 1 to day 9 ($P = 0.28$; Table 3). The expression of *IL-1β* mRNA at the highest concentration (309.47 μg/L) even exceeded the positive control value, which, in the relative density scale, had a value of 0.8. No induction of *TNF-α* mRNA expression was detected except sporadically low expression responses statistically significant observed at some intermediate concentrations, which should be considered of no biological relevance (Fig. 4).

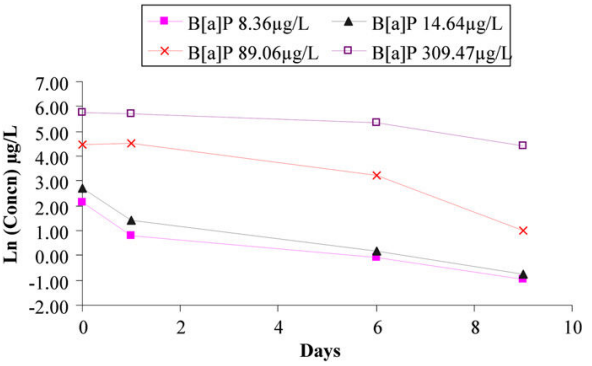

Fig. 1. Dissipation in water of benzo[a]pyrene at the four tested concentrations (8.36, 14.64, 89.06 and 309.47 μg/L). Water samples were analyzed immediately after toxic substance addition (time-zero samples) and subsequently at: 1, 6 and 9 days. The mean values of each concentration were obtained by two samples (replicate A and B) and two measurements in the same sample ($n = 4$).

Table 2
Concentration of benzo[a]pyrene in aqueous samples measured at different times.

B[a]P measured concentration (μg/l)		
Time-zero	Days 6 after expression	Days 9 after expression
8.36 ± 3.326[a]	0.93 ± 0.00	0.37 ± 0.001
14.64 ± 1.88	1.2 ± 0.00	0.47 ± 0.004
89.06 ± 22.24	24.59 ± 2.24	2.75 ± 0.58
309.47 ± 40.52	212.99 ± 21.03	81.89 ± 12.37

[a] The mean content ± standard deviation values of each concentration were obtained by two samples (replicate A and B) and two measurements in the same sample.

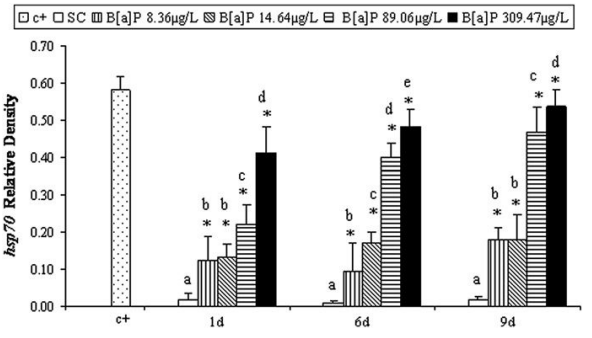

Fig. 2. Effects of exposure to 8.36, 14.64, 89.06 and 309.47 μg/L benzo[a]pyrene (B[a]P) on the mRNA expression of heat shock protein 70 (*hsp70*). Data are shown as mean ± standard deviation of gel densitometric evaluation of cDNA obtained from abdominal regions of tadpoles ($n = 6$). The house-keeping *β-actin* was used as internal standard and the amounts of amplified *hsp70* were normalized for the differences obtained by the PCR products of *β-actin* (relative density). Letter "c+" symbolizes the positive control, which was obtained by the exposure of 3 tadpoles (stage 47) to 33 °C temperature for 1 h. Acronyms 1d, 6d and 9d are used to represent the corresponding exposure times: 1, 6 and 9 days. Asterisks indicate a significant increase in band densities relative to the corresponding solvent (SC) control ($p < 0.001$) that was triethylene glycol 0.1% *v/v* (triethylene glycol/FETAX (frog embryo teratogenesis assay-*Xenopus*) solution). Different letters indicate differences among treatments ($P < 0.05$; LSD intervals) at different times.

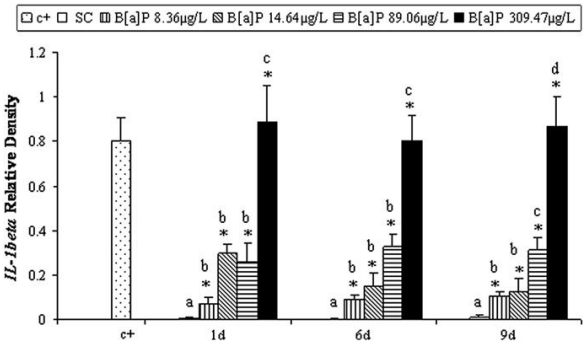

Fig. 3. Induction of interleukin-1β (*IL-1β*) mRNA caused by 8.36, 14.64, 89.06 and 309.47 µg/L benzo[a]pyrene (B[a]P) exposure. Data are shown as mean ± standard deviation of gel densitometric evaluation of cDNA obtained from abdominal regions of tadpoles ($n = 6$). Since, the house-keeping *β-actin* was used as internal standard, the amounts of amplified *IL-1β* were normalized for the differences obtained by the PCR products of *β-actin* (relative density). Letter "c+" symbolizes the positive control, which was obtained by intraperitoneal injection of a *Xenopus laevis* adult with 200 µg/L lipopolysaccharide dissolved in 0.2 ml phosphate buffered saline. Acronyms 1d, 6d and 9d are used to represent the corresponding exposure times: 1, 6 and 9 days. Asterisks indicate a significant increase in band densities relative to the corresponding solvent (SC) control ($p < 0.001$) that was triethylene glycol 0.1% *v/v* (triethylene glycol/FETAX (frog embryo teratogenesis assay-*Xenopus*) solution). Different letters indicate differences among treatments ($P < 0.05$; LSD intervals) at different times.

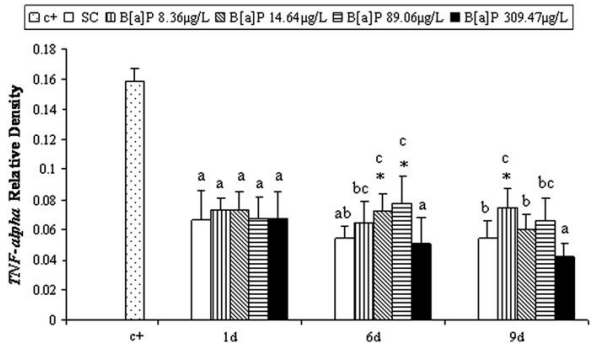

Fig. 4. Effects of 8.36, 14.64, 89.06 and 309.47 µg/L benzo[a]pyrene (B[a]P) exposure on tumor necrosis factor alpha (*TNF-α*) mRNA expression level at 1,6, and 9 days. Data are shown as mean ± standard deviation of gel densitometric evaluation of cDNA obtained from abdominal regions of tadpoles ($n = 6$). The house-keeping *β-actin* was used as internal standard and the amounts of amplified were *TNF-α* normalized for the differences obtained by the PCR products of *β-actin* (relative density). Letter "c+" symbolizes the positive control, which was obtained by intraperitoneal injection of a *Xenopus laevis* adult with 200 µg/L lipopolysaccharide dissolved in 0.2 ml phosphate buffered saline. Acronyms 1d, 6d and 9d are used to represent the corresponding exposure times: 1, 6 and 9 days. Asterisks indicate a significant increase in band densities relative to the corresponding solvent (SC) control ($p < 0.001$) that was triethylene glycol 0.1% *v/v* (triethylene glycol/FETAX (frog embryo teratogenesis assay-*Xenopus*) solution). Different letters indicate differences among treatments ($P < 0.05$; LSD intervals) at different times.

In summary, a dose–response increase in the expression of *hsp70* and *IL-1β* mRNA was shown (Figs. 2 and 3), while the increase in the response of *TNF-α* mRNA expression was negligible (Fig. 4). Moreover, in the case of *hsp70* is possible to observe an increasing expression of mRNA not only related with concentration, but also with time (Fig. 2; Table 3).

4. Discussion

The immunotoxicity of B[a]P has been extensively studied in mammalian and fish systems (Carlson et al., 2004; Hornung et al., 2007). Apparently, mechanisms involved in B[a]P-induced immunosuppression have been phylogenetically conserved due to similar metabolic pathways almost species. For example, in mammals and Japanese medaka (*Oryzias latipes*) B[a]P-induced suppression of humoral immunity relies upon the CYP1A-catalyzed production of immunotoxic B[a]P metabolites, and B[a]P metabolites may be created *in situ*, directly by specific cells within kidney lymphoid tissue (Carlson et al., 2004). Several authors have suggested that B[a]P and in particular its activated metabolite 7,8-diol-B[a]P, exert their immunotoxic effects by binding to a specific aryl hydrocarbon receptor (De Buck et al., 2005; Hornung et al., 2007). Obviously, the degradation products of B[a]P in test media were contributing to the effects, although in the present study they were not analyzed, since the objective was to investigate the immunotoxic effects of B[a]P that are regulated by cytokines in general, rather than each metabolic pathway of the compound.

Ligation of aryl hydrocarbon receptor (AhR) also up-regulates the phase 1 enzymes cytochrome P450 responsible for B[a]P activation to adduct forming metabolites and the generation of immunotoxic metabolites (Schellenberger et al., 2009). Moreover, effects of B[a]P or its reactive metabolites may be mediated by disruption of cell membranes, production of various interleukins, or alteration of intracellular calcium mobilization (Baken et al., 2008).

Considering that proper immune system functionality depends on cytokine production, and that cytokine release is considered very relevant to investigate the toxicity towards the immune system (Carfi' et al., 2007), *hsp70, IL-1β* and *TNF-α* mRNA levels alterations through a dose–response assessment could be an early and useful tool to identify potential immunotoxic compounds. A previous study in this laboratory has suggested the possible use of *hsp70* and *IL-1β* mRNA expression as new early warning endpoints that may be used for investigating the immunotoxic potential of environmental contaminants (Martini et al., 2010). To our knowledge, this is the first study where alterations of mRNA expression of *hsp70, IL-1β* and *TNF-α* are used to study the immunotoxicity of B[a]P on *X. laevis* in a dose–response pattern at different times. The sublethal concentrations of B[a]P used in the current study have been selected according to a study where FETAX standardized assay was used to determinate mortality and malformation incidence (Saka, 2004), taking into account that the metabolic activation of B[a]P by cytochrome P450 may increase the potential teratogenic hazard of the compound in FETAX assay (Fort et al., 1989; Propst et al., 1997).

As shown in Table 2, low percentages from the initial B[a]P concentrations were detected at the end of the assay, probably because of dissipation by photolysis and evaporation (the main metabolites and the degradation products were not measured in water) (Seo et al., 2007). The calculated DT_{50} for B[a]P in this assay (Fig. 1) was similar to the value reported by Świetlik et al. (2002), with a half-life of B[a]P of 3–4 days in water taken from a depth of 3 m, in studies carried out in a controlled ecosystem enclosure.

Table 3
Results of the three way ANOVA (factors: time, concentration and replicate nested within each concentration) for the relative expression of heat shock protein 70 (*hsp70*), interleukin 1-β (*IL-1β*), tumor necrosis factor α (*TNF-α*) with respect to the negative controls.

Factor	df	*hsp70*		*IL-1β*		*TNF-α*	
		F	*P*	*F*	*P*	*F*	*P*
Concentration	5	271.10	<0.001	404.59	<0.001	11.27	<0.001
Replicate (Concentration)	6	0.35	0.908	0.47	0.826	0.82	0.559
Time	2	20.01	<0.001	1.29	0.28	6.17	<0.05
Time × Concentration	10	6.56	<0.001	2.78	0.051	1.43	0.181

This study revealed a concomitantly increased expression, in a dose–response pattern, of *hsp70* and *IL-1β* mRNA (Figs. 2 and 3), which confirmed that these endpoints can be consider as useful indicators of a potential immunotoxic effect of chemicals and their possible use as a biomarker for detecting potential immunotoxic compounds. From day 1 to day 9, *IL-1β* mRNA induction remained constant at all concentrations (Fig. 3), and it may be due to B[a]P metabolites and degradations products effects, or to the attainment of the expression threshold top. On the other hand, the sublethal concentrations of B[a]P used in the current experiment did not result in a significant increase of *TNF-α* mRNA expression (Fig. 4). The sporadically low expression responses statistically significant observed at some intermediate concentrations and the differences among treatments at different times (Fig. 4; Table 3) should be considered of no biological relevance. In this case, it could be assume that the mRNA induction threshold has not been reached. Although, TNF-α and IL-1β are involved in innate immune responses (Morales et al., 2010), the results of this study with B[a]P did not show a similar induction of mRNA for *IL-1β* and *TNF-α* (Figs. 3 and 4), suggesting different induction patterns and/or sensitivities.

Tumor necrosis factor α is a cytokine produced by activated macrophages, T cells, and some other cells. It is a member of a group of cytokines that stimulate the acute-phase reaction in mammals and fish. Recent studies in mammals suggest that TNF-α has strong antiviral effects, and it is also involved in antiviral defence in invertebrates (Morales et al., 2010). Furthermore, TNF-α induces anti-tumor immunity through direct cytotoxic effects on cancerous cells and by stimulating anti-tumor immune response (Commins et al., 2010). In the absence of specific antibodies for *Xenopus* macrophages, it is not possible to determine whether these cells are the main producers of TNF-α (Morales et al., 2010).

Interleukin-1β is primarily produced by cells of the mononuclear phagocytic lineage but is also produced by numerous other cells. Interleukin-1β production is stimulated by a variety of agents, including endotoxin, that stimulate molecular pattern receptors. Moreover, IL-1β has an anti-inflammatory function. One of the most important biologic activities of IL-1β is its ability to activate T lymphocytes by enhancing the production of IL-2 and the expression of IL-2 receptors. In the absence of IL-1β, a diminished immune response or tolerance has been observed (Commins et al., 2010). Although TNF-α and IL-1β share several biological activities, the major distinction is that TNF-α has no direct effect on lymphocyte proliferation (Commins et al., 2010).

Several chemicals have deleterious effects on cytokine production, depending on that the response of the cells to chemical exposure is chemical-specific with respect to the expression of cytokines and their receptors (Haddad et al., 2005). Preliminary results, obtained by the study of different xenobiotics (Martini et al., 2010), have suggested that mRNA expression for HSP70 and IL-1β is not dependent from the action mechanism of compounds. On the other hand, the explanation of mRNA expression for TNF-α may be found in a different response of biological mechanisms over the time, therefore further research on specific pathways with other chemicals are necessary.

5. Conclusion

Although significant progresses have been made in the last years to promote the establishment of new sensitive methods to assess immunotoxicity, at present there are no validated *in vitro* tests that can replace *in vivo* methods to evaluate immunotoxicity (Carfi' et al., 2007). For identification of immunotoxicological properties of chemicals, exposure of experimental animals to xenobiotics is usually followed by evaluation of body and organ weights, histopathological features of lymphoid organs and tissue, lymphoid cell populations, and immune function. A more recent method to study immunotoxicity is the examination of changes in gene expression profiles in immunologically relevant organs, also referred to as immunotoxicogenomics (Baken et al., 2008). The results of this study, considering the evident dose-dependent induction in response to the concentration (Figs. 2 and 3), corroborate the possibility of the use of *hsp70* and *IL-1β* mRNA expression in pre-screening protocols to identify immunotoxic compounds; however the response in the induction of *TNF-α* would require more studies to better understand its behavior. Further research about the correlation of the expression of these parameters and phagocytosis capacity also should be considered. In future, by performing tests with more substances and more species for the validation of the endpoints for a more general use of them, these parameters might be used in screening protocols to identify immunotoxic compounds, especially since the use of gene expression profiles or biomarkers may allow an earlier identification of toxic effects compared to the traditional immunotoxicity tests.

Acknowledgments

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CAPÍTULO 7

Discusión

Conclusiones

DISCUSIÓN

Desde mediados de los años 90, el declive generalizado sufrido por las poblaciones de anfibios ha recibido atención tanto de los medios de comunicación como de la comunidad científica. Algunas de las razones consideradas para explicar dicho fenómeno se asocian directa o indirectamente a las actividades humanas, tales como la destrucción de los hábitats naturales de los anfibios o la contaminación (Birge y cols., 2000; Venturino y cols., 2003; Ankley y cols., 2004; Marcogliese y cols., 2009). Debido a sus particularidades anatomofisiológicas y funcionales, a la capacidad de adaptación al medio terrestre y al acuático, al proceso de metamorfosis y a la respiración cutánea, los anfibios se pueden considerar organismos centinelas de la salud medioambiental con una susceptibilidad frente a los contaminantes superior respecto a otros organismos acuáticos ampliamente usados en ensayos ecotoxicológicos, como por ejemplo los peces (Burkhart y col., 2003). Los protocolos de evaluación de riesgo medioambiental para contaminantes puros o mezclas complejas incluyen ensayos ecotoxicológicos en peces para estudiar los efectos de las exposiciones agudas o crónicas en larvas o en adultos. Del mismo modo, en el caso de los anfibios, sería de especial interés definir los efectos agudos o crónicos en los diferentes estadios de desarrollo.

Con el objetivo de comprobar la posible integración de los ensayos con anfibios en los protocolos de evaluación de riesgo ambiental de contaminantes puros y mezclas complejas se ha desarrollado el ensayo de toxicidad aguda con las larvas del anfibio *X. laevis* descrito en el capítulo 2 de la presente tesis. En la puesta a punto de este nuevo ensayo de toxicidad aguda se comparó por un lado la sensibilidad de las larvas de *X. laevis* respecto a los embriones del ensayo FETAX y, por otro lado, la sensibilidad en una misma fase de desarrollo pero en especies diferentes utilizando larvas de *X. laevis* y larvas de peces.

En la presente tesis, *X. laevis* fue elegido como modelo animal debido a su facilidad de manejo y de cría en cautividad, así como por ser la especie más

estudiada y mejor conocida en investigación (Sparling y cols., 2000, Robert y Ohta, 2009). No obstante, en contra de esta elección, existían estudios previos, como los de Birge y colaboradores (2000), donde *X. laevis* aparecía como una de las especies más tolerantes entre las usadas en las pruebas de toxicidad en fases tempranas de desarrollo, que se realizaron para clasificar 25 especies de anfibios como muy sensibles, sensibles, moderadamente tolerantes o tolerantes a los metales y contaminantes orgánicos, teniendo siempre a la trucha arcoíris (*Oncorhynchus mykiss*) como organismo de referencia.

Sin embargo, muchos de estos estudios sobre *X. laevis*, al igual que otros más recientes como por ejemplo el estudio de Carlsson y Norrgren (2014) en dos especies de anfibios (*Xenopus tropicalis* y *Rana arvalis*) y una de peces (*Danio rerio*), eran realizados usando el ensayo FETAX que utiliza la fase embrionaria, y una de las hipótesis que la presente tesis pretende comprobar es el hecho de que pudiera existir diferente sensibilidad frente a la exposición de sustancias toxicas en función del estadio de desarrollo del organismo; por ejemplo en un estudio de Howe y colaboradores (1998) se formula la hipótesis de que las fases larvarias más tardías de los anfibios podrían ser más sensibles que los bioindicadores acuáticos tradicionales para detectar los efectos de determinados contaminantes medioambientales.

No obstante, aunque era de esperar que la fase larvaria fuera más sensible respecto a la fase embrionaria (Howe y cols, 1998), la comparación de nuestros resultados y los datos encontrados en la literatura para el ensayo FETAX mostraron una sensibilidad igual o superior de este ensayo con respecto a los resultados obtenidos en el ensayo agudo en larvas. Por ejemplo, en las gástrulas de *X. laevis* expuestas al selenito de sodio ($NaSeO_3$) la concentración letal en el 50% de los casos a 96 horas (96h-LC_{50}) fue 2,3 mg/L (Browne y Dumont, 1979), lo que no difiere sustancialmente del valor obtenido en larvas, que fue de 6,2 mg/L. En el caso del dicloruro de cobre ($CuCl_2$), la 96h-LC_{50} del ensayo con larvas (2,3 mg/L) superó ligeramente el límite superior del rango establecido por Buchwalter y colaboradores (1996) para los embriones (0,042- 1,180 mg/L). Finalmente, la sensibilidad de las larvas al sulfato de zinc ($ZnSO_4$) fue sustancialmente inferior respecto a la sensibilidad

del ensayo FETAX frente al mismo compuesto; la 96h-LC_{50} en larvas fue 56,44 mg/L, mientras que para el ensayo FETAX se referenciaban unos valores de 3,6 mg/L(página web 8).

Sin embargo, una de las ventajas de este ensayo frente a la exposición con la fase embrionaria fue la posibilidad de detectar efectos subletales, tales como los descritos en el capítulo 2, por la exposición a compuestos como la ivermectina y las sales de zinc y cobre (tabla 2- capítulo 2) que dejan claras evidencias de la acción de dichos tóxicos sobre la viabilidad de las larvas de *X. laevis*. Las larvas expuestas al $CuCl_2$ eran inmaduras y despigmentadas, las expuestas a la ivermectina presentaban alteración de la locomoción y de la orientación (hiperactividad), y finalmente el $ZnSO_4$ provocó edema cráneo-facial y abdominal en los renacuajos (figura 1- capítulo 2). Dichos efectos, en el medio natural, podrían ser causa de un aumento de la susceptibilidad de los renacuajos a la depredación, como en las larvas despigmentadas por la acción del trifeniltin descritas por Yuan y colaboradores (2011), o reducir su capacidad de nutrición como consecuencia del retraso de desarrollo y del crecimiento (Yuan y col., 2011). Del mismo modo, los cambios en las funciones cognitivas y fisio-motoras, como la hiperactividad producida por la ivermectina, están generalmente relacionadas con neuropatías tóxicas (Moser, 2007), así como la disfunción renal o cualquier alteración metabólica pueden ser las causantes del edema presentado por los animales expuestos al $ZnSO_4$.

Por otro lado, revisando los datos de toxicidad aguda descritos en peces se comprueba que no fueron siempre suficientemente protectores cuando se trasladaban a los anfibios. Por ejemplo, comparando la sensibilidad de las larvas de *X. laevis* expuestas a $NaSeO_3$ (96h-LC50= 6,2 mg/L) respecto a los resultados del estudio de Buhl y Hamilton (1991) en trucha arcoíris, donde la 96h-LC_{50} fue de 118 mg/L, se demostró una sensibilidad mayor para *X. laevis*. Del mismo modo, en el estudio de sensibilidad a los metales y a los contaminantes orgánicos de Birge y colaboradores (2000) la comparación entre los valores de LC_{50} en anfibios y peces en un total de 573 muestras demostró una mayor sensibilidad de los anfibios en un 67% de los casos.

Comparando los datos considerados más interesantes del mismo estudio de Birge y colaboradores (2000) (tabla 3 - capítulo 2) con los obtenidos en el ensayo agudo en larvas (tabla 2- capítulo 2) se observó como *X. laevis* no siempre fue la especie más tolerante. Por ejemplo, *Xenopus* fue más sensible que la lubina negra (*Micropterus salmoides*) a los efectos letales del selenio, más sensible que el sapo de Fowler (*B. fowleri*) al zinc y generalmente es más sensible que los peces (a excepción de la trucha arcoíris, *Oncorhynchus mykiss*), que la salamandra *Ambystoma opacum* o que el sapo de Fowler (*Bufo fowleri)* a los efectos letales del cobre. Si bien para los antibióticos ensayados en el capítulo 2 (oxitetraciclina, tetraciclina y sulfamida asociada a trimetoprim) y para el manganeso no se observaron efectos letales o subletales, los datos obtenidos para ivermectina, zinc, selenio y cobre demostraron que el ensayo con las fases larvarias de *X. laevis* podría ser una buena alternativa de elección a la hora de evaluar la toxicidad de contaminantes acuáticos, tanto orgánicos como inorgánicos, sobre el grupo taxonómico de los anfibios.

El enfoque tradicional para definir la calidad de las aguas se basa en su análisis químico y físico. Sin embargo, la asociación de dichos parámetros con ensayos biológicos ofrece una mejor caracterización ecotoxicológica de los contaminantes. Una vez desarrollado el nuevo ensayo de toxicidad aguda en *X. laevis* y comparada su sensibilidad respecto a los ensayos con embriones y a larvas de otras especies de referencia para el medio acuático, se quiso, primero incorporar este ensayo en una batería de ensayos convencionales para la caracterización biológica de residuos peligrosos y mezclas complejas potencialmente peligrosas (capítulo 3), y posteriormente usarlo con los Residuos Sólidos Urbanos (RSU) (capítulo 4). Dicha incorporación pretendía confirmar la eficacia del ensayo de toxicidad aguda en larvas de *X. laevis* a la hora de valorar efectos tóxicos de mezclas complejas, y evaluar la posibilidad de ser incluido en cualquier batería de ensayos para realizar las primeras fases de seguimiento ecotoxicológico de contaminación del medio acuático para estos tipos de residuos, así como poder facilitar la gestión del los RSU.

Las baterías de ensayos utilizadas en los trabajos de investigación descritos en los capítulos 3 y 4 combinaron pruebas *in vivo* e *in vitro* sobre

diferentes grupos taxonómicos y consideraron diversos parámetros ecotoxicológicos. Los bioensayos han sido incorporados a la evaluación de riesgo de los residuos para estimar de manera global, y por tanto de un modo más realista, la toxicidad de las muestras complejas comparada con la caracterización química (Słomczyńska y Słomczyński, 2004; Thomas y col., 2009; de Lapuente y cols., 2014). Este tipo de enfoque se denomina Evaluación Directa de la Toxicidad (DTA) y permite cuantificar la toxicidad de muestras complejas medioambientales a través del estudio de las diluciones de dichas muestras en bioensayos que cubran diferentes grupos taxonómicos (Tinsley y col., 2004). La DTA ofrece una medición combinada entre todos los compuestos presentes en la muestra y toma en consideración los posibles sinergismos y antagonismos, así como la biodisponibilidad de cada compuesto (Pivato y Gaspari, 2006). Si bien la DTA ha sido usada para la evaluación de la toxicidad de los RSU (Słomczyńska y Słomczyński, 2004; Thomas y col., 2009), en la práctica actual la gestión de dichos residuos se basa exclusivamente en una caracterización físico-química.

Para la elección de las baterías de ensayos, el de toxicidad en *Daphnia magna* fue seleccionado como referente debido a que es uno de los ensayos convencionalmente usados en ecotoxicológia (ISO 6341, 1996), además de tener una sensibilidad a los compuestos tóxicos bien conocida. Con el objetivo de incrementar el conocimiento acerca de como diferentes ensayos *in vivo* pueden ser usados para la identificación aguda de los contaminantes del compartimiento acuático, también se tomaron en consideración bioensayos alternativos, no comúnmente utilizados, como el ensayo agudo en larvas de *X. laevis*. De este modo, se cubrió la reconocida necesidad de incluir en las baterías de ensayos organismos que puedan abarcar los diferentes niveles tróficos (Słomczyńska y Słomczyński, 2004; Thomas y col., 2009). Además, entre las pruebas *in vitro* se consideraron el ensayo DR-Calux®, prueba diseñada para la detección de la activación del receptor aril hidrocarburo (AhR) producida por los compuestos coplanares, y los ensayos de citotoxicidad en la línea celular RTG-2 de trucha arcoíris, que incluyeron: la prueba de viabilidad celular medida a través de el ensayo "*neutral red assay*", la prueba de

activación del citocromo CYP1A medido a través de la actividad EROD (7-etoxiresorufina-O-desetilasa) y la prueba de predicción de la defensa celular medida mediante la actividad beta-galactosidasa.

Los resultados del estudio descrito en el capítulo 3 clasificaron como toxicas el 60% de las muestras de residuos peligrosos ensayadas en las larvas de *X. laevis* (tabla 3- capítulo 3). Además, en comparación con las otras pruebas (tabla 2 y figura 2- capítulo 3), el ensayo con *Xenopus* detectó el porcentaje más elevado (46,67%) de muestras clasificables dentro de la categoría 7, o sea la más tóxica según la clasificación realizada por Manusadžianas y colaboradores (2003), seguido por un 40% de toxicidad detectada por la prueba *in vivo* con *D. magna* y por el ensayo DR-Calux®. Los porcentajes más bajos de detección de toxicidad fueron aquellos obtenidos con las pruebas *in vitro* con la línea celular RTG-2 de trucha arcoíris: un 20% en la prueba de viabilidad celular, un 6,67% por la prueba de activación del citocromo CYP1A y finalmente un 6,67% la prueba de predicción de la defensa celular.

Por otro lado, comparando el ensayo *in vivo* de *X. laevis* con el ensayo de *D. magna* se comprobó que el ensayo con el anuro no detectó toxicidad en muestras que resultaron toxicas en el ensayo con el microcrustaceo (tabla 2 y figura 2- capítulo 3). Por tanto, esa doble capacidad del ensayo con *X. laevis* de detectar tanto las muestras más tóxicas, como de no detectar toxicidad en un algunos de los casos en los que el ensayo con *D. magna* sí lo había hecho podría indicar la selectividad del ensayo con el anfibio a la hora de detectar compuestos tóxicos presentes en las mezclas complejas descritas en el capítulo·3. Esa mayor selectividad justificaría la inclusión de este ensayo en las baterías de seguimiento ecotoxicológico, conjuntamente con el ensayo de *D. magna*, porque permitiría reducir la incertidumbre y mejorar la caracterización de las muestras de composición no conocida, además de su ya comentada capacidad para detectar no sólo efectos agudos sino también subletales.

Cuando se utilizaron estas mismas baterías de ensayos para la determinación de la toxicidad de los RSU (capítulo 4), los resultados de los ensayos con *Daphnia* y *Xenopus* volvieron a ser más sensibles que los ensayos

in vitro con la línea celular RTG-2 de trucha arcoíris, obteniéndose con estos ensayos *in vivo* los valores más elevados de Unidades de Toxicidad (TU) (tabla 2- capítulo 4), calculados a través de la formula de Sprague y Ramsay (1965):

$$TU= [1/L(E)C_{50}]* 100$$

Si como se ha comentado, el ensayo con *Daphnia magna* es el referente en varios programas de seguimiento ecotoxicológico (Clément y cols., 1996; Isidori y cols., 2003 Bortolotto y cols., 2009), nuevamente los resultados del ensayo agudo con larvas de *X. laevis* mostraron toxicidades muy similares para las mismas muestras de lixiviados que el ensayo con *D. magna* (tabla 2- capítulo 4), lo que vuelve a remarcar la sensibilidad del ensayo con *Xenopus*. Este trabajo sin embargo daba un paso más allá y trataba de correlacionar la caracterización fisicoquímica de muestras complejas con la caracterización biológica, para permitir, mediante un análisis discriminante, clasificar la toxicidad de los residuos en base a sus propiedades físico-químicas. Los resultados obtenidos mostraron correlaciones muy altas entre propiedades fisicoquímicas y toxicidad tanto para la caracterización biológica realizada con *Daphnia* (0,85) como con *Xenopus* (0,86) y algo menor cuando se realizaba con el test *in vitro* con la línea RTG-2 (0,55). Además, el análisis discriminante también determinó que la caracterización biológica realizada con *X. laevis* presentaba un 0% de falsos positivos (muestras no tóxicas que por sus propiedades fisicoquímicas se considerarían tóxicas), frente a porcentajes también pequeños de los otros ensayos (4,8% en los casos de *D. magna* y el ensayo RTG-2). Las detecciones de falsos negativos (muestras tóxicas que se clasificaban como no tóxicas) fueron también pequeñas (menores del 10% para *Xenopus* y del 15% para las otras pruebas). Todo ello representa una ventaja económica añadida para la gestión de los residuos, evitando el tratamiento como tóxicos de residuos que en realidad no lo son.

En conclusión, en escenarios de exposiciones a corto plazo, la incorporación del ensayo con el género *Xenopus* en las baterías de seguimiento ecotoxicológico que se han estudiado en la presente tesis contribuyó a una

mejor caracterización temprana de muestras complejas, ampliando la información al grupo taxonómico de los anfibios que todavía no está adecuadamente valorado en ecotoxicología. Además, el ensayo agudo con larvas de *X. laevis* puede ofrecer la posibilidad de detectar no solo la mortalidad sino también las anormalidades determinadas por exposiciones a corto plazo (ej: edema, malformaciones esqueléticas). Su uso está siendo ampliado a otros escenarios de evaluación; por ejemplo, en las evaluaciones de los efectos a largo plazo, animales del género *Xenopus* han sido incorporados a diferentes baterías de ensayos para la determinación de los efectos genotóxicos (Perrodin y cols., 2006); asimismo se han propuesto como modelo para investigar mecanismos de acción específicos, como por ejemplo en la identificación de disrupción química del eje hipotálamo-hipófisis-tiroideo (Opizt y cols., 2005).

Una vez demostrado que el ensayo de toxicidad con *X. laevis* era capaz de poner en evidencia efectos subletales, los trabajos de investigación de la presente tesis se centraron en el desarrollo de ensayos novedosos que pudieran ser capaces de detectar efectos más específicos relacionados con la respuesta inmune de los organismos (capítulos 5 y 6). El estudio del sistema inmune está reconocido como un punto crítico en los procesos de evaluación de riesgo (Woolhiser y cols., 2005), y la selección de los criterios de valoración inmunotoxicológica es una prioridad para la adecuada interpretación de los efectos inmunes subletales que de otro modo no serían fácilmente cuantificables por los índices de salud de referencia (Kacmár y cols., 1999).

El estudio de la liberación de citoquinas se considera de gran utilidad para poder definir la toxicidad de las sustancias químicas a nivel del sistema inmune (Carfí y col., 2007). En mamíferos, la acción de las citoquinas como reguladores de la respuesta inmune, tanto innata como adaptativa, ha sido bien documentada. Algunas citoquinas típicas de la respuesta inmune innata, como las citoquinas pro-inflamatorias (por ejemplo IL-1β, IL-6, IL-8 y TNF-α), podrían ser universales o tener funciones equivalentes en vertebrados e invertebrados (Kaiser y col., 2004). Los cambios que se pueden llegar a producir en la expresión de los genes por la exposición a contaminantes ambientales contaminantes han sido objeto de interés tanto por parte de la

industria como de las agencias reguladoras (Luebke y cols., 2006). No obstante, hasta la fecha solo unos pocos laboratorios han investigado sistemáticamente la expresión génica como una herramienta de detección potencial para compuestos inmunotóxicos. Si bien estudios previos han demostrado la capacidad de las sustancias químicas en producir una respuesta de naturaleza proteica al estrés (inducción de HSP70), que en algunos casos se asocia a la producción de citoquinas pro-inflamatorias tales como la IL-1β (Cruz-Rodríguez y Chu, 2002; Gornati y cols., 2002; Robert, 2003), los resultados de esta tesis mostraron por primera vez cómo la expresión de ARNm para la HSP70 e la IL-1β producida por la exposición a contaminantes ambientales, puede ser empleada como posible indicador temprano del riesgo asociado a alteraciones del sistema inmune de *X. laevis*. La respuesta inmune de las larvas expuestas en el ensayo descrito en el capítulo 5 a tiabendazol, fluoxetina y cipermetrina pone en evidencia dicha relación a través del aumento del ARNm para ambas proteínas. Dicho aumento fue muy marcado en el caso del tiabendazol (figura 3-capítulo 5) y menos evidente en el caso de la cipermetrina (figura 4- capítulo 5). Estos resultados fueron sucesivamente corroborados y ampliados en el estudio presentado en el capítulo 6 donde, además de la HSP70 y la IL-1β, se tomó en consideración también el factor de necrosis tumoral α (TNF-α). En este caso el aumento la expresión de ARNm para la HSP70 y la IL-1β no solo se relacionó con la exposición a contaminantes a unos niveles relevantes desde el punto de vista ambiental, que en este caso fue el B[a]P, sino que también se encontró una relación entre dichos efectos y la concentración del contaminante.

Para poder relacionar las respuestas observadas con los compuestos estudiados en los capítulos 5 y 6 es interesante describir algunos parámetros relevantes del comportamiento de dichos compuestos, como por ejemplo sus incorporaciones al agua, sus concentraciones y sus grados de disipación o degradación. Los compuestos fueron añadidos al agua en una única administración al principio del ensayo y la vida media de disipación se correspondió con lo esperado para cada uno de ellos. La fluctuación de la fluoxetina entre el 73 y el 53% de las concentraciones nominales iniciales (0,3

y 30 µg/L) (figura 1a- capítulo 5) se explicó en los primeros muestreos por fenómenos de adsorción a partículas orgánicas y posteriormente por degradación y disipación. Consecuentemente, la expresión génica del *hsp70* y del *IL-1β* presentó una respuesta fluctuante en relación al tiempo y a la concentración; a la concentración más baja de fluoxetina (0,3 µg/L) la expresión génica del *hsp70* mostró un pico a las 24 horas y el *IL-1β* aumentó lentamente hasta alcanzar su valor máximo en el día 9, mientras que a la concentración de 30 µg/Ll la expresión de ambos genes fue muy evidente desde el principio (figura 2- capítulo 5). El tiabendazol mantuvo a lo largo de todo el ensayo unas concentraciones cercanas a los niveles nominales iniciales (figura 1b- capítulo 5), lo que se reflejó en una expresión sostenida de la HSP70 y de la IL-1β (figura 3- capítulo 5), mientras que la cipermetrina se disipó rápidamente (figura 1c- capítulo 5). No obstante, el tiempo de disipación medio fue de 24 horas, valor muy similar al calculado por González-Doncel (2004). La cipermetrina indujo la expresión del ARNm de ambas proteínas: la expresión de la HSP70 alcanzó el pico máximo a las 24 horas para después volver a aumentar lentamente en los tiempos siguientes, mientras que la expresión de la IL-1β fue bastante elevada a lo largo de todo el ensayo (figura 4- capítulo 5). Este hecho puede confirmar la capacidad de respuesta de los genes *hsp70* e *IL-1β* a exposiciones puntuales del contaminante.

En los diferentes muestreos a lo largo del ensayo concentración-respuesta con B[a]P (capítulo 6), cuanto más baja era la concentración inicial del B[a]P tanto menos persistente fue el compuesto en el agua (tabla 1- capítulo 6). En todos los casos, las concentraciones medidas al final del ensayo fueron sustancialmente menores respecto a las iniciales, probablemente por la disipación debida a la fotolisis y a la evaporación, hecho previamente descrito en otros estudios (Seo y cols, 2007). El tiempo de disipación medio fue de 73 horas (figura 1- capítulo 6), siendo parecido al calculado por Swietlik y col. (2002) (3,2 días) en un estudio de microcosmos realizado con condiciones ambientales controladas. De todos modos, las inducciones del ARNm para la HSP70 y la IL-1β presentaron una relación concentración-respuesta muy clara en el caso de la exposición a B[a]P (figuras 2 y 3- capítulo 6), confirmando la

posibilidad del uso de estos parámetros como biomarcadores en la detección de compuestos con efectos inmunotóxicos. A las dos concentraciones más altas de B[a]P, el incremento de la expresión de ARNm de la HSP70 no sólo estuvo relacionado con la concentración sino también con el tiempo (figura 2 - capítulo 6), mientras que los niveles de inducción para la IL-1β no mostraron cambios significativos entre el día 1 y 9 (figura 3- capítulo 6). Esto pudo ser debido a que los metabolitos o los productos de degradación del B[a]P seguían induciendo la expresión génica del *hsp70*, o bien a que se había alcanzado el umbral máximo de inducción en el caso del *IL-1β*. Por último, la exposición a las diferentes concentraciones subletales de B[a]P no se asoció a una variación significativa en la expresión del ARNm para el TNF-α, con algunas excepciones que no se consideraron biológicamente relevantes (figura 4- capítulo 6), por lo que se concluyó que la exposición no alcanzó los niveles suficientes para la inducción del gen.

Debido a la importancia del sistema inmune para la conservación del buen estado de salud los individuos, se ha dado mucha importancia al desarrollo de indicadores que puedan predecir los efectos inmunotóxicos de las exposiciones a los contaminantes ambientales. Actualmente, existe una batería de ensayos bien caracterizada, que fue originariamente desarrollada en roedores y organizada en niveles (*tier I* y *tier II*) para ensayar parámetros funcionales e histopatológicos (Krzystyniak y cols, 1995; Galloway y Handy, 2003), que lamentablemente sigue siendo inespecífica para los anfibios. Además, por la complejidad del sistema inmune, ningún indicador inmunológico debería ser utilizado como único parámetro para determinar la inmunotoxicidad inducida por los xenobióticos. La clave en la predicción de la inmunotoxicidad en las poblaciones silvestres de los organismos acuáticos está en el uso de baterías de ensayos que engloben indicadores bioquímicos, fisiológicos y histopatológicos. La presente tesis ofrece nuevos ensayos agudos y subagudos con el anfibio *Xenopus laevis* que podrían ser incluidas en las baterías convencionales de ensayos ecotoxicológicos. Los resultados de este trabajo de investigación sugieren que la expresión de *hsp70* y de *IL-1β* no dependen del mecanismo de acción del compuesto, siendo por tanto buenos modelos para el estudio de la

inmunotoxicidad. Además, los ensayos de inmunotoxicidad con *X. laevis* podrían incluso ser propuestos como indicadores de peligro subletal en estudios de inmunotoxicidad para salud humana, debido a la alta conservación de las HSP entre los vertebrados, además de la asociación de HSP e IL-1β con los procesos de inmunidad no específica. Las hipótesis de este trabajo se deberían confirmar con estudios de correlación entre estos ensayos y otros más específicos y más estandarizados, como por ejemplo los estudios de inhibición de la capacidad de fagocitosis.

Los Comités Científicos de Evaluación de Riesgos de la Unión Europea han señalado recientemente las limitaciones de las metodologías de evaluación de riesgos ambientales actuales y la necesidad de desarrollar nuevas metodologías que consideren no solo la especie más sensible sino la relevancia ecológica de las especies afectadas y de los efectos observados (página web 9; página web 10); las prioridades incluyen el desarrollo de nuevas metodologías para valorar la vulnerabilidad de los ecosistemas acuáticos y el desarrollo de metodologías que permitan valorar las consecuencias reales para comunidades y ecosistemas, incluyendo los efectos indirectos. Los resultados de esta tesis contribuyen a estas prioridades, con el desarrollo de nuevos ensayos sobre un grupo taxonómico de alta relevancia pero escasamente cubierto por los protocolos actuales, y la inclusión de efectos inmunotóxicos cuya valoración es esencial para evaluar efectos indirectos que incluyen el incremento en la sensibilidad de las poblaciones frente a agentes patógenos, y que en determinadas ocasiones pueden originar efectos ambientales catastróficos (e.g., Muñoz y col., 1994).

CONCLUSIONES

1. Al comparar los efectos producidos por los contaminantes en dos ensayos con diferentes fases de desarrollo de *Xenopus laevis*, la larvaria y la embrionaria, se evidenció que frente a la mayoría de los compuestos estudiados la sensibilidad con el ensayo FETAX fue igual o superior que la detectada por las larvas. Sin embargo, estas últimas mostraron efectos subletales que en muchos casos podrían llegar a ser incompatibles con la vida. Por lo tanto, el ensayo agudo con larvas de *X. laevis* desarrollado en este trabajo no solo fue capaz de detectar mortalidad, sino también alteraciones subletales que derivan de las anormalidades asociadas a las exposiciones a corto plazo.

2. Al comparar los resultados de los ensayos agudos con larvas del género *Xenopus* con otros ensayos agudos con fases larvarias en peces, se comprobó que en algunos casos los valores obtenidos en peces pueden no proteger suficientemente a los anfibios, que representan una clase especialmente sensible al estrés químico. Por tanto, se destaca la utilidad de la inclusión del ensayo agudo con larvas de *X. laevis* en las baterías clásicas de ensayos de seguimiento ecotoxicológico, siguiendo además las directrices de los nuevos desarrollos de modelos ERA que requieren la consideración de diferentes organismos relevantes incluso cuando éstos no sean los más sensibles.

3. Los resultados de la caracterización biológica de los residuos tóxicos y peligrosos y los residuos sólidos urbanos corroboraron que el ensayo agudo con larvas del anfibio *X. laevis* es una prueba eficaz a la hora de valorar los efectos tóxicos de mezclas complejas. Por tanto, podría ser incluido dentro de cualquier batería de ensayos de

seguimiento ecotoxicológico para estos tipos de muestras. Además, este ensayo permitió detectar toxicidad de manera selectiva y favoreció la clasificación de la toxicidad de las muestras complejas en función de sus propiedades fisicoquímicas con el menor número de falsos positivos y negativos.

4. En los estudios de efectos subletales inmunotóxicos en los que se asoció la variación de expresión génica con la toxicidad de los contaminantes emergentes y persistentes, los resultados obtenidos con tiabendazol, fluoxetina, cipermetrina y B[a]P sugieren que la expresión de ARNm para la HSP70 y la IL-1β no depende del mecanismo de acción de los compuestos, lo que las hace más fiables en su uso como biomarcadores de inmunotoxicidad. Debido al hecho de que las proteínas TNF-α e IL-1β son mediadores de la respuesta inmune innata, en la exposición con B[a]P cabía esperar un mismo comportamiento para ambas proteínas. Sin embargo, la proteína TNF-α no se expresó de la misma manera que la IL-1β, lo que lleva a suponer que la TNF-α necesita un tipo de estimulo diferente, por ejemplo por naturaleza o intensidad, respecto a aquél que se logró con el B[a]P.

5. Demostrada la relación concentración-respuesta entre la exposición a concentraciones subletales de B[a]P y la expresión génica de *hsp70* e *IL-1β,* estos parámetros se ofrecen como indicadores precoces de riesgo que podrían ser incluidos en las baterías inmunotoxicológicas para el medio acuático y en futuros estudios de las rutas *Adverse Outcome Pathways* (AOP).

6. La conservación de la proteína HSP70 y la asociación de la misma y la IL-1β a la respuesta inmune no específica indican que HSP70 y IL-1β podrían ser útiles como indicadores de peligro subletales incluso para la salud humana.

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