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TOXICOGENOMIC ANALYSIS OF THE ENVIRONMENTAL IMPACT IN AQUATIC SYSTEMS

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Director

Dr. Benjamin Piña Capó

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ABSTRACT

Field biomonitoring based on molecular biomarkers detects early warning signals of stress suffered by organisms exposed to contamination. The lower part of the Ebro River basin has a long pollution history related to the presence of a chlor-alkali plant adjacent to Flix. In addition, the Ebro River has been affected by the invasion of alien species that damage its natural ecosystem. In this thesis, *Cyprinus carpio* (common carp) and *Dreissena polymorpha* (zebra mussel) were used as a model species in laboratory and field studies intended to the development of a multi-molecular biomarker approach to identify the most relevantly effects of pollutants in the field. Gene expression techniques were used to study specific physiological defensive mechanism. The application and improvement of a well–established markers in different aquatic species and following different approaches allowed to analyze modes of action and to outline effects of persistent organic and inorganic pollutants found in the field. The results showed that chronic exposure to mercury of common carp results in increased levels of metallothionein in kidney, in scales (albeit at lower extent), but not in liver, considered as the primary detoxification organ. The measure of gene expression in scales provides the possibility of a new non-lethal method of study. Studies of toxicant effects in zebra mussel revealed that this bivalve could be used as potential sentinel specie for freshwater monitoring. Analysis of its detoxicfication mechanisms, and particularly of its ABC membrane transporters in adult and the early life stages could help understanding the survival of this species in highly contaminated areas.

Keywords: Molecular biomarkers, gene expression, Common carp, Zebra mussel, mercury, metallothionein, ABC transporters

PROLOGUE

The Ebro River is one of the Spanish longest and most important rivers, and probably the most studied and monitored one (Lacorte, S., et al 2006; Olivares, A. et al. 2010). Despite its great economic, social and ecological importance, several sources of pollution have been identified along its course. Probably the most important one, located just 90 km from the river mouth, is the Flix chlor-alkaly plant, whose continuous operation during more than 100 years resulted in the presence of about 200000 tons of contaminated mud in the river bed adjacent to the factory. This mud contains high concentrations of heavy metals (mercury, cadmium, chromium and nickel) as well as organochlorine compounds (hexachlorobenzene, polychlorinated biphenyls, etc., Grimalt, J.O. 2009). These pollutants are highly bioaccumulable and biomagnificable through the food chain in aquatic systems. Due to the potential high toxicity of the factory residues and the importance of the Ebro River, several monitoring campaigns have bee launched to assess the ecological status of the river. In this thesis we participated in two of these campaigns, the AquaTerra project, founded by the European Union and constituting one of the most ambitious studies of freshwater monitoring in whole Europe, and the MOBITROF (*Movilización de contaminantes a través de las cadenas tróficas*), an agreement between the Spanish Ministry of Environment and the Government of Catalonia (2005) to evaluate the impact in the lower part of the river as well as the assessment of the effects through the food chain The results showed the need for extension of this kind of monitoring campaigns to measure temporal tendencies in the aquatic systems (Navarro-Ortega, A. and Barceló, D. 2011), and extend the recommendation, afterwards honored by the Spanish Government, to remove the residues from the Ebro River bed (Grimalt, J.O. 2009).

On the other hand, many water bodies, in Spain and elsewhere, Spanish water bodies like many other countries have been suffering the invasion of new species able to change the equilibrium of the native ecosystems, and Ebro River basin was not an exception. The last recruitment was the bivalve *Dreissena polymorpha*, commonly known as zebra mussel, native from the Caspian Sea. This bivalve has a great colonization capacity into new freshwater bodies, as it has been spread all over Europe and in freshwaters from the East Coast of the United States. This colonization success is due to its high reproductive output, reaching its sexual maturity at one-two years, when reaching sizes of only 3 to 5 mm) (O'Neill, C.H. and MacNeill D.B. 1991). Besides its reproductive strategies, Zebra mussel is a byssal specie that can attach to any hard structure present in the water, like pipes

and other water conduction systems. As a consequence, it compromises water intake facilities for electrical generation, industry and urban water, generating enormous costs in cleaning. Finally, its very high filter-feeding ratio and its very highly dense populations consume all food available in the area, entering in competition with native species.

The study done in this thesis started in 2006 as part of a project studying the effect in fish of the pollutants in Flix area and down-stream. In 2007, two new projects from the Spanish Ministry, ZebraPop and ZebraGest, of the Environment allowed the study of the new invasive bivalve *Dreissena polymorpha* in order to obtain more information about its sensitivity to the pollutants.

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Acronym & Symbol List

ABD: ATP- Binding Domain
AhR: Aril hydrocarbon receptor
ARE: Antioxidant response element
ATP: Adenosine triphosphate
Act: Acetone
B: Half body
BFR: Brominated Flame Retardant
B-NF: ß-naphthoflavone
C: Control
Ca_AM: Calcein AM
CAT: Catalase
Cd: Cadmium
CDD: Conserved Domain Database
COI: Cytochrome c Oxidase
COP: Contaminant organic persistent
Ct: Cycle threshold
Cp: Crossing point
Cq: Quantification cycle
Cu: Copper
CYP1A: Cytochrome P450 1A
DBD: DNA Banding Domain
DCPA: Dacthal
DG: Digestive Gland
dpf: days post-fertilization
EF1: Elongation Factor gene
ERA: Environmental risk assessment
EROD: Ethoxyresorufin-O-deethylase
F: female
Fe: Iron
Feb: February
Fw: Forward
GPx Glutathione peroxidase
GSH: Reduced glutathione
GST: Glutathione-S-transferase
Hg: Mercury
HKG: Housekeeping
hpf: hour post-fertilization
HSP: Heat shock protein
IC50: 50% Inhibition concentration
Jan.: January
G: Gonad
L1/L2: Larvae
LBD: Ligand Banding Domain
LC50: 50% lethal concentration
M: Male
MEPC: Marine Environmental Protection Committee
mRNA: messenger RNA
MRP: Multidrug resistance associated protein
MT: Metallothionein
MXR: Multixenobiotic resistance
NI: Non –injected
NR: Nuclear receptors
NS: Non significant variations
PAH: Polycyclic aromatic hydrocarbons
PBS: Phosphate saline buffer
PCB: Polychlorobiphenyls
PCR: Polymerase chain reaction
ppb: part per billion
ppm: part per million
PCR: Polymerase Chain Reaction
P-gp: P-glycoprotein
qRT-PCR: Quantitative Real-Time PCR
RhB: Rhodamine B
Re: Reverse
Ref: Reference gene
ROS: Ractive oxygen species
RxR: Retinoid x Receptor
SC: Solvent Control
SD: Standard deviation
Sept: September
SOD: Superoxide dismutase
TBT: Tributyltin

Tg: Target gene

THg: Total mercury

TMD: Transmembrane Domain

UNEP: United Nations Environmental

Program

VI: Vehicle injected

Wt: Weight

WW: wet weight

ZM: *Dreissena polymorpha*

Zn: zinc

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Chapter 1: General Introducción

1. Introducción general

1.1. Evaluación ambiental en sistemas acuáticos

El desarrollo tecnológico e industrial ha significado en general prosperidad y bienestar para el ser humano. Sin embargo, el crecimiento de las poblaciones, el acelerado desarrollo de la industria junto al incremento de las actividades agrícolas y el consumo de medicamentos han introducido miles de nuevos compuestos químicos en los ecosistemas. Estos compuestos pueden generar perturbaciones en el medio y romper así con el equilibrio del ecosistema que se mantiene por un intercambio abierto de materia y energía. En algunos casos, después de unas perturbaciones severas, el ecosistema no se puede recuperar y entra en un estado de degradación.

En el 2006, el EINECS (*European Inventory of Existing Commercial Chemical Substances*) identificó cerca de 100.000 sustancias químicas, de las cuales 30 o 40 están actualmente bajo monitorización. Estas sustancias pueden llegar a los ecosistemas por tres vías diferentes: por vertido no intencionado como es el caso de los accidentes y escorrentías o *run-off*; los vertidos intencionados de aguas residuales de las zonas industriales y urbanas; y por deposición atmosférica (Walker et a. 1997). La mayor parte de las sustancias químicas tienen como destino final los sistemas acuáticos.

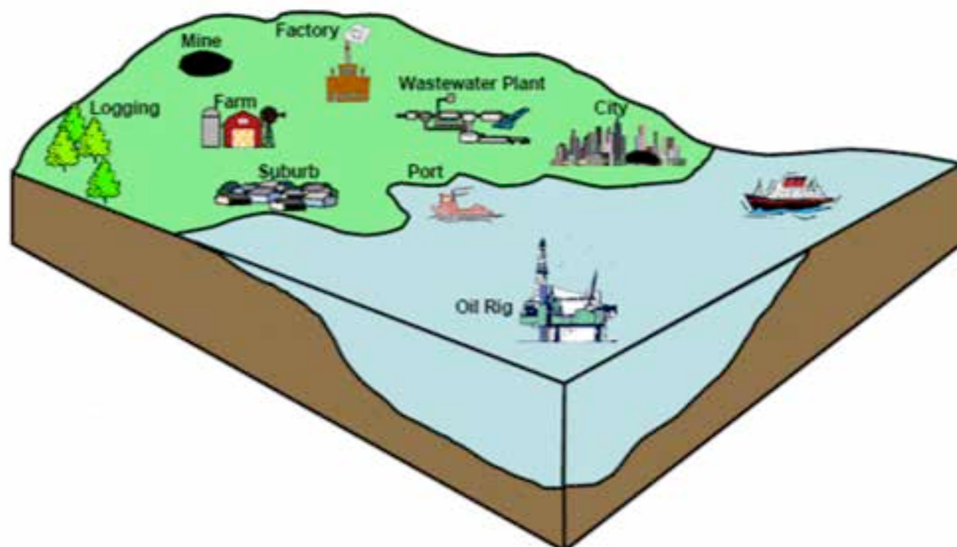


Fig 1.1 Representación de las principales fuentes de emisión de contaminantes en los sistemas acuáticos

El agua es uno de los recursos naturales más importantes para el ser humano, el cual la utiliza para beber, para el regadío, recreación y como fuente de alimentos, al ser el hábitat de muchas especies importantes en la dieta como peces y bivalvos. El hecho de que los sistemas acuáticos sean el destino final de muchas de las sustancias químicas puede afectar seriamente la calidad del agua y de los organismos que en ella habitan, pudiendo llegar a limitar severamente o impedir algunos de sus diferentes usos. El alto nivel de interacción de la población humana con el medio acuático y el alto deterioro que este está sufriendo en los últimos años ha hecho ver la necesidad de realizar controles de calidad de aguas con la finalidad de proteger tanto la salud humana como el medio ambiente.

Según la "*International Organization for Standarization*", la monitorización de la calidad del agua es un proceso en el cual se incluyen el muestreo, la medición y registro de los diferentes parámetros físico-químicos del agua para poder evaluar si el agua es apta para sus diferentes usos. En términos generales se pueden diferenciar 3 tipos vigilancia (Bartram, J. and Helmer, R. 1996):

- *Monitorización* es una evaluación a largo plazo donde se estandarizan las medidas y observaciones del medio acuático para poder definir su estatus y la tendencia de este.
- *Inspección* se da en un medio acuático que ya pasó el control de calidad y se quiere comprobar que todavía reúne las propiedades necesarias para poder realizar un uso específico. Consta de un programa intensivo de medidas y observaciones, pero tiene un tiempo de duración finito.
- *Vigilancia* continua, consta de medidas y observaciones específicas que permiten ver las tendencias a lo largo de un periodo establecido de estudio y así obtener una evaluación real del ecosistema.

Los procesos de evaluación proporcionan información sobre un ecosistema y ayuda en la toma de decisiones a la hora de la utilización y protección de recursos. Durante este proceso se identifican tanto los problemas actuales de contaminación como los futuros o emergentes, pudiendo así formular planes efectivos de prioridades, desarrollo y evaluación del medio.

1.2. Contaminación ambiental

Los xenobióticos son compuestos químicos que no pertenecen a la bioquímica normal de los seres vivos ni de los ecosistemas, y su presencia como contaminantes puede generar alteraciones tanto en las vías metabólicas de los organismos como en las características físico-químicas del medio. Esta definición también incluye aquellas sustancias que se producen de forma natural, pero que por motivos antropogénicos o causas naturales puntuales se encuentran a concentraciones mucho más elevadas de las consideradas normales, generando también perturbaciones.

La toxicología ambiental es una ciencia multidisciplinar que mide el grado de toxicidad de compuestos contaminantes a través del estudio de sus efectos adversos en los organismos. También se encarga de analizar sus mecanismos de acción y las vías metabólicas afectadas por estas sustancias.

Bro-Rassmussen, en 1996, afirmó que la toxicidad de un compuesto no viene establecida por su presencia en el medio, sino por sus características químicas como por ejemplo:

- *Lipofilicidad:* Se refiere a la afinidad que presentan algunos compuestos por las grasas y lípidos, y tienden a acumularse en los tejidos de los organismos (bioacumulación). Como resultado los compuestos lipofílicos se bioconcentran en los organismos y se acumulan a lo largo de la cadena trófica encontrando concentraciones mayores a niveles altos del nivel trófico (biomagnificación).
- *Movilidad:* Es la capacidad de un compuesto a desplazarse a través de diferentes compartimentos ambientales. Esta característica esta relacionada con la volatilidad y el transporte atmosférico. Las sustancias semi-volátiles una vez entrados en la atmosfera se unen al aerosol y pueden ser transportadas largas distancias. Este proceso explica la aparición de compuestos en zonas remotas y a larga distancia de su foco de emisión (Barrie et al. 1992).
- *Presencia de material particulado:* la cantidad de material particulado en el medio determina el grado de absorción e ingesta de los contaminantes, así como su biodisponibilidad a través de la cadena trófica. También afecta a la capacidad de retención de la sustancia por los sedimentos, que afecta generalmente a los organismos filtradores.

1.2.1 Biotranformación y bioactivación de los compuestos

Los contaminantes, una vez se encuentran dentro del organismo, pueden sufrir diferentes procesos metabólicos que, en algunos casos, cambian sus propiedades. Este proceso se conoce como biotransformación. En este caso, los xenobióticos son transformados en formas más solubles en agua para facilitar su excreción. Todas las células del organismo tienen la capacidad de transformar y detoxificar sustancias xenobióticas, aunque algunos tejidos son más eficientes que otros, como por ejemplo el hígado o riñón (Valavanides, A., et al. 2006; Lock, E.A. and Reed, C.J. 1998)

El proceso de biotransformación y eliminación de los contaminantes presenta 4 pasos, o fases, diferentes:

- Fase 0: Algunos xenobióticos pueden ser excretados de la célula en el mismo momento en el que entran sin pasar por el proceso de transformación gracias a la acción de proteínas de transporte de la membrana.
- Fase I: Esta fase incluye reacciones químicas como oxidación, reducción e hidrólisis, teniendo como resultado la modificación química de las moléculas añadiendo nuevos grupos funcionales (-OH; -COOH; $-NO^2$; etc). Esta reacción generalmente produce metabolitos más polares, más reactivos y más soluble en agua, los cuales pueden ser conjugados durante la fase II. Este proceso es catalizado mayormente por enzimas monooxigenasas, como el Citocromo P450. Entre todas las clases de enzimas que presenta el Citocromo P450, la familia CYP1A tiene gran interés en estudios de impacto ambiental debido a su importante papel en la biotransformación de xenobióticos (Van der Oost, et al 2003)
- Fase II: En esta fase se facilita la conjugación de los metabolitos resultantes de la fase I con moléculas polares como el glutatión, sulfato o aminoácidos para formar compuestos más solubles en agua y así facilitar su excreción (Commandeur, JN, 1995).
- Fase III: Los metabolitos obtenidos en la fase II son excretados de la célula a través de la membrana utilizando las proteínas de transporte. Este transporte de xenobióticos conlleva un gasto energético en forma de hidrólisis de ATP, por lo cual este tipo de transportadores pertenecen al grupo de *ATP binding proteins* (Commandeur, JN, 1995).

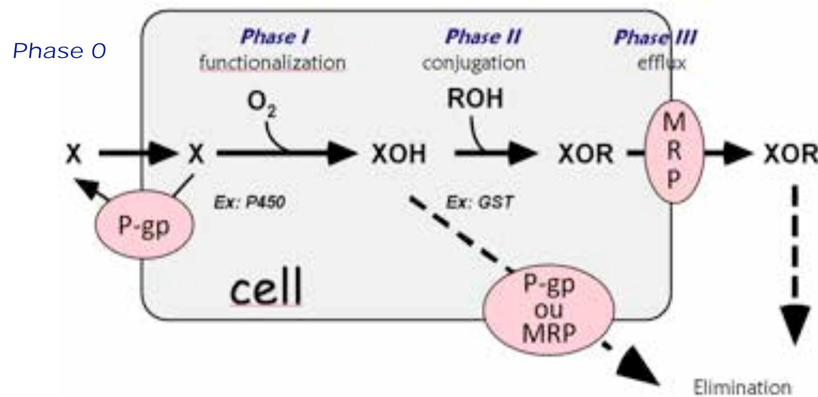


Fig 1.2 Representación del proceso de detoxificación de un compuesto xenobiótico (X) donde se observa las diferentes fases: Fase 0 es la cual toma acción la proteína de membrana P-gp para excretar directamente el contaminante. Fase I y II donde el compuesto sufre una transformación para facilitar su excreción, y por último la fase III donde el compuesto transformado es excretado de la célula.

En algunas ocasiones durante el proceso de biotransformación, y debido al propio metabolismo de las moléculas, se pueden llegar a originar especies químicamente más reactivas. Este caso se puede dar como consecuencia de las reacciones catalizadas por los citocromos P450 de la fase I del proceso de detoxificación. El resultado es por lo tanto una reacción de bioactivación del compuesto (Castell, J.V et al 2005).

Los organismos acuáticos están continuamente expuestos a contaminantes presentes en el agua, sedimentos y comida. Dependiendo del tipo de xenobiótico este afectará de manera diferente al funcionamiento normal del metabolismo, interaccionando con funciones celulares específicas, incrementando la producción de moléculas activas, induciendo enzimas (Stegeman et al 1995), alterando el sistema nervioso (Beauvais et al 2000), interaccionando con el receptor de hormonas actuando como disruptores endocrinos (Denslow, N. et al 1999) o con los inmunoreceptores (Luebeke, et al 1997). A continuación se describe los diferentes efectos sufridos por la interacción de los contaminantes usados durante este estudio.

1.2.2 Mecanismos de acción de los metales pesados

Los metales pesados son elementos con elevado peso molecular que se pueden encontrar en los diferentes compartimentos de la bioesfera como, por ejemplo, la comida, la corteza terrestre, etc. Algunos metales pesados como el Cu, Zn, Mn, Cr y Fe se consideran elementos esenciales ya que son necesarios para un buen funcionamiento del metabolismo (Carson, et al 1986). Sin embargo, variaciones importantes de sus concentraciones pueden producir efectos nocivos en los organismos. Efectos nocivos similares, o peores, pueden ser causado por otros metales como el Cd, Hg y Pb, los

cuales no tienen ninguna función biológica conocida y pueden acumularse en los tejidos de los organismos (Canli and Atli, 2003).

Los metales pesados se encuentran tanto en la naturaleza (normalmente como sales u óxidos) como en diferentes productos manufacturados, como los pesticidas, pinturas y en residuos industriales (minería, fábricas de papel e industrias de fabricación de productos clorados). El uso de productos con metales pesados genera movilización y acumulación de estos compuestos, incrementando su concentración en el medio ambiente. Metales como el mercurio, cobre, cadmio, cromo, níquel, zinc, arsénico y plomo han recibido más atención por la comunidad científica debido a sus propiedades tóxicas que afectan los organismos, pudiendo llegar a ser una amenaza importante para la salud humana (Raymond A. Wuana and Felix E. Okieimen, 2011).

La toxicidad y biodisponibilidad de los metales presentes en el medio ambiente dependen de una serie de factores. Estos factores se pueden dividir en bióticos y abióticos. Se consideran factores bióticos aquellas funciones orgánicas (metabolismo, asimilación, etc.) que intervienen en la solubilización o insolubilización de los elementos inorgánicos y generan alteraciones en los minerales condicionando la formación de depósitos. Los factores abióticos incluyen aspectos como la naturaleza del metal, su abundancia y tiempo de permanencia en el medio; factores físico-químicos como el pH y el potencial redox, la temperatura y la cantidad de materia orgánica presente (Thayer, J.S. 2004). Todas estas variables hacen difícil de establecer relación directa entre las concentraciones de los metales y su efecto biológico.

Cuando el metal es biodisponible, la primera vía de acceso al organismo es a través de la ingesta de comida contaminada o, en el caso de los organismos acuáticos, a través de las branquias (Taylor, E.W. 1996). Existen diferentes mecanismos de toxicidad por metales pesados, los cuales afectan las funciones biológicas normales de los organismos. Su toxicidad es debida, mayoritariamente, a su unión a diferentes macromoléculas y al desplazamiento de metales esenciales (Zn, Fe, Cu) de sus sitios de unión fisiológicos (Vasak, M. 1991). Los individuos expuestos a altas concentraciones de metales pesados pueden disminuir los efectos potenciales tóxicos reduciendo la entrada de metales al organismo, aumentando la excreción y/o secuestrando el metal por proteínas especializadas, como es el caso de las metalotioneínas (Kito, H., et al 1982). El exceso de metales pesados puede estimular la formación de radicales libres y especies reactivas de oxígeno (ROS), generando estrés oxidativo por diferentes mecanismos: reacción de Fenton, disminución de los niveles intracelulares de glutatión,

inhibición de enzimas antioxidantes o por alteración de la cadena de transferencia de electrones (Hansen, B.H. et al. 2007)

A lo largo de esta tesis se ha estudiado el efecto de algunos metales pesados en organismos acuáticos, los cuales se describen a continuación.

1.2.2.1 Mercurio

El mercurio (Hg) es uno de los metales más antiguos conocidos y también uno de los más tóxicos. Se puede encontrar en diferentes compartimentos ambientales (litosfera, hidrosfera, atmósfera y biosfera), como también en un amplio rango de formas químicas, desde sales inorgánicas a organomercurio (Zhanga, L. and Wong M.H. 2007). Aunque el mercurio es un elemento natural, diferentes actividades antropogénicas han aumentado notablemente las concentraciones y cambiado la distribución de este metal en el medio ambiente.

El mercurio presenta diferentes usos, como pesticidas mercuriales; también en industria, particularmente en la industria de fabricación de productos clorados, usos actualmente restringidos en la UE pero vigentes en muchos países y todavía residuales (en "phase out") en la misma UE. La principal fuente de emisión de mercurio en el medio ambiente es por procesos de combustión, como combustibles fósiles, incineración de residuos médicos entre otras, seguido de la producción de productos clorados ([a]EPA, 1997).

La toxicidad y biodisponibilidad del mercurio está estrechamente vinculada a su forma química. Este forma sales en dos estados iónicos diferentes, mercurio (I) y mercurio (II), las cuales son solubles en agua y disponibles para entrar en los organismos. Por otro lado el mercurio como elemento, Hg(0), da lugar a un gas que es poco soluble en agua. En sistemas aerobios, el mercurio se encuentra en forma iónica libre (Hg^{2+}) formando compuestos orgánicos. El ión Hg^{2+} tiende a ser absorbido por partículas sólidas, llegando a acumularse en él material en suspensión de los sistemas acuáticos y dando valores más elevados que los que existen en el agua. Los organomercurios son el resultado de la combinación de mercurio con materia orgánica. Estos compuestos son una de las formas más peligrosas de los derivados del mercurio ya que son más estables y más liposolubles (Repetto, M. 1995).

El mercurio puede ser transportado por la atmosfera y llegar a ecosistemas tanto terrestres como acuáticos por precipitación o deposición seca (Swackhamer, D.L. et al 2004). Las mayoría del mercurio que entra en los sistemas acuáticos lo hace en su forma inorgánica, que presenta una toxicidad relativamente baja (Eisler, R, 1987). Sin embargo, mediante la actuación de las bacterias sulfato reductoras, el mercurio inorgánico es transformado a metil-mercurio. El metil-mercurio es fácil de absorber por los organismos acuáticos debido a su capacidad de pasar a través de membranas, y es de lenta excreción permitiendo que se biomagnifique en los niveles altos de la cadena trófica (Elhassani 1983, citado por Eisler, R. 1987).

La eficiencia de transferencia en el proceso de bioacumulación del mercurio en sistemas acuáticos es lenta en los niveles bajos de la cadena trófica, pero incrementa en niveles superiores como, por ejemplo, en peces y aves (Eisler, R. 1987). Muchos autores han estudiado la absorción de mercurio por parte de los peces. Alguna de estas investigaciones muestra que el metil-mercurio es absorbido 5 veces más rápido que las formas inorgánicas (Stopford, W. y Goldwater, L.J, 1975). La dieta en este caso es una de las rutas principales en la absorción del metil-mercurio, y debido a que en muchas partes del mundo los peces son una base importante en la dieta de los humanos, ha crecido el interés sobre la monitorización de este metal para prevenir casos de toxicidad. El metil-mercurio afecta al sistema nervioso central humano, con consecuencias devastadoras. Un claro ejemplo es el accidente de 1950 en Minamata, Japón, donde un alto número de personas se intoxicaron al comer pescado contaminado por metil-mercurio procedente de una industria química (Hoffman, D.J. et al 2002). En organismos acuáticos, el mercurio se ha visto que afecta al crecimiento y comportamiento de los peces (Canli, M. and Atli, G. 2003)

1.2.2.2 Cadmio

El cadmio (Cd) entra en el medio ambiente generalmente debido a actividades humanas como la minería y procesos industriales. Este entra en los sistemas acuáticos por vía de lixiviados de cenizas de carbón, por la agricultura (fertilizantes fosfatados y pesticidas que contengan cadmio) y aguas residuales. El cadmio es muy persistente en el medio ambiente y tiende a bioacumularse en la biota. Estudios en organismos acuáticos muestran que los principales órganos de acumulación son las branquias y el riñón seguido del hígado y músculo (Radhakrishnan, M.V, and Hemalatha S. 2011). Además

se considera un metal peligroso ya que es similar al zinc, un metal esencial para el metabolismo de los organismos.

Diversos estudios sobre los efectos tóxicos del cadmio han demostrando que los organismos acuáticos son especialmente a la contaminación por este metal. En peces, se estima que las concentraciones letales para el 50% de la población (LC50) están entre 2 y 50 ppm a las 96h (Sorense, E.M.B. 1991). Incluso en concentraciones bajas, como por ejemplo 0.5mg/l, puede ser letal para algunos teleósteos (Lacroix, A. and Hontela, A. 2004). Además, algunos de sus efectos subletales, como la disminución del crecimiento, la inhibición de la reproducción y la alteración en poblaciones, puede ser detectada a 0.7 ppb (Eisler, R., 1985). En bivalvos, el cadmio interfiere con el sistema antioxidante de especies como *Mytillus galloprovincialis*, *Ruditapes decussatus* y *Bathymodiolus azoricus*, incluyendo la disminución de los niveles de glutatión y la inhibición de la actividad del SOD, GPx y Catalasa. A nivel molecular, el cadmio puede interferir con el metabolismo energético, transporte de membrana y la síntesis de proteínas en los organismos acuáticos (Company, R. et al. 2006).

En el ser humano, el cadmio puede crear daños en el riñón e hígado, además de enfermedades pulmonares y cáncer. Una exposición crónica a bajas concentraciones puede contribuir en problemas de hipertensión, alteración de niveles de colesterol en sangre y del metabolismo del calcio, como también disminución del crecimiento (Wolverton, B.C. et al 1978; Laws, E.A. 2000)

1.2.2.3 Cobre

El cobre (Cu) es un nutriente esencial que se requiere en pequeñas cantidades (5-20 µg/g) para un buen funcionamiento del metabolismo de todas las células, tanto procariotas como eucariotas. Es un cofactor de enzimas importante para el metabolismo como es el caso del superoxido dismutasa (Cu/Zn-SOD) y el citocromo c Oxidasa (COX), y su esencialidad viene dada por su capacidad de participar en las reacciones de intercambio de electrones (Arredondo, M. and Núñex, M.T 2005). Está implicado en procesos de formación de glóbulos rojos, del metabolismo del colesterol y glucosa, absorción y utilización del hierro, en la síntesis y liberación de proteínas y enzimas y producción de energía (Sorensen, E.M.B. 1991). El cobre, procedente de la dieta, es absorbido a través de la mucosa intestinal y transportado por la corriente sanguínea y liberado en diferentes tejidos. Las concentraciones más elevadas de cobre se han

encontrado en hígado (Gaetke, L.M et al. 2003), seguido del cerebro, riñón y corazón (Turnlund, J.R. 1998).

El cobre es utilizado industrialmente como material de aleación en industria, en la construcción y transporte, y es un componente importante en joyería. Se utiliza además en productos dentales y cosméticos. Aunque el contacto con estos productos no es perjudicial, una exposición a concentraciones elevadas de este metal puede llegar a ser peligroso (Gaetke, L. M. et al. 2003). El cobre también se puede encontrar como ingrediente de las pinturas antiincrustantes de las embarcaciones, que se suelen utilizar para matar algas, hongos y pequeños moluscos debido a su toxicidad para los organismos acuáticos. Además también tiene uso en la agricultura ya que sus propiedades antifunguicidas para proteger las semillas. El cobre es moderadamente soluble en agua y se une fácilmente a los sedimentos y materia orgánica, donde se suele bioconcentrar dando concentraciones más elevadas en animales que en el agua y sedimentos. Sin embargo, el cobre no se biomagnifica a lo largo de la cadena trófica.

La vía de entrada más importante para el cobre en sistemas acuáticos es la dieta, pero se ha demostrado que su biodisponibilidad y toxicidad están relacionadas con su concentración total en el agua (Eisler, R. 2000). La exposición al cobre de salmones afecta el sentido del olfato, las funciones cerebrales, la respuesta inmunológica y crea disrupción en el sistema de osmoregulación (Woody, C.A., 2007).

1.2.3 Mecanismo de acción y efecto de los disruptores endocrinos

El sistema endocrino es uno de los sistemas encargados de controlar y coordinar el funcionamiento de todo el organismo, incluyendo el crecimiento y el desarrollo, la reproducción, los niveles de energía, etc. Ejerce esta función por medio de una red de glándulas y órganos que producen, secretan y almacenan ciertas hormonas (fig 1.3).

Las hormonas son sustancias químicas segregadas por células especializadas y pertenecen al grupo de mensajeros químicos, que incluye también neurotransmisores y feromonas. Son transportadas por el torrente sanguíneo o se difunden entre los espacios intersticiales hasta llegar a órganos o tejidos diana donde desempeñan su función.

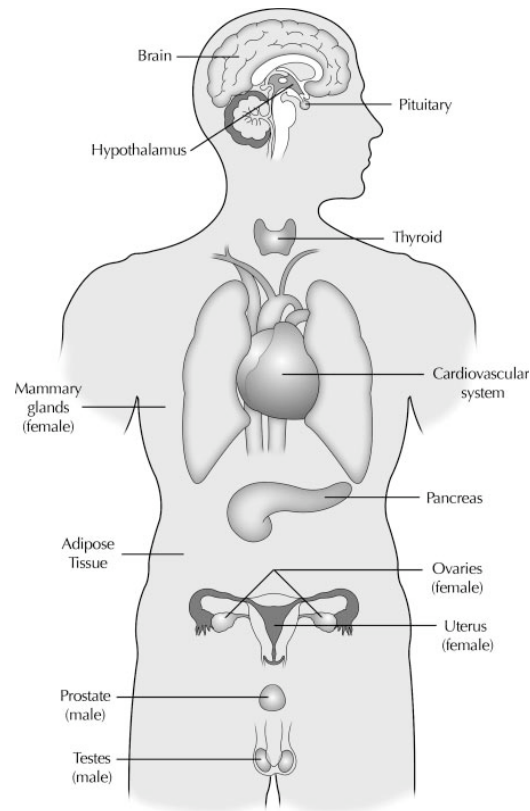


Fig 1.3 Representación del sistema endocrino humano

Debido a la complejidad del funcionamiento del sistema endocrino, y su importancia para un correcto funcionamiento del organismo, las sustancias que pueden alterarlo, como es el caso de algunos contaminantes orgánicos, generan una preocupación creciente. En 1997, la agencia de protección del medio ambiente de US, ([b]EPA, 1997) describió a los Disruptores Endocrinos Ambientales como aquellos agentes exógenos que son capaces de interferir con la síntesis, secreción, transporte, unión o eliminación de las hormonas naturales. Entre los posibles compuestos que pueden actuar como disruptores endocrinos encontramos:

- *Hormonas naturales:* Estas pueden ser de origen animal o humano y son liberadas/excretadas al medio ambiente, pudiendo actuar en el sistema endocrino de los organismos expuestos a ellas. Un ejemplo es el efecto feminizante observado en peces por hormonas procedentes de aguas residuales y en algunos casos reactivadas durante el proceso de depuración de aguas (Rodgers-Gray, T.P. et al 2001; Tyler, C.R. and Jobling, S. 2008)
- *Sustancias naturales:* como los fitoestrógenos, que son toxinas producidas por plantas o hongos cuya estructura química mimetiza a la del estradiol. La ingestión de estos fitoestrógenos puede alterar directa o indirectamente los

mecanismos responsables de la segregación de hormonas (Faber, K.A. and Hughes, C.L. Jr. 1991).

- *Esteroides sintéticos:* Son productos farmacéuticos o de cuidado personal con alto contenido de hormonas activas, como por ejemplo las pastillas anticonceptivas y algunos tratamientos para el cáncer. Estos compuestos son liberados al medio ambiente por medio de un tratamiento incompleto de aguas residuales o vertidos.
- *Compuestos químicos antropogénicos y derivados:* En este grupo se incluyen pesticidas (DDT y otros organoclorados), productos químicos, como plásticos y aditivos, algunos compuestos industriales (PCB's y dioxinas) y los alquilfenoles (Jobling, S. et al, 1995).

Se pueden distinguir cuatro vías de acción por parte de los disruptores endocrinos: 1) como agonistas, que presentan efectos aditivos y/o sinérgicos sobre la acción de las hormonas naturales; 2) antagonistas que contrarrestan su acción y disminuyen su actividad efectiva, actuando por ejemplo, como antiestrógenos o antiandrógenos; 3) alteradores del metabolismo hormonal modificándolas rutas metabólicas de síntesis o degradación de hormonas y alterando a su vez los niveles en sangre y 4) modificando los niveles de los receptores hormonales o su actividad (Weybridge, 1996; Argemi, F., et al 2005). Entre las substancias que pueden causar directamente o indirectamente disrupción endocrina se encuentran los contaminantes orgánicos persistentes.

1.2.3.1 Contaminantes orgánicos persistentes o COPs

Los contaminantes orgánicos persistentes, o COPs, son sustancias químicas que, como su propio nombre indica, persisten en el medio ambiente. Esto es posibles gracias a su resistencia a la degradación por procesos biológicos, químicos o fotolíticos (Kannann, N. Et al 1988). Estos compuestos son generalmente solubles en lípidos y tienden a bioacumularse en los tejidos adiposos y biomagnificarse a través de la cadena alimenticia, causando efectos adversos en el ecosistema y en la salud humana.

Los COPs se han convertido en una amenaza global debido a su capacidad para moverse a grandes distancias desde su punto de emisión, incluyendo regiones remotas alejadas de las emisiones locales, como en mar abierto, las zonas de alta montaña y las zonas polares. La movilización de contaminantes a lugares remotos se explica por el transporte atmosférico global de la tierra (Fernández, P. and Grimalt, J.O, 2003). Por ejemplo, se ha medido niveles de PCBs en el aire, encontrado concentraciones de más

de 15 ng/m^3 en todo el mundo, independientemente de la distancia a los focos emisores (Ritter, L. et al 1995). Frente a esta evidencia, la comunidad internacional ha diseñado un plan de acción global urgente para disminuir y eliminar la emisión de este tipo de compuestos. El Programa Ambiental de las Naciones Unidas (UNEP) desarrolló en 2001 una lista en la cual aparecía los 12 COPs más importantes cuyas emisiones han de estar controladas. Durante la última reunión, en el 2009, a esta lista se le añadieron 5 compuestos más.

Plaguicidas	Industrial	Derivados	Retardante de llama	Antiadehrentes
DDT	Bifenilos Policlorados	Dioxinas	Pentabromdifenileter	Perfluorooctosulfanatos
Aldreina	Hexaclorobenceno	Furanos	Hexabromobifenilo	PFOS
Clordano				
Dialdrina				
Endrina				
Heptacloro				
Mirex				
Toxafeno				
Clordecona				
Lindano				

Tabla 1.1 Listado de los 17 COPs negociados por la UNEP

Los hidrocarburos policíclicos aromáticos (PAHs) son los contaminantes orgánicos más extendidos en el medio ambiente y se caracterizan por tener dos o más anillos de benceno unidos entre sí. Su estructura química varía dependiendo del número y la posición de los anillos, afectando a sus propiedades físico-químicas como solubilidad, movilidad, volatilidad y lipofilicidad (Bulder, A.S et al. 2006). En general, cuanto más anillos aromáticos posean los PAHs, mayor es su lipofilidad y menor su volatilidad. Los PAHs se encuentran en la naturaleza formando parte del crudo del petróleo, en depósitos de carbón y como resultado de incendios, pero también se crean de forma antropogénica por la combustión incompleta del carbón o combustibles. En sentido estricto, no son compuestos persistentes, ya que son susceptibles de biotransformación, oxidación y fotolisis; sin embargo, y dada su continua producción por medios tanto naturales como antropogénicos, su presencia en el medio ambiente es continuada y relativamente estable, cuando no creciente. Hoy en día, las emisiones de combustibles fósiles y las emisiones de PAHs están reguladas en los países desarrollados (ATSDR, 2009).

Los retardantes de llama bromados (BFR) son compuestos organobromados que tienen un efecto inhibitorio en la ignición de materiales orgánicos. Se utilizan en plásticos, tejidos y productos electrónicos. Los BFR son productos persistentes en el medio ambiente, y tienen la capacidad de acumularse en los sedimentos y en los tejidos animales a niveles relativamente altos. Estos pueden llegar a causar disrupción de las hormonas tiroideas y disfunciones en el desarrollo neuronal (USGS, 2004).

Por otro lado, los compuestos organometálicos son productos químicos que contienen un enlace entre un carbón y un metal. Estos compuestos se comportan de manera diferente a las formas inorgánicas de los metales cuando se encuentran dentro de un organismo, debido a su naturaleza orgánica. Los organometales presentan mayor solubilidad en lípidos y una elevada capacidad de penetrar en las membranas celulares (Manahan, S.E. 2007). Debido a estas características, se incrementa la toxicidad y biodisponibilidad de los metales que forman el compuesto. Los compuestos organometálicos tienen orígenes variados, algunos pueden ser sintetizados para usos industriales, como es el caso del tributilestaño, mientras que otros son producidos por transformaciones realizadas por microorganismos, como el metilmercurio.

A continuación se describen con más detalles dos contaminantes orgánicos que se han estudiado durante esta tesis, el Dacthal y el tributilestaño:

El dactal (DCPA), también conocido como dimetil tetraclorotereftalato o clortal-dimetil (fig 1.4), se utiliza como herbicida en el control de plantas y semillas. El DCPA y sus metabolitos tienen una toxicidad baja a niveles de exposición aguda y crónica. Sin embargo, presenta algunas impurezas de manufacturación como el hexaclorobenceno con unas propiedades toxicas crónicas (Mountfort, R.F. 1988).

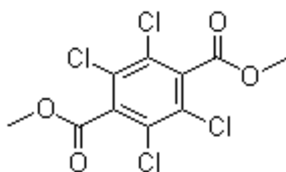


Fig 1.4 Estructura molecular del Dacthal

Existe poca información sobre la distribución interna del dactal en los organismos, pero se sugiere que el hígado, riñón, tiroides y pulmones son los tejidos más importantes. En invertebrados se ha visto que el dactal inhibe el mecanismo de transporte de membrana del P-gp en *Mytilus californianus* (Strum, A. And Segner, H. 2005)

El tributil estaño (TBT) es un compuesto organometálico que se utiliza en las pinturas antiincrustante de las embarcaciones para impedir que se adhieran algas y otros organismos vivos (Clark, E.A. et al 1988). Se considera un compuesto extremadamente tóxico para los organismos de sistemas acuáticos, y se puede bioconcentrar a través de la cadena trófica (Fent, K. 1998)

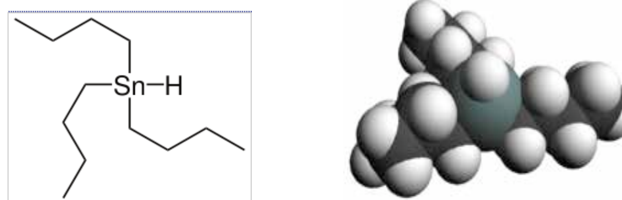


Fig 1.5 Estructura y modelo de la molécula de TBT

Muchos organismos pueden degradar el TBT, metabolizándolo a especies menos tóxicas mediante sistemas enzimáticos dependientes del citocromo P450 (Viglino, L et al 2006). Como la actividad P450 es relativamente baja en moluscos, estos organismos son particularmente sensibles al TBT y tienden a acumularlo en sus tejidos (Lee, R.F. 1991), un efecto especialmente evidente en gasterópodos y bivalvos recolectados en puertos y marinas (Héral, M. et al. 1989; Alzieu, C. 1991;Becker, K. et al. 1992; Strand, J. and Asmund, G, 2002; Strand, J. et al 2009). En *Dreissena polymorpha*, por ejemplo, se ha observado uno de los valores más elevados de bioacumulación de TBT en cualquier especie, superando los 9 µg/g de peso fresco. En peces, el rango de bioacumulación está entre 0.06 y 0.3 µg/g tanto en agua dulce como marina (Fent, K. 1998).

El TBT puede causar disrupción endocrina en organismos acuáticos, especialmente en moluscos. La primera efecto negativo fue detectado en gasterópodos con la aparición de estructuras masculinas externas en hembras de caracolas afectadas por TBT (Smith, et al 1981). Este fenómeno se conoce como *imposex* (Imposición del sexo), causado por una exposición directa a elevados niveles de testosterona libre, que en caracoles se ha visto asociado a la presencia de TBT (Gooding, M. et al 2003). A partir de aquí, el concepto de imposex se ha descrito en diferentes especies de gasterópodos y hoy en día se utiliza como bioindicador de exposición a TBT.

En bivalvos, la exposición de TBT genera malformaciones en las valvas y reduce la tasa reproducción, ya que las larvas sobreviven solo unos pocos días en ambientes contaminados por TBT. En estudios con *C. gigas* se ha visto una reducción en la tasa de

fecundación a concentraciones de 35 µg/L, mientras que a concentraciones de 0.35 µg/L se observaron larvas en fase velígera malformadas (Alzieu, C. 2000).

Como consecuencia del elevado número de estudios probando los efectos tóxicos del TBT en organismos acuáticos, su uso ha sido regulado en muchos países desarrollados. La aplicación de estos controles resultó en un descenso de los niveles ambientales del TBT en diferentes áreas, en la bahía de Arcachon, Francia, donde sus niveles se dedujeron de 5 a 10 veces en 3 años (Alzieu, C. et al 1986). Las medidas de control fueron adoptadas por el Comité de Protección Ambiental Marina (MEPC) en el 1990, prohibiendo el uso de los antiincrustantes que liberaran más de 4 µg de TBT por día. En 1999 el comité hizo un llamamiento a la prohibición en la aplicación de pinturas que contengan productos organoestánicos en embarcaciones desde el 1 de Enero del 2003, y una prohibición completa para 2008 (IMO, 2001).

1.3 Biomarcadores ambientales

La constante liberación de contaminantes en los ecosistemas ha hecho que aumente la preocupación sobre los posibles efectos que puedan tener en los organismos, y se ha visto en la necesidad de implantar estudios de toxicología y gestión ambiental. En un primer paso, los estudios de toxicología ambiental se basan en la identificación y cuantificación a través de análisis químicos de los compuestos que se encuentran en el medio. Sin embargo, saber el nivel de concentración al que se encuentran estas sustancias en un ecosistema, o en un organismo, no proporciona suficiente información sobre los efectos que estas sustancias pueden tener sobre los animales y plantas que están expuesto. La introducción de análisis biológicos en el estudio de un ecosistema bajo estrés proporciona información sobre estos y sirven de indicación del daño potencial que puede sufrir el ecosistema.

En el medio acuático, los peces fueron los primeros organismos en ser utilizados en los protocolos de evaluación de los efectos toxicológicos, y aún hoy siguen siendo una de las primeras elecciones en diversos ensayos de toxicidad (Rand, G.M 1995). Junto a esta práctica se han ido añadiendo nuevas tecnologías, como la utilización de embriones de pez, micro-invertebrados, algas, líneas celulares y tisulares y las mediciones de reacciones bioquímicas y del nivel de ácidos nucleicos como biomarcadores (Ferré, M., et al. 2009).

La respuesta bioquímica, o biomarcadores, fue definida por primera vez en 1989 por el NCR como las alteraciones en procesos celulares o bioquímicos inducidos por xenobióticos, y que se pueden cuantificar en un sistema biológico o muestra. Actualmente el termino "*Biomarcador*", se define como las respuestas generadas por un organismo expuesto a contaminantes que se pueden detectar y medir para establecer una relación causa efecto, como es el caso de los cambios en la actividad de proteínas o en el nivel de expresión génica (Peakall, D.B. 1994).

Según la Organización de salud mundial (*World Health Organization*, WHO, 1993), y dependiendo de su relevancia biológica, los biomarcadores pueden ser divididos en:

- Biomarcadores de exposición: Indican la presencia de sustancias exógenas o sus metabolitos. Estos marcadores pueden ser productos de la interacción entre una agente xenobiótico y una molécula o célula diana.
- Biomarcadores de efecto: Son indicativos de cambios fisiológicos, en los organismos expuestos a xenobióticos y que se pueden interpretar como un deterioro de la salud o fertilidad.
- Biomarcadorres de susceptibilidad: Indican la sensibilidad de un organismo a un contaminante específico.

El potencial uso de los biomarcadores en ecotoxicología fue promovida en 1990 (Adams, 1990; McCarthy,J. F. and Shugart, L.R 1990), basándose mayormente en la medida de parámetros bioquímicos como actividades enzimáticas y en perfiles histológicos para ver cambios morfológicos (Adams, S.M. et al 2001). Aunque los biomarcadores han sido, históricamente, muy útiles a la hora de identificar áreas contaminadas o potenciales estresores del medio, en algunos casos sus resultados han sido malinterpretados. Por ese motivo es necesario desarrollar métodos de validación de las técnicas, para mejorar el conocimiento de los mecanismos de toxicidad y establecer una mejor relación entre las respuestas de exposición y los contaminantes ambientales.

Los efectos adversos producidos por contaminantes en poblaciones naturales son la prueba más directa de su potencial toxicológico, aunque es difícil detectarlos antes de que los daños en el ecosistema sean irreversibles (Van der Oost, 2003). Por esta razón, es importante el desarrollo de metodologías para la detección de las primeras señales de cualquier efecto en los organismos como respuesta a la exposición de contaminantes. Estas señales se pueden utilizar como biomarcadores para controlar el estado del ecosistema durante las evaluaciones de calidad ambiental (Cajaraville, M.P et al 2000).

El uso de los biomarcadores en la evaluación de la calidad ambiental, genera información sobre la biodisponibilidad de los contaminantes y el tipo de contaminante, ya que son respuestas específicas a determinados grupos de sustancias. Los biomarcadores, además pueden ser utilizados en especies de diferentes hábitats o niveles de la cadena trófica, tanto en muestras de laboratorio como de campo (Livingstone, D.R. 1993). Los recientes avances en genómica molecular permiten desarrollar biomarcadores basados en el análisis de transcripción de genes relacionados con mecanismos de detoxificación, estrés o madurez sexual (Piña, B., et al. 2007)

1.3.1 Biomarcadores moleculares

La presencia de contaminantes o de agentes de estrés en el medio ambiente genera perturbaciones en los organismos a diferentes niveles, desde niveles moleculares y/o bioquímicos hasta a niveles de población y comunidad (Van de Oost, 2003; fig1.6).

A nivel molecular, la regulación de transcripción del DNA es muy sensible a la influencia de agentes de estrés ambiental o de contaminantes, ya que en muchos casos la regulación génica es el primer paso en los procesos de detoxificación o aclimatación. Además, su análisis genera información importante sobre el modo de acción de los contaminantes. Por ello, la medición de niveles de transcripción de genes se utiliza como biomarcador en los estudios de valoración ambiental (Fent, K. 2001). Por otro lado, los biomarcadores analizados a niveles superiores responden más lentamente en condiciones de estrés, presentando una significancia toxicológica baja, pero una mayor significancia ecológica (Adams, S.M. et al 1989)

La aplicación de las técnicas de medición de los cambios en expresión génica debidos a la presencia de tóxicos o estresores se conoce como *Toxicogenómica* (Tennant, R.W., 2002). Esta rama de la toxicología proporciona a los investigadores una herramienta para identificar y cuantificar los efectos producidos por contaminantes, ya sea con técnicas invasivas como no-invasivas, y proporciona información valiosa de los mecanismos de acción de los diferentes tóxicos.

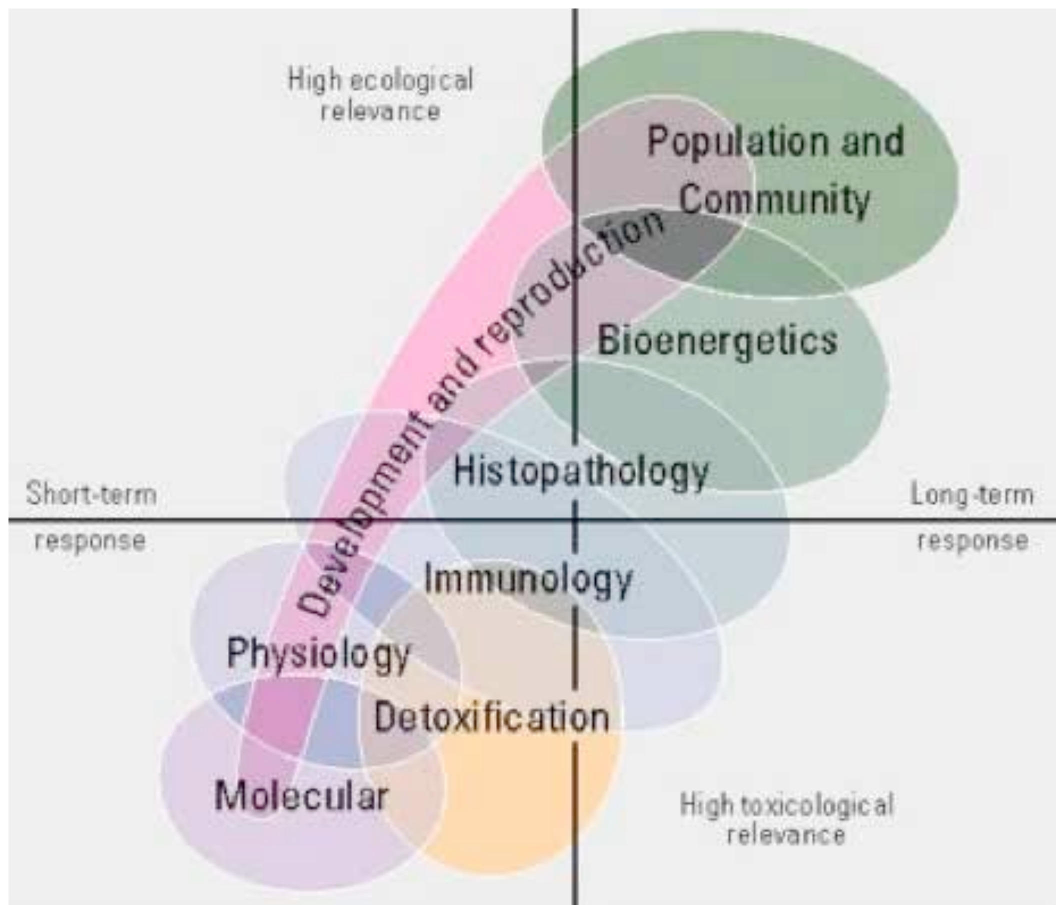


Fig 1.6. Diagrama de los diferentes niveles de respuesta biológica a la polución. Esta imagen muestra la continuidad de respuesta a lo largo de los diferentes niveles de organización en función del tiempo de respuesta y de la relevancia toxicológica y ambiental (Adaptación de Van der Oost, 2003).

La respuesta a nivel molecular a la presencia de tóxicos se inicia cuando en el interior de una célula uno de los receptores detecta la presencia de un agente (ya puede ser endógeno o exógeno como hormonas, metales, etc.) y se activa. Este receptor activo se une a una secuencia específica de DNA, en el promotor de los genes diana, y modifica su tasa de transcripción o bien activándola o inhibiéndola. Como resultado de esta acción se produce una variación del número de moléculas de RNA mensajero (mRNA) del gen diana que son exportadas al citoplasma de la célula, donde su información se traduce para sintetizar la proteína correspondiente en el ribosoma (fig 1.7; Janosek, J., et al 2006; Piña, B., et al 2007).

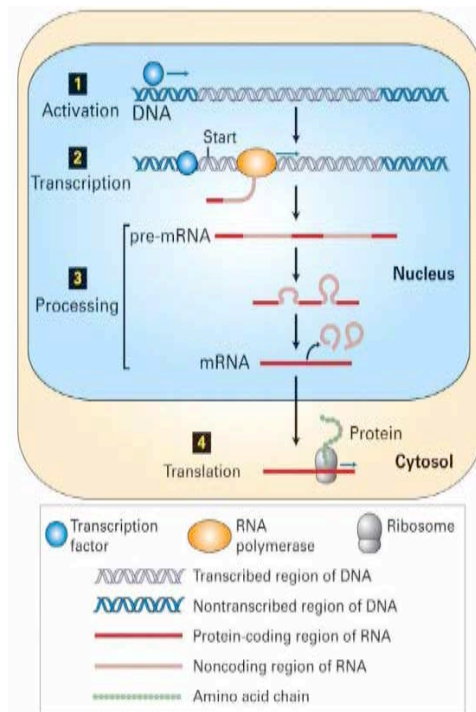


Fig 1.7: Representación gráfica del proceso de expresión génica. En esta figura se muestran los pasos de activación (1) Unión a un fragmento especifico de DNA, denominado elemento de respuesta (2) Activación (en algunos casos, inhibición) de la transcripción, que genera moléculas de RNA complementarias a la secuencia del gen de respuesta (3) Procesado de las moléculas de RNA para dar lugar a moléculas de mRNA y (4) Exportación al citoplasma y traducción a proteínas del mRNA, que tiene lugar en los ribosomas.

Existe una gran variedad de contaminantes ambientales capaces de interaccionar con diferentes receptores endógenos, debido a sus características estructurales que los hacen semejantes al ligando o ligandos naturales. En algunos casos, el contaminante es indistinguible del ligando natural, y si se encuentra en concentraciones elevadas se convierte en un agente tóxico al alterar el equilibrio homeostático. Entre ellos se pueden destacar algunos compuestos orgánicos que se unen a los receptores de estrógeno o hidrocarburo de arilo, o diferentes metales pesados.

Los cambios de expresión génica son considerados marcadores sensibles porque tienen una rápida respuesta a la exposición de contaminantes, incluso a concentraciones bajas (McClain J.S., et al 2003). Protocolos avanzados de extracción de mRNA de tejidos y la implantación de técnicas como la de la Reacción en Cadena de la Polimerasa (PCR) han hecho que la medición de variaciones de niveles de mRNA sea considerada una herramienta muy útil en estudios de toxicología, ya que puede llegar a detectar entre 10 y 100 moléculas de mRNA. Por ese motivo, la aplicación de esta técnica permite analizar los niveles de expresión génica en organismos pequeños o utilizar porciones pequeños de tejidos. Así pues, se puede reducir la cantidad de muestras recolectadas

en campañas de muestreo e incluso evitar el sacrificio de los individuos del estudio, según el tejido y la especie seleccionada ([a]Quirós L. et al 2007).

1.3.2 Selección de biomarcadores

Uno de los puntos claves para la utilización de la expresión génica en muestras ambientales es la elección de la especie de estudio, el tejido diana y los genes que puedan proporcionar una mejor información sobre los efectos tóxicos esperados. Un biomarcador apropiado será aquel que indique los efectos específicos de una sustancia en concreto o de un tipo de contaminantes. En general, cada tipo de xenobióticos afecta a un ruta metabólica específica y por tanto, presenta un modo de acción diferenciado.

La metodología de cuantificación de RNA se basa en la utilización de fragmentos de secuencias de genes conocidos. Hoy en día existe un número limitado de especies secuenciadas cuya información está recogida en la base de datos GeneBank. Entre las especies mejor conocidas se incluyen humanos, animales de laboratorio "clásicos" (rata, ratón, pez cebra, *Drosophila*, *C. elegans*) y diversas especies con interés ambiental, pero para muchas otras la información es aún muy incompleta. Poco a poco esta situación está cambiando dada la importancia de cubrir más niveles tróficos y más ecosistemas en los estudios de impacto ambiental. La base de datos se ha ido incrementando con información de nuevas especies, como especies invasivas o de nuevos ecosistemas para poder evaluar nuevas zonas geográficas.

La selección y aplicación de los biomarcadores se realiza dependiendo de los objetivos que tienen que cumplir y se basa en diferentes factores. Los biomarcadores han de ser un indicador de exposición, del efecto biológico o del tipo de contaminante. Adams, S.M. y su grupo, en el 2001, realizaron una lista de características que se han de tener en cuenta a la hora de escoger el biomarcador apropiado:

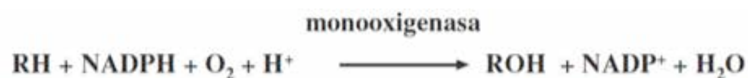
- Caracterizar los mecanismos de estrés que dan respuesta biológica a la presencia de contaminantes en los niveles superiores de organización.
- Ayudar a establecer una relación de causa-efecto.
- Indicar la presencia o ausencia de un grupo determinado de contaminantes.
- Indicar la ausencia de efectos biológicos o ecológicos.
- Proporcionar suficiente información de la respuesta biológica para ser utilizado como evidencia de problemas ambientales.
- Uso para la monitorización de la salud ambiental y para documentar mejoras en la gestión de riesgo ambiental.

Actualmente existen una serie de marcadores moleculares bien caracterizados, como es el caso de los estudios de citocromo P450A1 para evaluar la presencia de contaminantes orgánicos (Rotchell, J.M., et al 2000), las metalotioneínas para contaminación de metales (Moshe, T. Et al 2000) y la vitelogenina en peces para contaminación por disruptores endocrinos (Denslow, N.D., et al 1999). Los biomarcadores más importantes utilizados durante este trabajo están descritos a continuación.

1.3.2.1 Citocromo P450 1A

El citocromo P450 1A (CYP1A) pertenece a un grupo numeroso y diverso de enzimas asociadas a proteínas de unión a membrana, los citocromos P450. Se encuentra principalmente en el retículo endoplasmático del hígado (Siroka, K. and Drastichova, J., 2004), aunque también está presente en muchos otros tejidos. Es uno de los enzimas más estudiados debido a su importancia en el proceso de eliminación de medicamentos y otras sustancias del organismo. También es uno de los biomarcadores más usados en programas de monitorización para evaluar la presencia de contaminantes orgánicos.

El enzima CYP1A es inducido tras la unión de los contaminantes orgánicos persistentes al receptor de hidrocarburo de arilo (AhR), donde una de sus funciones principales es la catalizar la oxidación de sustancias orgánicas por monooxigenación (fase I del proceso de detoxificación). La monooxigenación consiste en la inserción de un átomo de oxígeno en la sustancia orgánica (fig 1.8), que se transforma en una especie más hidrofílica, lo que facilita su excreción y/o conjugación con otras sustancias (fase II del proceso de detoxificación).



$$RH + NADPH + O_2 + H^+ \xrightarrow{\text{monooxigenasa}} ROH + NADP^+ + H_2O$$

Fig 1.8. Reacción de monooxigenasa que se producen durante la fase I del proceso de detoxificación.

En peces, el CYP1A es el responsable de la biotransformación de un gran número de compuestos como los PAHs, PCBs, dioxinas, etc.. Y la inducción de los niveles de mRNA del CYP1A se han utilizado como biomarcador para la identificación compuestos de tipo-

dioxina y se ha utilizado en programas de evaluación de riesgo ambiental (Van der Oost, et al. 2003).

1.3.2.2 Metalotioneínas

Las metalotioneínas (MT) son proteínas de bajo peso molecular (6-7 kDa) ricas en cisteínas, descubiertas en 1957 por Margoshes and Vallee. Las MT participan en procesos de regulación de las funciones de los metales esenciales como en el caso del Cu^{2+} y Zn^{2} y en procesos de detoxificación de metales no esenciales como el Cd^{2+} y el Hg^{+}, ya que el grupo tiol (-SH) de las cisteinas residuales permiten que estas proteínas se unan a metales pesados (Roesijadi et al 1994; Thirumoorthy,N. et al 2007).

Las metalotioneínas están encargadas de la captación de una gran variedad de metales pesados y su inducción, mediada por factores de regulación de unión a metales (MTR), es un indicativo del incremento de metales en el interior del organismo (Thiele, D.J. 1992). Haq y su grupo, en el 2003, propusieron un modelo de inducción de las metalotioneínas, en el cual el incremento de la concentración de metales pesados desplaza la unión del Zn de las metaloproteínas reguladoras (MP). Al desplazarse el Zn, este quedaría libre y se podría unir al factor de transcripción (MTF-1), un factor dependiente del Zn. Este a su vez incrementa la capacidad de unión a los elementos de respuesta a metales (MRE) y la inducción de la transcripción del gen de la metalotioneína (fig 1.9). El estrés oxidativo también es capaz de inducir la expresión de la MT utilizando dos vías: desplazando el Zn de las MP reguladoras o activando otras regiones promotoras conocidas como elementos de respuesta a antioxidantes (ARE) (Dalton et al. 1996)

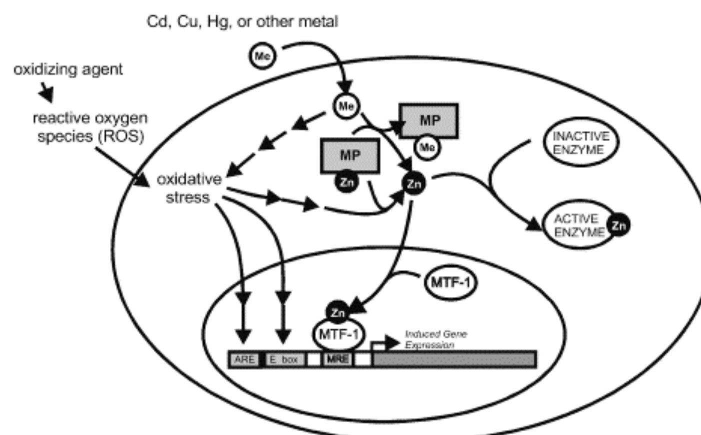


Fig 1.9. Representación de los mecanismos de inducción de la metalotioneína por parte del Zn. MTF: Factor de transcripción de metales; MP: Metaloproteína; MRE: Elemento de respuesta a metales (Haq et al 2003)

A diferencia de la mayor parte de los teleósteos, que solo presentan una isoforma de MT, salmónidos y ciprínidos disponen de dos isoformas, denominadas MT1 y MT2, una situación semejante a la de los mamíferos (Hermesz, et al 2001). En la carpa común (*C. carpio*), la isoforma MT1 está regulada por metales y radicales libres, mientas que la MT2 está regulada solo por metales (Hermesz, et al 2001). Los niveles de MT se han revelado como un buen biomarcador de exposición a metales pesados en procesos de Evaluación de Riesgo Ambiental (ERA) (Van der Oost, R., et al 2003).

1.3.2.3 Enzimas de estrés oxidativo

El oxígeno es una molécula esencial en el control fisiológico de las funciones celulares de los sistemas biológicos y esencial en la respiración celular de los organismos aeróbicos. El metabolismo básico celular de los organismos aeróbicos genera especies reactivas de oxígeno (ROS), como el anión superóxido (O^{-}_2), los radicales de hidroxilo ($^{\cdot}OH$) o el peróxido de hidrogeno (H_2O_2) (Dröge, W. 2003).

Los organismos regulan la producción de radicales libres para mantener el equilibrio redox celular, utilizando para ello enzimas antioxidantes. Si por algún motivo la cantidad de radicales libres presente en la célula supera su capacidad de neutralizarlos, se generan daño en lípidos, proteínas y DNA. La superóxido dismutasa (SOD), la catalasa y las peroxidasas son los enzimas encargados de defender el organismos de la acción de los ROS (Bandyopadhyay, U et al 1999).

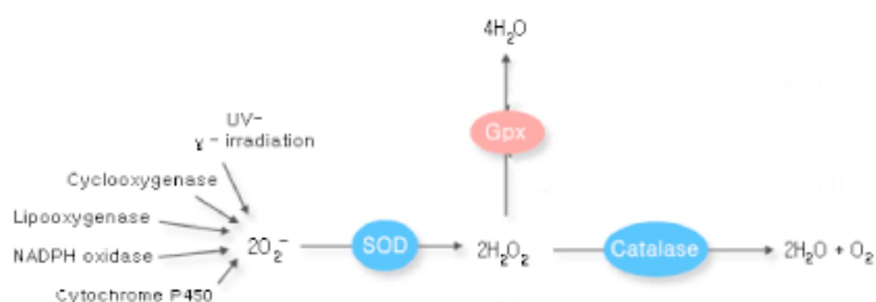


Fig 1.10. Representación de la acción de los enzimas antioxidantes que encargados de la reducción de las especies ROS.

- La superoxido dismutasa (SOD) es una metaloproteína considerada como el primer paso de la defensa antioxidante. Este enzima cataliza la dismutación del anión superóxido a una molécula de oxígeno y a peróxido de hidrógeno

(fig 1.10). Los metabolitos creados por la SOD son posteriormente detoxificados por otros enzimas como la catalasa y la glutatión peroxidasa.

- La glutatión peroxidasa (GPx) es un enzima con actividad peroxidasa cuya función biológica es reducir los hidroperóxidos de lípido al alcohol correspondiente y al peróxido de hidrógeno a agua (fig 1.10). Las GPx son principalmente enzimas selenio dependientes que emplean GSH como cofactor (Van der Oost et al. 2003).
- La catalasa es un metaloenzima tetramérico que se encarga de convertir el peróxido de hidrógeno en agua y oxígeno (fig 1.10; Boon, E.M. et al 2001).

Algunos contaminantes ambientales pueden inducir estrés oxidativo en los organismos de sistemas acuáticos, como por ejemplo los hidrocarburos policíclicos aromáticos (PAHs), las dioxinas, la radiación UV etc. (Di Giulio et al. 1995). Por esta razón, el estudio de la inducción de enzimas antioxidantes se considera como diagnóstico de exposición a diversos contaminantes o agentes de estrés (Winston, G.W. 1991; Company, R., et al 2006).

1.3.2.4 Transportadores de membrana

Los transportadores de membrana son proteínas encargadas de facilitar el flujo de sustancias entre el interior y exterior de la célula. Su función principal es el transporte de nutrientes o sustancias endógenas como azúcares, amino ácidos y vitaminas. Sin embargo, al tener una baja especificad para la sustancia transportada, también transportan sustancias exógenas, como medicamentos y xenobióticos. Se ha comprobado que debido a su inespecificidad, estos transportadores tienen un papel importante en la determinación de la biodisponibilidad, en la eficacia terapéutica y en la cinética de diversos fármacos, incluyendo antitumorales. Además junto a los procesos de detoxificación de xenobióticos, como la acción de los enzimas CYP1A, se pueden convertir en una barrera de protección para riñón, tracto intestinal, hígado y placenta, entre otros, ya que evita la acumulación de xenobióticos en el interior de la célula (Rollo, C.D. 2007).

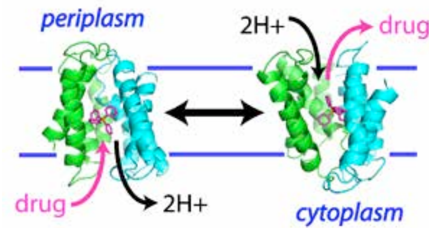


Fig 1.11. Modelo de un transportador de membrana

Los transportadores pueden dividirse en pasivos, que permiten que las moléculas se muevan a través de la membrana celular mediante gradientes electroquímicos facilitando su transporte, y activos, capaces de transportar moléculas en contra del gradiente electroquímico generando un gasto energético. El transportador activo puede ser tanto primario (hidrolizando moléculas de ATP para mover las moléculas) como secundario (utilizando gradientes de iones o protones).

Existen diferentes tipos de transportadores activos primarios, uno grupo de los más importantes son los transportadores ABC (de sus siglas en inglés : *ATP Binding Cassette*), que necesitan de la unión e hidrólisis del ATP para realizar su función. Los transportador ABC más estudiados son el MDR (de sus siglas mulltidrug resistance protein), el MRP (de las siglas multidrug resistance-associated protein) y las BCRP (breast cancer resistance protein las proteínas resistentes a medicamentos (MDR, multidrug resistance protein) (You, G, and Morries ME. 2007).

-*P-glicoproteina:* Proteína también conocida como ABCB1 o P-gp. Es una de las mejores caracterizadas de la familia de transportadores ABC en mamíferos. Se encuentra principalmente en la membrana plasmática de las células multiresistentes del intestino, hígado y riñón (Honorat, M. et al. 2009). El P-gp se compone de una cadena larga (170 kDa) que se pliega en dos mitades (fig 1.12). La mayoría de los sustratos para P-gp son hidrofóbicos, e incluyen tanto moléculas tóxicas como medicamentos importantes como ciclosporina y diferentes antitumorales. Así pues, y desde un punto de vista terapéutico, la acción del P-gp protege el organismo de las toxinas pero a la vez reduce la efectividad de los medicamentos, especialmente antitumorales (Stephen, G., et al. 2009). En condiciones naturales, su actividad es importante para la resistencia a diversos tóxicos, incluyendo pesticidas.

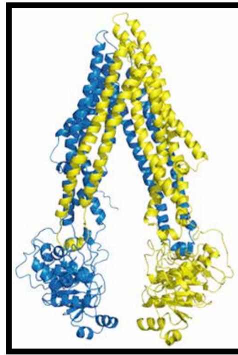


Fig 1.12. Estructura de las P-glicoproteínas

- *MRP (Multidrug resistance-associated protein)* Después del descubrimiento del P-gp, las investigaciones terapéuticas contra el cáncer descubrieron un nuevo grupo de transportadores de membrana denominado ABCC. Esta subfamilia tiene un total de 13 proteínas, la mayoría de ellas identificadas como transportadores activos dependientes de ATP y que se activan por medio de aniones orgánicos y componentes terapéuticos. Los transportadores ABCC tienen un papel importante en el control de la concentración de sustancias tanto exógenas como endógenas en el citoplasma celular.

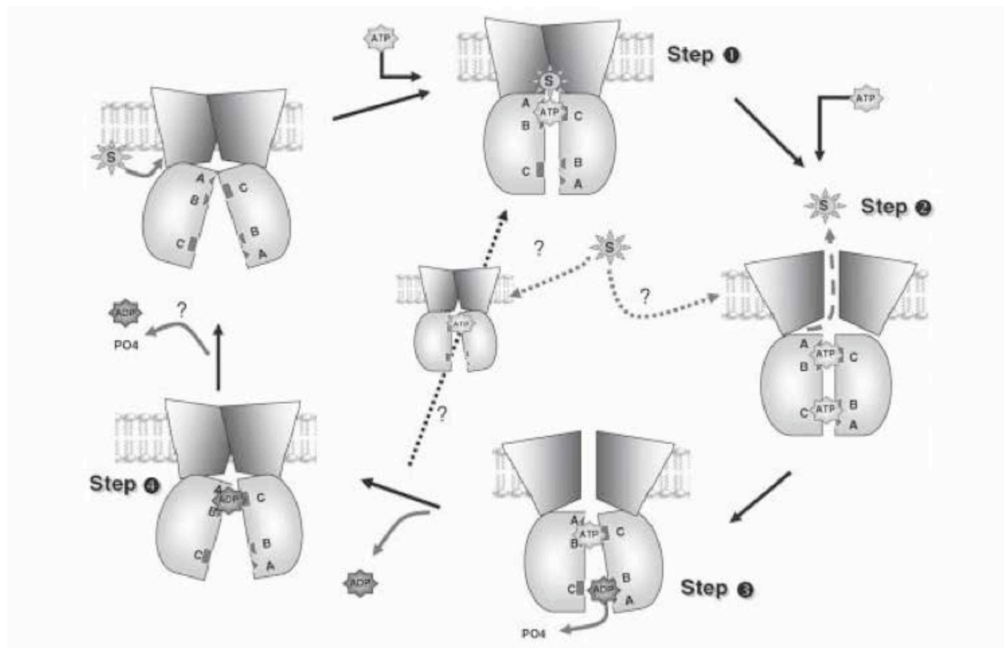


Fig 1.13. Modelo de transporte de la familia ABCC. Paso 1: Unión al sustrato. Paso2: La unión inicial facilita la unión de la segunda molécula de ATP, la conformación cambia, el sustrato queda expuesto extracelularmente y se reduce la afinidad al sustrato. Paso 3 y 4: después de la hidrólisis de los ATPs, la conformación se restablece y la proteína pasa a estar de cerrada a abierta, preparada para el siguiente ciclo de transporte.

Como en el caso de los ABCB, muchos de los miembros de esta subfamilia están asociados con diferentes funciones fisiológicas, como por ejemplo procesos de detoxificación, defensa contra compuestos xenobióticos y estrés oxidativo.

Muchos organismos acuáticos son capaces de vivir en áreas consideradas contaminadas, sugiriendo que deben poseer algún mecanismo que les permite tolerar y resistir la presencia de xenobióticos. El mecanismo de transporte de la familia ABC, muy bien caracterizada en mamíferos, también se ha estudiado en especies acuáticas como *Mytilus californianus* (Cornwall, R. et alt. 1995), *Corbicula fluminea* (Achard, M. et alt 2003) y *Dreissena polymorpha* (Pain, S. and Parant, M. 2007), entre otros bivalvos, y *Fundulus heteroclitus* (Bard, BM. et alt 2002) como ejemplo de vertebrados. Se ha observado que estos transportadores tienen un papel importante en los mecanismos de defensa a contaminantes de estas especies. El P-gp puede exportar tanto xenobióticos en su forma sin modificar como xenobióticos conjugados, mientras que el MRP solo exporta glutatión y xenobióticos conjugados (Sharom, F.J. 2008)

Existen diferentes metodologías para medir la actividad de las proteínas de membrana, como por ejemplo el uso de colorantes como la rodamina 123 o la calceína-AM (Ca-AM) para evaluar la entrada, acumulación y salida de determinadas sustancias. Tanto la rodamina como la Ca-AM se utilizan como sustrato para las subfamilias ABCB y ABCC. En el caso de la calceína, esta se hidrolizan al entrar en la célula por mediación de las esterasa citosólicas y forma una molécula fluorescente verde. La rodamina, por otro lado ya presenta una fluorescencia de color rojo, y no es necesarioactivarla. La cantidad de estas moléculas acumuladas en el interior de la célula se considera que es la cantidad de Ca-AM o rodamina que ha entrado en la célula y se puede considerar por lo tanto como medida de la actividad de transporte. En el caso de una elevada actividad de los transportadores de membrana es indicado por una medición leve de fluorescencia, sin embargo, en el caso de una actividad baja de los transportadores la señal es más fuerte (Luckenbach, T. 2008).

1.3.2.5 Receptor del ácido 9-cis-retinoico, o RXR

La vitamina A y sus derivados activos son conocidos como retinoides, dentro de la categoría de hormonas no esteroides. Los retinoides desempeñan un papel muy importante en el desarrollo y la homeostasis de la mayoría de tejidos, regulando aspectos como la diferenciación, proliferación celular, apoptosis y reproducción.

El receptor del ácido 9-cis-retinoico, o RXR es miembro del tipo II de la superfamilia de los receptores hormonales nucleares (NR), y presenta 3 isotipos diferentes (α,β,γ). Actúa como factor de transcripción, ya que se une a secuencias específicas de DNA en el promotor de sus genes diana, regulando su transcripción dependiente de la presencia y la naturaleza de ligandos específicos ([a]Mangelsdrof, et al 1995).

Fig 1.14 Estructura general del receptor nuclear donde se puede observar las tres partes principales de un receptor: un dominio de amino-terminal (N), el dominio de unión a DNA (zona muy conservada) y el dominio de unión a ligando (LBD) en la zona terminal. También se puede ver el dominio de activación 1 y 2 (AF) y bisagra o hinge en inglés (H)

Como la mayoría de receptores nucleares, los receptores de retinoides comparten dominios modulares que pueden ser intercambiados sin perder su función. Los dominios N-terminal A/B presentan la función de activación (AF-1), y junto al dominio C (AF-2), son los dominios más variables del gen. Por otro lado cuenta con el dominio DBD (unión al DNA) y LBD (unión al ligando) que están más conservados ([b]Mangelsdorf, DJ et al 1995).

Otro receptor de retinoides es el ácido retinoico (RAR), que forma heterodímeros con el RxR (RAR/RXR). Este heterdímero se une a secuencias específicas del DNA regulando la transcripción de diferentes genes. La secuencias a las que se unen se conocen como elementos de respuesta al ácido retinoico (RARE), y han sido identificadas en una larga lista de genes, presentando una gran variedad de funciones. Los receptores RxR también pueden formar heterodímeros con la vitamina D (VDR) y el receptor activador de la proliferación peroxisomal (PPAR), desempeñando un papel importante en otras vías metabólicas (Soria-Gili, M and Ribera-Pibernat, M. 2005)

El ligando fisiológico natural conocido del RxR es el 9-cis ácido retinoico, al cual se une con una elevada afinidad (Mangelsdrof, D.J. et al 1992). Además, se ha comprobado que el RxR se une a diversas sustancias de la familia de los organoestánicos, como el TBT, un efecto ligado a la aparición de *imposex* en gasterópodos (Nishikawa, J-I, et alt 2004). Por tanto, la inducción del gen RxR se puede considerar un buen candidato para detectar posibles casas de disrupción endocrina en invertebrados (Nishikawa, J-I, et alt 2004; Sousa, A.C.A, et al 2010)

1.4 Análisis de expresión génica

La medición de cambios en la transcripción de genes específicos para evaluar los efectos de contaminantes u otros agente de estrés sobre las diferentes vías metabólicas, se ha convertido en una herramienta importante en los estudios de impacto ambiental. Existen varias técnicas que se utilizan para la cuantificación de los niveles de mRNA, como el *Northern blot* y la PCR a tiempo real (qRT-PCR), adecuados para estudiar un número limitado de genes o los chip de DNA (*microarrays*), cuando se quiere estudiar los niveles de transcripción de muchos genes a la vez (perfiles de expresión). Las técnicas utilizadas en este trabajo han sido los chips de DNA y qRT-PCR, que son descritas a continuación.

1.4.1 Chips de DNA

El chip de DNA consta de una soporte sólido al cual, y siguiendo un patrón geométrico definido, se le une una serie de fragmentos de DNA llamados sondas, cuya secuencia es específica para cada uno de los genes a estudiar. Los soportes que se utilizan para fijar estas sondas pueden ser muy variados, como vidrio, plástico o silicio, y la técnica permite monitorizar los niveles de transcripción de miles de genes simultáneamente.

El funcionamiento de los chips se basa en la capacidad que tienen las moléculas complementarias de DNA a unirse entre sí. En un experimento tipo, se extrae el RNA de las células o del tejido que se desea estudiar, se retrotranscribe a DNA complementario (cDNA) y se marca con un fluoróforo. La muestra marcada se enfrenta a las sondas fijadas en los chips de DNA. Las diferentes moléculas marcadas de cDNA se mueven por difusión y, al encontrar la sonda complementaria, se hibridan y quedan fijadas a las mismas. Después de un tiempo de incubación, el chip se lava y se miden los niveles fluorescencia de los fragmentos hibridados a cada una de las sondas, tomándose estos niveles como indicadores de la cantidad relativa de moléculas de RNA presentes en la muestra original para cada uno de los genes correspondientes. Así se determina qué genes se transcribes más o menos o si su transcripción cambia en diferentes condiciones experimentales o ambientales (fig 1.15).

Las aplicaciones de los chips de DNA se van ampliando, y uno de los usos es el análisis cuantitativo de la expresión génica, que se utiliza para identificar genes que cambian de nivel de expresión tras la influencia de un factor en particular (Drăghici, S., 2003)

Hoy en día existen dos tipos mayoritarios de plataformas de chips de DNA (Ju, Z et al. 2007). El sistema de Affimetrix GeneChip®, utiliza la síntesis de oligonucliótidos *in situ* sobre una superficie de cristal mediante fotolitografía. Es un chip muy fiable, poco flexible, ya no permite cambios sobre un patrón preestablecido, pero lo que es solo útil para organismos modelo, muy bien conocidos y muy usados en este tipo de estudios. En cambio, existen chips personalizables, como por ejemplo los sistemas de Agilent®, que consisten en la deposición mecanizada de sondas pre-sintetizadas (60-meros en general) en soportes de vidrio. Estas plataformas pueden usarse tanto para especies y modelos conocidos como crear chips de DNA de especies poco conocidas, usando las secuencias disponibles en cada momento.

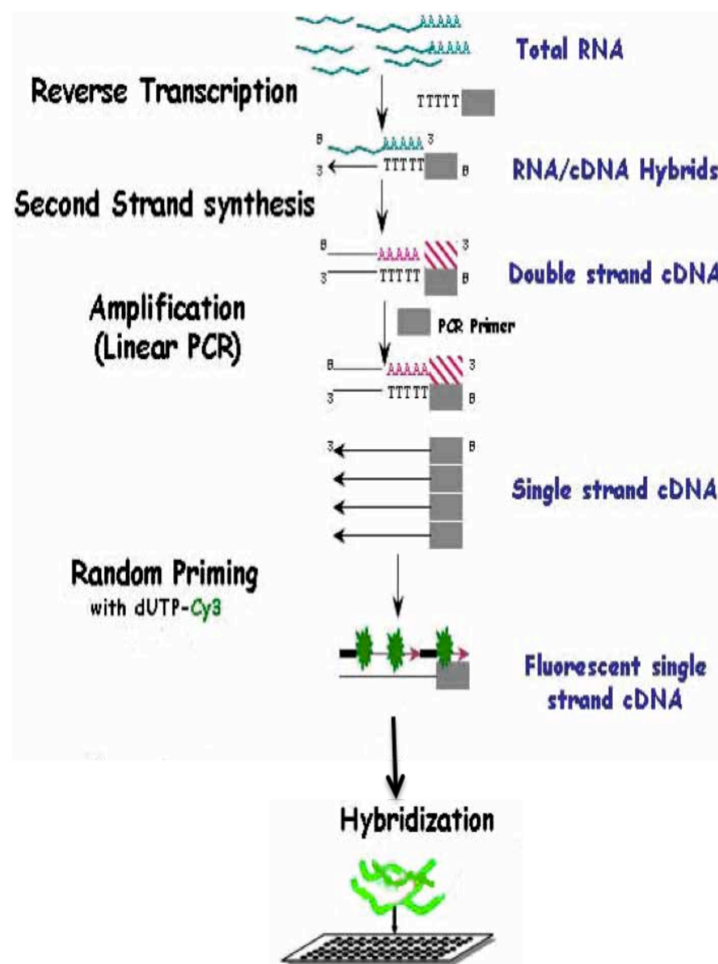


Fig 1.15 Esquema del procedimiento de hibridación para el análisis de la técnica de chips de DNA de un color . Cuando se realiza la técnica de un color del chip de DNA solo se utiliza el fluorófo CY3 (verde).

La utilización de los chips de DNA permite obtener información de miles de genes a la vez, aunque presenta numerosas fuentes de variabilidad, siendo necesaria la aplicación

de réplicas y de técnicas estadísticas y de análisis de datos para obtener resultados de alta calidad (Kerr, M.K. and Churchill G.A. 2001). Es una técnica útil para estudiar el efecto que pueden causar nuevos contaminantes y descubrir nuevos modos de acción.

1.4.2 PCR cuantitativa a tiempo real (qRT-PCR)

La PCR cuantitativa a tiempo real (qRT-PCR) es una de las técnicas más potentes para la detección y cuantificación de mRNA debido a su alta sensibilidad, buena reproducibilidad y su amplio rango dinámico de cuantificación. Esta técnica es una variante de la reacción en cadena de la polimerasa (PCR: Polymerase Chain Reaction) que combina la amplificación exponencial de PCR con la cuantificación simultánea del producto final de amplificación en cada ciclo.

La PCR fue desarrollada por Kary Mullis en el 1980. Este método se basa en la capacidad del enzima DNA polimerasa para duplicar indefinidamente cadenas de DNA *in vitro* de manera precisa y repetitiva. Este proceso necesita la presencia de cebadores, pequeños fragmentos de DNA (oligonucleotidos) complementarios para ambas cadenas (sentido y antisentido) de la molécula DNA parental. Estos cebadores, una vez hibridados a sus secuencias complementarias, sirven de substrato que la DNA polimerasa añada nucleótidos complementarios a su extremo 3' hasta copiar (idealmente) toda la cadena de DNA que sirve de patrón. Esta reacción requiere un proceso cíclico de cambios de temperatura ya que para la separación de las hebras de DNA (desnaturalización) se requiere una temperatura más elevada que para la hibridación de los cebadores (*annealing*) y el proceso de extensión del fragmento (extensión del cebador). El factor clave de la PCR es la existencia de DNA polimerasas termorresistentes, que no se desnaturalizan durante estos cambios de temperatura y que se obtienen a partir de microorganismos termófilos. Así pues, en cada ciclo de PCR se genera una nueva copia de la secuencia diana, obteniendo una amplificación exponencial del fragmento deseado (fig 1.16).

En la PCR estándar, el producto final de la reacción se detecta al final del proceso, pero en el caso de la qRT-PCR, el DNA amplificado se detecta y cuantifica a cada ciclo del proceso de amplificación a tiempo real. La detección se lleva a cabo mediante la monitorización de la emisión de señal de una molécula fluorescente añadida a la mezcla de la reacción. Esta fluorescencia está directamente relacionada con la cantidad de moléculas de DNA acumuladas en cada ciclo (Higuchi et al, 1992). Existe una gran variedad de métodos de detección, como por ejemplo la fluorescencia generada por

fluoróforos específicos de ADN bicatenario (SYBR Green®) o por sondas fluorescentes específicas de secuencia (sondas TaqMan®, MBD, Molecular Beacons, entre otras).

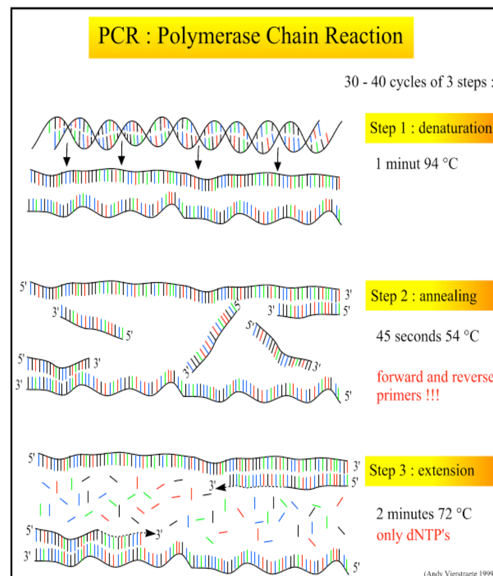


Fig 1.16 Representación de los pasos de la reacción de la PCR. La secuencia de DNA que se ha de amplificar junto al enzima y los cebadores es calentada a 94°C para desnaturalizar las hebras de DNA (Step 1 Denaturation). A continuación, se enfría a 54°C para permitir la hibridación de los cebadores con la secuencia complementaria de DNA (Step 2, Anneling). La temperatura vuelve a aumentar a 72°C para que los enzimas extiendan la cadena complementaria (Step 3, Extension).

Durante esta tesis se ha utilizado el método de SYBR Green®, que proporciona una metodología simple y económica de detección y cuantificación de fragmentos de PCR. El SYBR Green® es una cianina asimétrica que se une a las moléculas de DNA formando un complejo DNA-fluoróforo que emite fluorescencia a 510nm. Sin embargo, como este fluoróforo puede intercalarse a cualquier cadena de doble hélice de DNA que esté presente en la reacción, es necesario diseñar métodos de control para la evaluación de la formación de productos no específicos, para evitar así errores de cuantificación o interferencia de señales con falsos positivos.

Existen diferentes métodos para calcular el número de ciclos necesarios para detectar la amplificación de una secuencia concreta, llamado ciclo de cuantificación (Cq). Por un lado, mediante la determinación en ciclo, o Ct (*threshold cycle*; fig 1.17). El Ct se define como el número de ciclos necesarios para que la intensidad de emisión del fluoróforo sea más elevada que el ruido de fondo. El otro método se basa en calcular la segunda derivada de la curva de amplificación, donde el Cq se identifica con el punto máximo de aceleración de fluorescencia, calculado a partir de la segunda derivada de la curva de cuantificación, y se denomina Cp (Luu-The, V. et al 2005).

Model of real time quantitative PCR plot

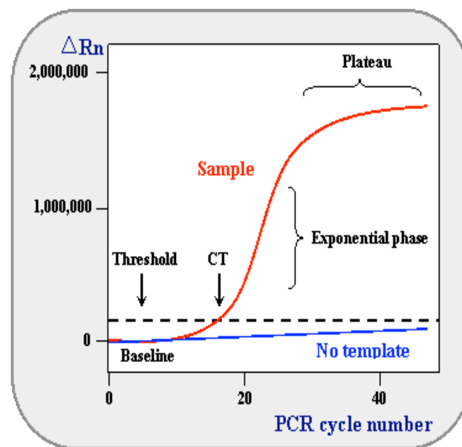


Fig 1.17. Resultados de detección de fluorescencia en una qRT-PCR dónde se muestra la curva de amplificación de una muestra en la fase de extensión. El valor de Ct es calculado determinado el punto en el cual la fluorescencia para el nivel del umbral del ruido de fondo.

Varios factores pueden alterar el cálculo por qRT-PCR del número de moléculas de mRNA presentes en una muestra, incluyendo el proceso de extracción y la retrotranscripción, por eso es aconsejable el uso de controles internos. El control interno ideal es aquel que se expresa a niveles constantes y no se ve alterado por tratamientos experimentales (Olsvik, P.A., et al. 2005). En este tipo de análisis, la expresión de un gen diana se cuantifica en relación al gene control, o de referencia, de cada muestra con la ecuación siguiente (Pfaff, M.W., 2001):

$$R = \frac{N_{0Diana}}{N_{0Ref}} = \frac{(1+E_{ref})^{Ct_{ref}}}{(1+E_{diana})^{Ct_{diana}}} x \frac{N_{diana}}{N_{ref}}$$

donde R representa el número de copias normalizadas por el gen de referencia.

Si se considera que la eficiencia de la reacción de PCR es óptima (100%), *E* es igual a 1 y se obtiene la siguiente ecuación:

$$R = K x 2^{(Ct^{ref} - Ct^{diana})}$$

donde K es el coeficiente entre el número de copias N del gen diana y de referencia, y el valor resultante de la diferencia es el exponente en base dos que representa la función de duplicación de la PCR.

En comparación con otras técnicas utilizadas para la cuantificación de mRNA, como por ejemplo el *Northern blot*, la qRT-PCR es mucho más sensible y precisa, suficiente para analizar el RNA procedente de una sola célula (Dharmaraj, S. 2011).

1.5 Especies centinela para el monitoreo de sistemas acuáticos

Para evaluar los efectos causados por la exposición a xenobióticos se han aplicado mayoritariamente dos tipos de estrategias diferentes: el uso de bioensayos o organismos estabulados, para analizar los efectos de contaminantes conocidos en condiciones controladas y el uso de organismos de poblaciones naturales, conocidas como especies centinela, para evaluar los efectos de exposición en condiciones naturales ([b]Quirós, L., et al 2007). Estas especies sirven para detectar, reconocer y monitorizar la presencia o ausencia de contaminantes en agua, sedimentos o alimentos, así como su biodisponibilidad y bioacumulación. Además, se utilizan para definir riesgos potenciales asociados a la exposición de contaminantes en el medio ambiente. La gran ventaja del uso de animales centinela en estudios de impacto ambiental es que detectan los efectos reales de cada contaminante bajo condiciones naturales, mientras que los análisis químicos o físicos solo determinan la exposición, pero no los efectos. También permiten medir los efectos de mezclas de contaminantes y determinar resultados de exposición a concentraciones bajas a lo largo del tiempo. Cada individuo analizado puede responder a efectos de contaminación de diferentes maneras y niveles, lo cual permite monitorizar cambios moleculares y bioquímicos, celulares, y/o fisiológicos que indican la presencia de contaminantes o posibles disfunciones de órganos tras la exposición a xenobióticos. Los análisis bioquímicos y celulares pueden ser útiles para la evaluación de efectos tóxicos a corto plazo y se pueden extrapolar a posibles consecuencias en humanos. Las especies centinela ofrecen diferentes tipos de información (Adams et al.1989):

- Primeras señales de daño ambiental;
- Efecto integrador de la variedad de estreses ambientales a nivel de organismo, especie, población, comunidad y ecosistema;
- Correlaciones entre respuestas individuales de organismos expuestos y efectos a nivel de población;
- Señal de alerta de daño potencial a la salud humana
-Evaluación de la efectividad de los esfuerzos de remediación en la descontaminación de los sistemas acuáticos.

Las especies centinela pueden ser animales salvajes, en el caso de estudios de campo, o animales de estabulario para estudios de laboratorio. Los estudios de campo permiten monitorizar diferentes hábitats ya sea por observación de animales residentes o a través de animales transplantados en jaulas (Van der Schalie et al 1999). Las especies centinelas han de ser sensibles a los compuestos del estudio, fáciles de capturar y representativos de la zona de estudio.

Entre las especies más utilizadas en programas de biomonitorización de sistemas acuáticos se encuentran comunidades microbianas, vegetales, insectos, macro-crustáceos, moluscos, anfibios, reptiles, peces y aves.

1.5.1 Peces

Los peces son un grupo importante desde la perspectiva de la toxicología al ser el grupo de vertebrados más diverso, con casi 25.000 especies diferentes y presentes en la mayor parte de sistemas acuáticos. Son especies susceptibles a la contaminación que responden con gran sensibilidad a los cambios en el medio acuático y a los efectos de contaminantes. Se pueden estabular con facilidad y exponer en el laboratorio en dosis similares a las encontradas en el medio, lo que permite comparar resultados experimentales con alteraciones observadas en peces que viven en hábitats expuestos a xenobióticos (Van der Oost et al. 2003). Su monitorización se considera especialmente relevante en el estudio del medio acuático gracias a que:

- Debido a la gran variedad de especies existentes, los peces cubren todos los niveles de la cadena trófica. Las especies depredadoras son muy susceptibles a la biomagnificación y bioacumulación de metales pesados y contaminantes orgánicos.
- Algunas especies tienen vidas relativamente largas, útiles para indicar cambios a largo plazo.
- Las comunidades de peces son muy sensibles a los cambios en factores ambientales debido a impactos antropogénicos, por lo que se utilizan para evaluar el estado de sistemas acuáticos.
- Son una fuente de alimento para humanos, lo cual da relevancia directa para la evaluación de potenciales tóxicos de contaminación en lugares de estudio.
- Los métodos de muestreo son baratos y sencillos. Además son especies relativamente fáciles de capturar y muchas veces se pueden usar métodos de análisis no destructivos (importante para especies grandes o en peligro de extinción). En muchos casos las especies elegidas como centinelas son las especies más abundantes

en el lugar de muestreo y por lo tanto el sacrificio de individuos no tienen tanto impacto sobre la comunidad (Melancon, 2004).

Durante este trabajo se ha utilizado como centinela:

- *Cyprinus carpio*: O carpa común, es una especie que pertenece a la familia de los Ciprínidos, representativa de los sistemas de agua dulce y originaria de Asia. Fue introducida en Europa entre el siglo XI y XII. Las poblaciones de carpa salvajes viven en la parte media y baja de los ríos, en las partes inundadas y en zonas de lagos y embalses encontrados a lo largo de la zona de estudio, río Ebro. Son animales omnívoros con tendencia a dieta carnívora como insectos, larvas, gusanos, moluscos y zooplancton (FAO, 2004-2011). Se utilizan comúnmente en exposiciones de laboratorio para el estudio de efecto de sustancias (Solé, M. et alt. 1999; Kono, T. et alt 2002), como también en poblaciones naturales para así poder estudiar el estado del río (Lavado, R. et alt. 2004).

Cyprinus carpio

Fig 1.18. Foto de la especie de pez utilizada como especie centinela

1.5.2 Bivalvos

Los bivalvos pertenecen a uno de los grupos más numerosos de organismos filtradores tanto de los sistemas acuáticos marinos como de agua dulce. Muchas especies tienen un gran interés comercial, como ostras, mejillones y almejas. Además, su estilo de vida sedentario en la fase de adultos y su estrategia de alimentarse por filtración hace que los bivalvos sean útiles como especies centinela para estudios de bioacumulación de contaminantes (Naimo, T.L 1995). Los bivalvos, presentan otra serie de ventajas como especie centinela: 1) su longevidad, que en algunas especies puede llegar a los 70 años; 2) su tolerancia a los contaminantes; 3) su sensibilidad a los cambios

ambientales y por último 4) son relativamente fáciles de muestrear y monitorizar (Grabarkiewiz JD y Davis WS. 2008).

Una buena parte de los estudios de esta tesis se han basado en el estudio de efectos de contaminantes y cambios ambientales en el mejillón cebra (*Dreissena polymorpha*). El mejillón cebra es una especie invasora original del mar Caspio, y es una de las especies de mejillones que ha tenido más éxito colonizando nuevos hábitats de agua dulce en todo el mundo. Este éxito de colonización es debido a (Borcherding, J. 1991):

- La habilidad de los adultos a adherirse a la superficie de rocas y demás objetos del lecho del sistema acuático utilizando su biso.
- El tener una fase larvaria velígera que nada libremente en el agua, característica única entre los bivalvos de agua dulce.
- Un alto índice de fecundación.

Además el mejillón cebra presenta alta resistencia a los cambios temperaturas y aunque se trata de un bivalvo de agua dulce puede vivir en hábitats de aguas salobres (8-10% de sal), como en el mar Caspio (Arajuzo, R., et al 2010). Estas características ayudan a que el mejillón cebra pueda sobrevivir en ecosistemas nuevos con facilidad y así colonizar y extenderse por casi toda Europa desde el siglo XIX (CSMA, 2007). El mejillón cebra se estableció en la cuenca del Río Ebro hace cerca de una década, ya que se vio por primera vez en el embalse de Riba –Roja en 2001. Ahora está presente en 7 ríos de la cuenca del Ebro (toda la cuenca. Con una rápida capacidad de crecimiento y un ciclo biológico corto, este bivalvo puede llegar a medir 3cm de largo y vivir alrededor de 3 años, llegando a la madurez sexual en el primer año de vida (Lucy, F. 2006).

El ciclo de vida de *D. polymorpha* consiste en una fase larvaria planctónica (velígera) de rápido crecimiento; una fase juvenil, que en condiciones óptimas pueden llegar a ser fértiles con menos de 5mm de longitud; y la fase adulta. Las hembras pueden comenzar a reproducirse durante su primer año de vida. Presentan una estrategia de reproducción externa y pueden llegar a producir cerca de 40000 huevos en cada puesta. Después de una fertilización externa se desarrolla una larva trocófora (6-96 horas después de la fertilización (hpf)) con forma de vida pelágica. Durante el estado de trocófora se desarrollan los órganos de locomoción, alimentación y pasa a ser una larva velígera. De los 2 a los 9 días después de la fertilización (dpf) la larva velígera forma una concha en forma de "D". De los 7 a los 9 dpf se distingue una región umbonal abombada y de contorno redondeado, propia del último estadío planctónico. A los 18 -90 dpf la larva acaba de desarrollar las valvas y aumenta de peso hasta que ya no puede permanecer suspendida en el agua, en la fase llamada pedivelígera. Una vez iniciada la fase

bentónica la larva sufre una fase de metamorfosis, pierde el velum (órgano que utiliza para alimentarse) y desarrolla las branquias. La segunda concha se sustituye por una nueva con forma triangular, pasando de la fase pedivelígera a juvenil. Los mejillones de consideran adultos cuando alcanzan la madurez sexual (fig 1.19). Algunos estudios muestran que en la cuenca del Río Ebro solo se pueden encontrar juveniles en los meses de Julio y Agosto (Araujo. R., et al 2010).

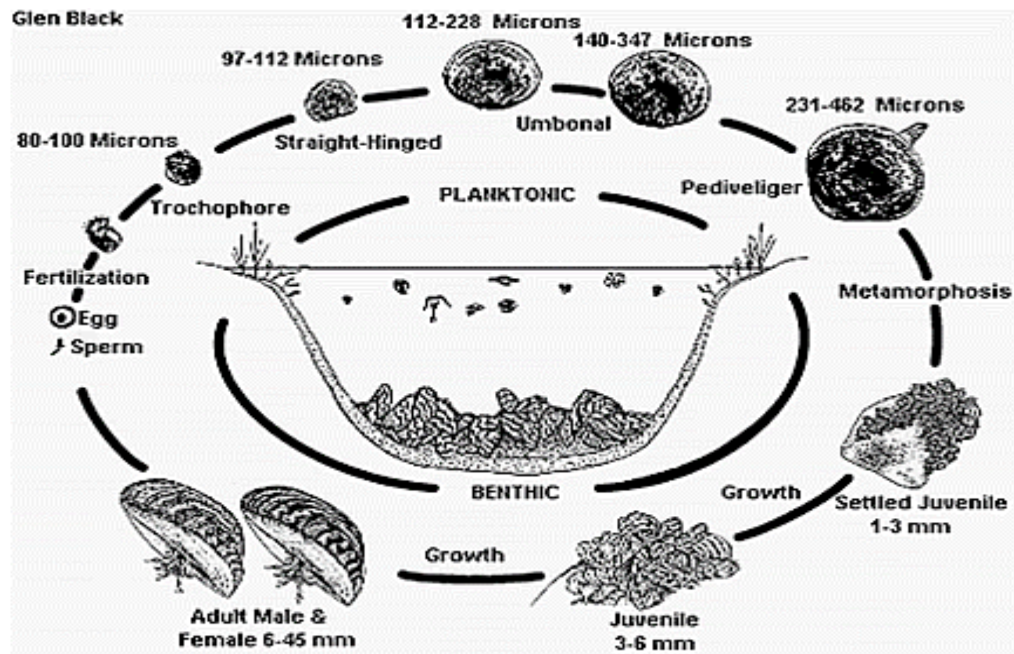


Fig 1.19 Representación de los diferentes estadios de vida del mejillón cebra.

1.6 El río Ebro

La cuenca del río Ebro se localiza en el noreste de la península Ibérica, con una superficie total de 85362 Km^2. Es uno de los ríos más caudalosos de España, con un caudal de $600m^3/s$, y con una cuenca del 17.3% de la superficie total de España. El canal principal del río es de 910 km, y su caudal circula de NW (de las montañas de Cantabria) al SE (al mar Mediterráneo), donde forma un delta. Recoge agua de los Pirineos y de diferentes afluentes como los ríos Gállego, Cinca y Segre (fig 1.20)

Fig 1.20 Mapa de la cuenca del Río Ebro mostrando la localización de los afluentes y ciudades más importantes

La cuenca del Ebro tiene paso por diferentes ciudades de diferentes provincias y durante la historia el río ha tenido un papel importante tanto como frontera como línea de comunicación. Los usos económicos principales del río han sido la irrigación y las plantas de energía hidroeléctrica. El desarrollo de la agricultura a lo largo de su cuenca implicó la construcción de muchas infraestructuras, como por ejemplo el Canal Imperial en 1446. Hoy en día se cuentan con 187 embalses, que retienen el 57% del agua de escorrentía anual (Romaní, A.M. et al. 2011). Debido a su importancia, el Río Ebro se utilizó como área de estudio para esta tesis, concentrando los estudios en su curso bajo, una zona con un largo historial de contaminación por compuestos orgánicos y metales pesados, así como también problemas derivados de la presencia de especies invasoras.

1.6.1 Contaminación en el río

El Ebro es probablemente uno de los ríos españoles más estudiados. La Confederación Hidrográfica del Ebro (CHE), encargada del gestión del río, ha estado realizando programas de monitoreo de la calidad de agua durante más de 30 años. Sin embargo, la introducción del Río Ebro en un proyecto de la Unión Europea "AquaTerra" en 1992 dio la oportunidad de evaluar los niveles de contaminantes a lo largo del río para detectar sus puntos más conflictivos. En el año 2009, la Confederación Hidrográfica del Ebro (CHE) detectó la presencia de niveles altos de mercurio, DDT y hexaclorobenceno en peces capturados en la parte baja del río, concretamente en el área de Flix, donde se encuentra operativa una planta de fabricación de compuestos clorados. Esta planta química se puso en marcha en 1897 y lleva operando más de un siglo. Durante todo

este tiempo ha estado utilizando el río Ebro como vertedero de algunos de sus residuos, acumulándolos en forma de sedimentos en el río, que han llegado a formar un semi-delta de residuos en frente de la planta (fig 1.21).

Fig 1.21. Foto aérea donde se muestra los residuos depositados por la planta de fabricación de productos clorados que opera en el área de Flix, aguas en el tramo bajo del río Ebro.

La detección de altos niveles de contaminantes en el área de Flix dio paso a un nuevo proyecto (RiscFlix), que incluyó el desarrollo de test toxicológicos para detectar el nivel de estrés de los organismos acuáticos debido al impacto producido por los fangos de Flix. Durante este proyecto se analizaron poblaciones naturales desde la zona de Riba-Roja (considerada control) hasta la desembocadura del río Ebro. Diferentes estudios han mostrado altos niveles de pesticidas aguas abajo de la zona de Flix, dónde el análisis de la respuesta biológica revela efectos severos en invertebrados (Barata et al 2007; Faria, M. et al 2010). Otros estudios con vertebrados (Barbus graellsii y Cyprinus carpio) muestran la presencia y efecto de contaminación por metales pesados aguas abajo de la zona de Flix debido a la movilización de contaminantes aguas debajo de la planta (Quirós, L., et al 2008; Navarro, A. et al 2009).

1.6.2 El problema de las especies invasoras

Otro problema importante que sufre la cuenca del río Ebro es la introducción de especies exóticas durante décadas. La entrada de especies nuevas en un ecosistema perturba las poblaciones naturales y los procesos biológicos del ecosistema, al crearse una competición por los mismos recursos, ya sean alimentarios o de hábitat. Además de

los problemas ecológicos, las especies invasoras también generan un alto impacto en la economía local, ya que pueden destruir especies autóctonas de consumo o también creando un gasto económico en su erradicación y restauración. En el casos del mejillón cebra, hay que añadir el gasto de mantenimiento de infraestructuras como limpieza de tuberías y canales para evitar su obstrucción. Sin embargo, la introducción de algunas especies exóticas pueden llegar a ser económicamente beneficiosas, como por ejemplo en el caso de especies de consumo humano que dan lugar a proliferación de industrias pesqueras, de procesado y comercialización. Actualmente se cuentan 18 especies introducidas de manera reciente en la parte baja del Ebro (incluyendo el Parque natural del Delta), como los siluros (como especie de pesca deportiva), el cangrejo americano (con finalidad de consumo), mejillón cebra y caracol manzana (introducción por accidente), entre otros (Balsells, F. 2009).

Chapter 2: Objectives and Structure

El objetivo principal de esta tesis es el de diseñar y validar nuevos marcadores moleculares, tanto en vertebrados como en invertebrados, para aplicarlos en el estudio de monitorización ambiental y así poder utilizarlos en la evaluación de impacto de contaminantes en sistemas acuáticos.

Los objetivos específicos de este trabajo son:

1. Optimizar el análisis de marcadores moleculares ya establecidos, como es el caso de la metalotioneína, para obtener información más precisa sobre el estudio de impacto ambiental por metales pesados.
2. Desarrollar una batería de marcadores moleculares de genes específicos en una especie invasiva, el mejillón cebra (*D. polymorpha*), tanto en larvas como adultos para evaluar su potencial como especie centinela y obtener más información sobre su tolerancia a contaminantes y agentes estresantes.
3. Estudiar poblaciones naturales del mejillón cebra para identificar diferencias variaciones durante el ciclo anual utilizando chips de DNA como técnica de estudio.

Los resultados obtenidos durante el estudio se presenta en diferentes capítulos, los cuales forman parte de artículos publicados, enviados o en preparación. Los capítulos se dividen en:

Chapter 3: Improvement of molecular biomarkers for better response to anthropogenic contamination

Los resultados de este capítulo han sido publicados.

Anna Navarro, Laia Quirós, Marta Casado, Melissa Faria, Luís Carrasco, Lluís Benejam, Josep Benito, Sergi Díez, Demetrio Raldúa, Carlos Barata, Josep M. Bayona, Benjamin Piña 2009. Physiological responses to Mercury in feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site. *Aquatic Toxicology*. 93 150-157

Chapter 4: Development and validation of new molecular biomarkers in order to assess the effect in adults and larvae of Zebra mussel by heavy metals

Los resultados de este capítulo han sido publicados.

Anna Navarro, Melissa Faria, Carlos Barata, Benjamin Piña 2011. Transcriptional stress genes to metal exposure in zebra mussel larvae and adults. *Environmental Pollution* 159. 100-107

Chapter 5: Development and validation of new molecular marker for endocrine disruption.

Los resultados de este capítulo forman parte de un artículo en preparación.

Anna Navarro, Carlos Barata and Benjamin Piña. Retinoid X Receptor gene expression study in adult *Dreissena polymorpha:* Laboratory and Field approach *RXR*

Chapter 6: Characterization of ABC membrane transporters proteins family in larvae and adult zebra mussel and the study of its role in detoxification mechanisms.

Los resultados de este capítulo han sido enviados a publicar.

Anna Navarro, Melissa Faria, Susann Weißbach, Till Luckenbach, Benjamin Piña and Carlos Barata. Constitutive expression and activity levels of ABCB and ABCC transporter homologs in larvae and adults of zebramussel and inducibility by chemicals.

Chapter 7: Development of microarray for non-model specie, Zebra mussel, to study the pathway of genes.

Los resultados de este capítulo forman parte de un artículo en preparación.

Anna Navarro, Bruno Campos, Carlos Barata and Benjamin Piña Seasonal variations in gene expression profiles in natural populations of zebramussel (Dreissena polymorpha) from the Ebro River (NE Spain)

CHAPTER 3: Improvement of molecular biomarkers for better response to anthropogenic contamination.

Physiological responses to Mercury in feral carp populations inhabiting the low Ebro River (NE Spain), a historically contaminated site.

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ABSTRACT

The low Ebro River course (Northeast Spain) is historically affected by mercury pollution due to a chlor-alkali plant operating at the town of Flix for more than a century. River sediments analyzed during the last 10 years showed high mercury levels in the river section starting just downstream the factory and spanning some 90 km, down to the river delta. The possible environmental impact was studied by a combination of field and laboratory studies. Mercury concentrations in liver, kidney and muscle of feral carp (Cyprinus carpio) sampled downstream Flix were one to two orders of magnitude higher than those from carps sampled upstream Flix. Elevated levels of mercury in these samples associated with significant increases on the concentration of reduced glutathione (GSH) in liver and on mRNA expression of two metallothionein genes, MT1 and MT2, in kidney and, partially, in scales, but not in liver. Conversely, no biochemical evidence for oxidative stress or DNA damage was found in these tissues. Non-contaminated carps subjected to intra-peritoneal mercury injection resulted in a 20-fold increase of MT1 and MT2mRNA levels in carp kidney, with minimal changes in liver levels. Our data suggests the coordinate increase of metallothionein mRNA in kidney and of GSH in liver constitutes an excellent marker of exposure to subtoxic mercury levels in carps. This study also demonstrates that apparently healthy fish populations may exceed the mercury contamination acceptable for human consumption.

Keywords: *Cyprinus carpio*, Mercury poisoning, Gene expression biomarkers, Metallothionein, Glutathione, Natural populations

3.1. INTRODUCTION

Mercury is a heavy metal with no known metabolic function. Due to its unique physico-chemical properties, mercury occurs in the environment in different physical and chemical forms (Zalups, 2000). Mercury and, particularly, its organic derivatives are considered strongly neurotoxic in humans and wildlife (Díez, 2008), whereas kidney is reportedly the primary target tissue for toxicity by inorganic mercury in fish (Zalups, 2000). Exposure to heavy metals, including mercury, induces formation of highly oxidative chemical species like peroxide or superoxide groups in the cell (De Flora et al., 1994; Huang et al., 1996; Arabi, 2004), which generate different types of cell damage, including lipid peroxidation (Huanget al., 1996) and DNA double-strand breaks (De Flora et al., 1994). The cell reacts to these aggressions by activating different enzymes like catalase (CAT) and glutathione-S-transferase (GST), directly related to detoxification and the reestablishment of the redox balance in the cell (Douglas, 1987; Sharma et al., 2004).

A substantial fraction of mercury toxicity is due to its avidity for thiol groups (Maracine and Segner, 1998; Zalups, 2000). A known mechanism of mercury detoxification is to increase the cellular levels of different sulfur-containing protective molecules, like glutathione (GSH) and metallothioneins (MT) (Zalups, 2000). GSH is the most abundant cellular thiol compound. It is involved in metabolic and transport processes and in the protection of cells against the toxic effects of a variety of endogenous and exogenous compounds, including reactive oxygen species (ROS) and heavy metals (Meister and Anderson, 1983; Peña-Llopis et al., 2001). Metallothioneins (MTs) are cysteine-rich, low-molecular weight proteins with high affinity for metals such as mercury, zinc, copper and cadmium. The expression of MT genes is induced in different tissues by the presence of divalent cations of heavy metals and by many other factors, such as glucocorticoids and cytokines (Kagi and Schaffer, 1988; Haq et al., 2003). Mice from which MT genes have been removed become hypersensitive to mercury and other metals (Satoh et al., 1997; Yoshida et al., 2004). GSH and MTs have been proposed to play cooperative protective roles against metal toxicity, the former as an initial defense and the latter acting at a second stage (Ochi et al., 1988).

In the present work, a combination of chemical analysis, quantitative analyses of mRNA and biochemical biomarkers was used to establish the biological effects of chronic mercury exposure in carp populations from the low Ebro River basin (NE Spain). This area is polluted by mercury due to the activities of a chlor-alkali plant located at Flix (fig. 3.1), which discharges large amounts of heavily polluted industrial sludge in the adjacent dam. The impact of these pollutants in local populations of molluscs, fish and birds has been evaluated only recently (Carrasco et al., 2008; Eljarrat et al., 2008; Quirós et al., 2008). The present work aims to study the biological effects on fish populations which can be associated with mercury pollution at the low Ebro River and to put these findings in the perspective of the toxification of surface waters by industrial activities occurring around the world.

The biomarkers selected for this study covered different kinds of toxicity markers commonly associated with mercury (De Flora et al., 1994; Sato and Kondoh, 2002). They include biomarkers for oxidative stress and genotoxic effects as well as analysis of mRNA levels for different stress genes related to mercury contamination. Biomarkers based on the precise quantitation of mRNA levels for different genes are becoming a powerful tool for environmental assessment, since they monitor the primary cell response to many stressors and are adequate to evaluate low levels of environmental stress. In the past, we identified some of these biomarkers for detection of feminizing and dioxin-like activities (Garcia-Reyero et al., 2004; Piña et al., 2007). The implementation of biomarkers for mercury toxicity is of utmost interest to monitor not only the presence of mercury in the animal tissues (which is relatively easy and cheap to perform) but also its bioavailability and capacity to elicit biological responses.

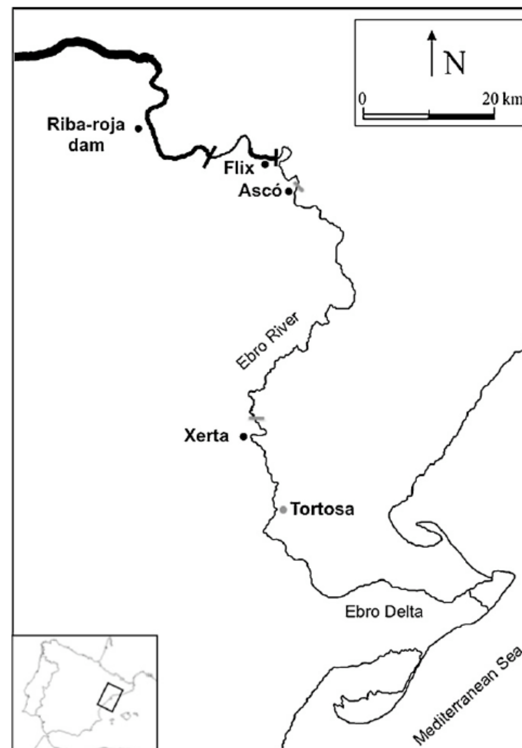

Fig. 3.1. Map of the low course of the Ebro River with indication of sampling sites. Approximate position of dams (black bars) and overflow dams (grey bars) are indicated to illustrate the mutual isolation of the four carp populations sampled in this study. The situation of Tortosa, not a fish sampling site, is provided to mark the geographical positioning of sediment sampling data shown in Table 3.2.

3.2. MATERIAL and METHODS

3.2.1 Environmental setting and sampling strategy

The Ebro River drains a watershed area of 85,362km2 constituting the largest river basin in Spain (http://www.chebro.es/) and receives discharges from the potential influence of three million inhabitants, including some heavily industrialized areas. One of these areas is the Flix site, where a chlor-alkali plant operates since the beginning of the 20th century. This long operational period has resulted in the accumulation of large amounts of heavily polluted industrial sludge (ca. 2.5×104 tonnes) in the adjacent riverbed, containing organochlorine compounds and mercury, among other pollutants (Llorente et al., 1987; Fernández et al., 1999). Nevertheless, both the low Ebro River course from Flix to the Delta (90 km, approximately) and the Delta itself have an enormous ecological, agricultural and recreational value. In addition, the input of nutrients from the Ebro River to the Mediterranean Sea drives the development of prosperous aquaculture activities (mussels, clams, oysters) and establishes one of the richest fishing areas in the

western Mediterranean Sea. Due to this social and commercial interest, and to the potential hazard associated to the Flix industrial waste, an environmental recovery program for the area has been implemented, involving the removal of the industrial waste to a controlled disposal site (more information in http://iagua.es/) Sampling sites were selected according to accessibility, availability of fish and mutual isolation regarding fish populations.

The dam at Riba-roja forms a large water reservoir (210 Hm^3, www.embalses.net) in the Ebro River, 13 km upstream Flix. The Flix dam itself forms a much smaller reservoir conceived to supply water for the electric plant included in the Flix industrial complex. Ascó and Xerta sites are placed in consecutive sections of the Ebro River, separated by an overflow dam, and situated 5.6 km and 37 km downstream Flix, respectively (see map in fig. 3.1.) To our knowledge, these four fish populations are essentially isolated one each other, as none of the dams has specific channels for allowing fish passage between them. We cannot exclude that some animals may be carried downstream in occasional high-flow episodes typical for Mediterranean rivers, especially throughout the overflow dam, although we consider it unlikely for the tall Riba-roja dam (60m high).

3.2.2 Fish sampling

Common carp (Cyprinus carpio) specimens were captured by direct current electric pulse, anesthetized in ice and its length and weight measured. They were sacrificed by decapitation and sexed by visual inspection of gonads. Samples of dorsal muscle, liver and posterior kidney were dissected, immediately frozen in liquid nitrogen, and stored at −80 °C for mercury and biochemical determinations. Samples of liver, kidney and dorsal scales (approximately 50 mg of each tissue) were placed in a cryogenic vial with 1mL RNAlater® (Sigma–Aldrich, St. Louis, MO), transported to the laboratory on ice, and stored at −80 °C for mRNA analysis.

3.2.3 Induction experiments

Carp specimens (10 individuals, mean total length 24.1±1.5 cm; mean body weight 256.2±53.7 g) were captured at irrigation channels in the Ebro Delta on January 2006; these channels are relatively free from contaminants. The specimens were placed by groups of five in 500 L flow-through tanks in the Centre of Aquaculture-IRTA, also in the Ebro Delta, and acclimatized to captivity for 5 weeks in dechlorinated tap water under natural conditions of photoperiod and temperature

and fed three times a week with commercial pellets. Fish were injected intra-peritoneally either with phosphate saline buffer (PBS, control group), or with a single dose of 20µg/kg of total Hg ion in PBS (injection volume, 5µl/gwetweight). No mortality occurred during exposure. All animals were sacrificed 72 h post-injection and processed as the animals captured in the field.

3.2.4 Mercury determination

Total mercury (THg) was determined using an Advanced Mercury Analyzer (AMA-254) from Leco Corp. (Altec, Praha, Czech Republic) following the method previously described (Díez et al., 2007). The entire analytical procedure was validated by analyzing a certified dogfish muscle certified reference material (CRM DORM-2) from the National Research Council Canada (NRCC,) at the beginning and at the end of each set of tissue samples. Detection and quantification limits were calculated from blank measurements, these values being 0.2 and 0.7 ng/g wet weight of Hg, respectively. A blank was analyzed periodically to verify that mercury was not being carried over between samples.

3.2.5 Quantitative analysis of MT1 and MT2 mRNA

Total RNA was extracted from tissue samples as previously described (Garcia-Reyero et al., 2004). Total RNA concentration was estimated by spectrophotometric absorption at 260nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE), treated with DNaseI (F. Hoffmann-La Roche Ltd., Basel, Switzerland), reverse transcribed to cDNA (Omniscript, Qiagen, Valencia, CA) and stored at −20 ◦C. Specific transcripts were quantified by real time PCR in a Abi Prism 7000 SDS (Applied Biosystems, Foster City, CA) using the SYBR Green chemistry (Power SYBR Green PCR Master Mix, Applied Biosystems). Metallothionein I and II, and ß-actin primers were designed from existing *C. carpio* sequences (Quirós et al., 2007a). Relative mRNA abundance values were calculated according to Eq. (1) using threshold cycle (Ct) values from triplicate assays as previously described (Pfaffl, 2001).

$$1)\quad \frac{mRNA_{\mathrm{Tg}}}{mRNA_{\mathrm{Act}}} = \frac{E_{\mathrm{Act}}^{\mathrm{Ct,Act}}}{E_{\mathrm{Tg}}^{\mathrm{Ct,Tg}}} \times 1000$$

PCR efficiency values for ß-actin and the target gene, E_{Act} and E_{Tg}, were calculated as described (Pfaffl, 2001). Results are given in copies of MTs mRNA per 1000

copies of ß-actin mRNA. The sequence of amplified PCR products was confirmed by DNA sequencing in Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and sequences compared to the corresponding references in GenBank (accession numbers AF002162 and AF249875 for Metallothionein I and II, and M24113 for ß-actin (Quirós et al., 2007a)). The suitability of ß-actin as a reference gene in all three tissues was assessed by the BestKeeper program (Pfaffl et al., 2004) (data not shown).

3.2.6 Biochemical determinations

For GST and CAT determinations, liver and kidney samples were weighted, flushed with ice-cold 1.15% KCl, and homogenized in 1:5 w/v cold 100mM phosphate buffer, pH 7.4, containing 100 mM KCl, and 1.0mM ethylenediaminetetraacetic acid (EDTA). Cytosolic supernatant fractions were prepared by sequential centrifugation steps as previously described (Andersson and Forlin, 1985; Raldúa et al., 2008). Protein content in the final supernatant was measured by the Bradford method, using bovine serum albumin as standard. Cytosolic glutathione-S-transferase conjugation activity versus 1-chloro-2, 4-dinitrobenzene (CDNB) and catalase activity were determined measuring the formation of S-(2, 4-dinitrophenyl)-glutathione conjugate as at 340nm and the decrease in absorbance at 240 nm due to H_2O_2 consumption, respectively, as described earlier (Raldúa et al., 2008). For determining glutathione content liver samples were homogenized in ice cold 5% trichloroacetic acid (TCA) and centrifuged at 10,000×g. Reduced (GSH) and oxidized glutathione (GSSG) levels were then measured in the supernatant fraction using the Ellman's reagent, 5,5-dithiobis (2-nitrobenzoic acid) assay at 412nm (Peña-Llopis et al., 2001). Lipid peroxidation and of DNA double-strand breaks in liver and kidney were quantified using the malondialdehyde (MDA) and DNA alkaline precipitation assay in the 10,000×g supernatant fraction, respectively, as previously described (de Lafontaine et al., 2000; Raldúa et al., 2008).

3.2.7 Statistical analysis

All statistics were performed using the SPSS 17 (SPSS Inc., 2002) package. Values are presented as means±SEM (standard error of means). Non-parametric tests were preferred when comparing very different sets of data; normality of data distribution was assessed by the Kolmogorov–Smirnov test. Statistical comparisons of mean values were made using a one-way analysis of variance (ANOVA) or the Kruskal–Wallis non-parametric test, depending on the properties of data sets.

Bivariate correlations between different parameters were analyzed by the non-parametric Spearman rank correlation test. Quantitative real-time PCR data from experimental induction of mRNA formation of metallothionein genes was analyzed by the non-parametric REST tool, using ß-actin as reference gene (Pfaffl et al., 2002).

3.3. RESULTS and DISCUSSION

3.3.1 Mercury levels in carp tissues from the low Ebro River course

Mercury concentrations in muscle, kidney and liver of carps from the Ebro River showed variations from one to two order of magnitude between different specimens. Maximal levels were found in kidney, followed by liver and muscle (table 3.1). Therefore, we concluded that kidney and liver accumulate mercury to higher concentrations than muscle. The lowest concentration of mercury in the three tissues corresponded to samples captured at the Riba-Roja site, upstream of Flix and considered a reference site (fig. 3.2A). Average levels increased progressively at Flix, Ascó and Xerta sites, evidencing a downstream transport of the mercury pollution originated at the Flix industrial dumping site (fig. 3.2A). These figures show that the 36% (10 out of 28) of the analyzed specimens presented mercury concentrations above the maximum permitted for consumption by the EU (0.5 mg/kg in muscle, fresh weight, EC regulation No. 1881/2006). Xerta was the site from which most non-consumable fish were captured (six individuals), followed by Ascó (three individuals) and Flix sites (a single specimen).

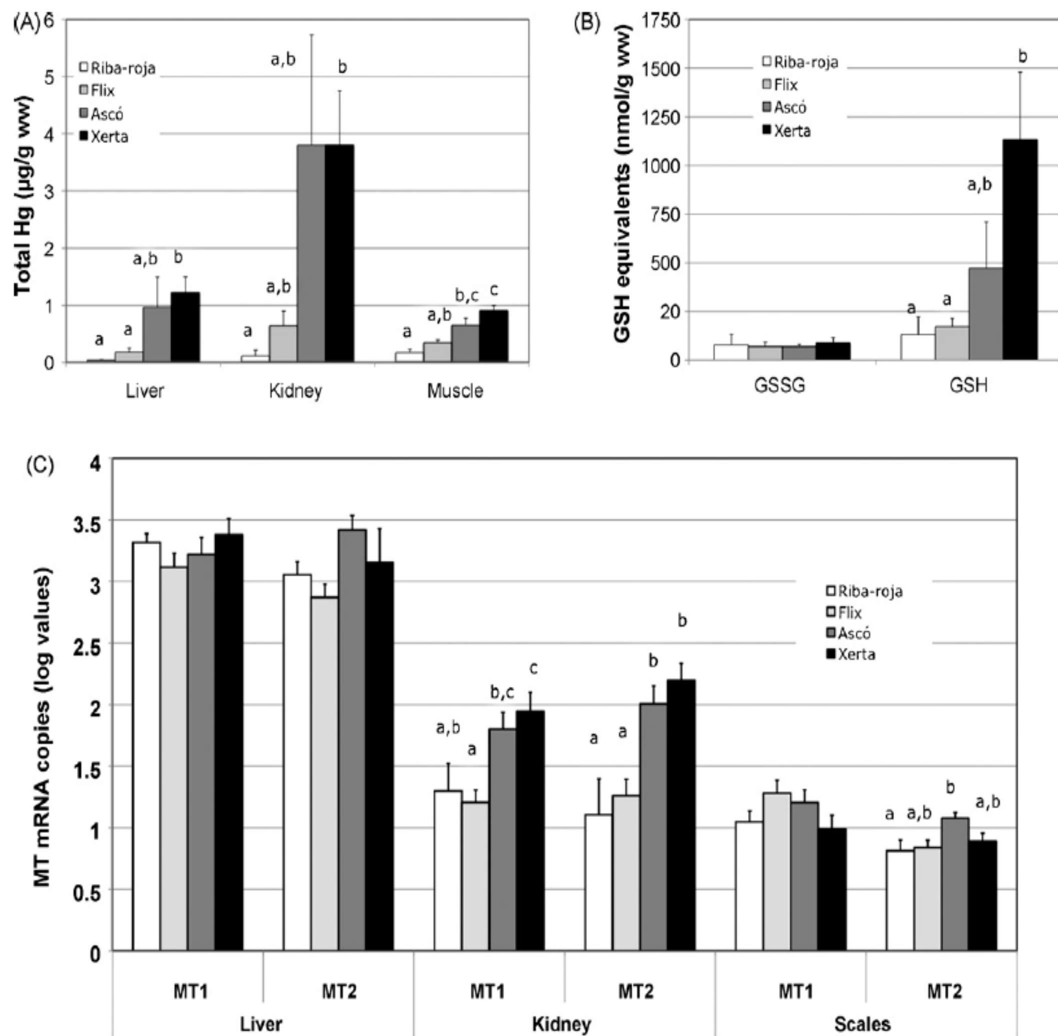

Fig 3.2 Representation of average values±SEM of different parameters in carp populations sampled at Riba-roja (empty bars), Flix (pale grey), Ascó (dark grey) and Xerta (solid bars). (A) Mercury levels in liver, kidney and muscle (µg/g wet weight). (B) Levels of reduced (GSH) and oxidized (GSSG) forms of glutathione in liver. (C) MT1 and MT2 mRNA abundance in liver, kidney and scales (mRNA copies per 1000 copies of ß-actin mRNA, logarithmic values). Where appropriate, different lower case letters indicate statistically different groups of samples (Tukey's test was used for comparisons).

	Riba-roja			Flix			Ascó			Xerta			Kruskal Wallis test[a]
	N	Median	Range	*N*	Median	Range	*N*	Median	Range	*N*	Median	Range	
Weight (kg)	7	4.00	3.20 – 6.40	7	3.35	2.7 – 4.60	7	2.30	2.10 – 4.60	7	3.56	2.56 – 4.55	0.050
Length (cm)	7	58.00	54.00 – 68.50	7	55.40	54.00 – 62.00	7	53.00	49.00 – 65.00	7	55.40	52.60 – 62.20	0.386
Total Hg (ppm, ww)													
Liver	6	0.03	0.03 – 0.05	6	0.10	0.03 – 0.52	7	0.34	0.08 – 4.01	7	1.27	0.43 – 2.20	0.001[***]
Kidney	6	0.07	0.06 – 0.31	7	0.37	0.11 – 1.80	6	2.43	0.19 – 12.82	7	3.84	0.65 – 6.92	0.001[**]
Muscle	6	0.17	0.07 – 0.24	7	0.36	0.17 – 0.57	7	0.77	0.19 – 1.03	6	0.96	0.52 – 1.09	0.001[***]
MT mRNA copies (referred to 1000 copies ß-actin mRNA)													
MT1, liver	7	1870	1292 – 5031	6	1136	750 – 3482	7	1406	384 – 3982	6	2477	621.48 – 5466	0.183
MT2, liver	7	966	694 – 4433	7	830	270 – 1974	7	2973	775 – 6967	7	2548	40.47 – 5200	0.050
MT1, kidney	5	24.45	2.50 – 32.44	7	19.05	6.33 – 31.89	6	83.80	17.94 – 137.62	7	79.13	22.47 – 232.64	0.005[**]
MT2, kidney	5	15.11	1.02 – 34.94	7	23.53	4.15 – 45.13	6	107.82	36.28 – 296.94	7	105.36	58.61 – 433.35	0.000[***]
MT1, scales	5	10.68	4.60 – 15.51	6	17.45	9.50 – 51.43	7	18.89	5.46 – 28.48	6	11.28	3.30 – 18.69	0.167
MT2, scales	4	6.25	3.27 – 8.25	6	6.94	4.52 – 11.33	6	11.95	8.47 – 18.41	6	8.48	4.75 – 11.76	0.019[*]
Enzymatic activities in liver													
Catalase (μmol min^{-1} mg Protein^{-1})	7	323	141 – 1027	7	250	146 – 350	7	280	203 – 801	7	286	183 – 433	0.430
GST (nmol min^{-1} mg Protein^{-1})	7	326	220 – 709	7	540	294 – 1422	7	570	473 – 817	7	454	210 – 865	0.248
Glutathione species (GSH equivalents, nmol per g fresh tissue)													
GSSG	7	47	37 – 189	7	49	29 – 208	7	55	31 – 128	7	81	27 – 207	0.908
GSH	7	117	16 – 242	7	127	74 – 500	7	263	77 – 1879	7	784	205 – 2953	0.006[**]
Lipid peroxidation (nmol malondialdehyde per g fresh tissue)													
Liver	7	14.38	9.85 – 18.30	7	19.24	15.06 – 29.71	7	11.91	10.98 – 25.52	7	19.49	12.94 – 29.54	0.016[*]
Kidney	6	29.87	11.20 – 63.65	7	35.01	18.68 – 40.47	7	23.66	20.70 – 105.98	7	24.83	16.42 – 116.28	0.643
DNA double strand breaks (μg damaged DNA per g fresh tissue)													
Liver	7	774	212 – 1166	7	867	311 – 1519	7	796	323 – 1047	7	1090	506 – 1650	0.573
Kidney	6	3941	3294 – 6868	7	4297	1518 – 6047	7	3584	1531 – 5334	7	3727	1825 – 5551	0.766

[a] Asymptotic sigma value.
[*] $p < .05$.
[**] $p < .01$.
[***] $p < 0.001$

3.1: Values obtained for different parameters in carp feral populations

The distribution of mercury levels in fish muscle is consistent with the current data on mercury pollution in the river sediments (table 3.2). Sediments sampled upstream the Flix factory, even at the Flix dam, showed mercury values typically below 0.5µg/g, whereas mercury concentrations above 1µg/g were common for sediments sampled downstream the Flix dam, up to the Delta. Variations in mercury contents were probably related to the composition and granulometry of the sediment samples and, consequently, to their ability to retain mercury. Maximal values, reaching up to 170 µg/g of mercury, were found at the same discharge point, on the factory residues (table 3.2). This situation seems to have changed very little during last years, as current mercury levels are very similar to the reported ones 10 years ago (table 3.2). The river water contains negligible amounts of mercury all along the low Ebro River, as determined by both current measures (www.chebro.com) and historical data (Ramos et al., 1999).

Site	Ramos et al. (1999)	Grimalt (2003)	Bosch et al. (un published)
Riba-roja (dam)	0.07, 0.10, 0.08	n.d.	n.d.
Flix (upstream factory)	0.4	n.d.	<0.5
Flix (factory residues)	n.d.	170, 67, 39, 29, 18	3, 15.1
Ascó-Xerta	0.9, 0.66	1.9	1.9, 1.4
Xerta-Tortosa	0.18	1.1, 0.83	<0.5
Tortosa-River mouth	1.46	0.1–1.7 (11 values)	<0.5

Table 3.2 Historical data on Hg content (µg/g) in sediments from the low Ebro River

3.3.2 Analysis of biomarkers of oxidative stress and DNA damage

Analysis of putative oxidative and DNA damage stresses due to mercury accumulation in liver and kidney showed no correlation between mercury contents and the activity of three oxidative stress-related enzymes (catalase, GST and MDA) or DNA breaks (tables 3.1 and 3.3). Moreover, the four sampled populations showed essentially identical levels for these biomarkers (table 3.1). These data show no significant oxidative or DNA damage to the exposed animals to mercury levels exceeding the maximal values permitted for food consumption.

Hepatic levels of reduced glutathione (GSH) showed a significant variation among the different carp populations (table 3.1), in a pattern resembling that of hepatic mercury content (fig. 3.2B). Bivariate analyses showed significant correlations between GSH content in liver and mercury levels in kidney, liver, and, to a lesser extent, in muscle (table 3.3, fig. 3.3A). In contrast, the correlation between mercury loads in different tissues and hepatic levels of oxidized form GSSG were very weak or absent (fig. 3.2B, table 3.3). Therefore, we concluded that GSH levels

responded to levels of mercury at which no other effects on oxidative stress or DNA damage were experimentally observable.

	Total mercury content					
	Hg (liver)		Hg (kidney)		Hg (muscle)	
	Rho[a]	*Sigma*[b]	*Rho*	*Sigma*	*Rho*	*Sigma*
Weight	−0.164	0.422	−0.363	0.069	−0.087	0.674
Length	−0.078	0.705	−0.267	0.187	−0.003	0.989
Total Hg						
Liver	1		0.909	0.000***	0.883	0.000***
Kidney	0.909	0.000***	1		0.761	0.000***
Muscle	0.883	0.000***	0.761	0.000***	1	
MT mRNA abundance						
MT1, liver	0.194	0.353	0.137	0.514	0.125	0.550
MT2, liver	0.311	0.122	0.296	0.141	0.290	0.150
MT1, kidney	0.704	0.000***	0.599	0.002**	0.654	0.001***
MT2, kidney	0.800	0.000***	0.745	0.000***	0.794	0.000***
MT1, scales	0.213	0.328	0.126	0.577	0.272	0.221
MT2, scales	0.515	0.017*	0.429	0.059	0.495	0.027*
Enzymatic activities and biochemical parameters						
Catalase	−0.015	0.941	0.067	0.746	0.001	0.996
GST	0.092	0.656	0.263	0.194	−0.187	0.360
GSH	0.773	0.000***	0.824	0.000***	0.575	0.002**
GSSG	0.314	0.119	0.473	0.015*	0.128	0.535
MDA, liver	0.064	0.758	0.014	0.946	0.014	0.944
MDA, kidney	−0.077	0.713	−0.034	0.870	−0.128	0.533
DNAdsb, liver	−0.024	0.909	−0.007	0.972	0.045	0.828
DNAdsb, kidney	−0.321	0.117	−0.286	0.156	−0.298	0.139

Units as in Table 1. MDA, malondialdehyde (lipid peroxidation); DNAdsb, DNA double strand breaks.
[a] Spearman rank correlation coefficient.
[b] Two-tailed sigma value.
* $p < .05$.
** $p < .01$.
*** $p < .001$

Table 3.3 Correlation between Hg concentration in carp liver, Kidney and muscle and different biomarkers

3.3.3 Quantitative analysis of metallothionein mRNA in kidney and liver from carp feral populations

Levels of mRNA for two metallothionein genes, MT1 and MT2, were analyzed in liver, kidney and scales. Liver showed the maximal mRNA levels for both genes, with no significant differences between the different carp populations (table 3.1, fig. 3.2C) or correlation between MT mRNA levels and mercury contents (table 3.3). This suggests that metallothionein genes did no respond to mercury in carp liver. Kidney MT1 and MT2 mRNA levels were significantly lower in the Riba-roja carp population than in Ascó or Xerta populations, in keeping with the average mercury loads found at the different sites (fig. 3.2C). Correspondingly, bivariate analyses showed a significant correlation between renal MT1 and MT2 mRNA levels and mercury loads (table 3.3). The correlation between mercury contents and MT1 and

MT2 mRNA levels in kidney is shown graphically in fig. 3.3B and C. Scales showed the lowest mRNA levels for both genes; only MT2 mRNA abundance showed significant differences among the four carp populations analyzed, corresponding to the Ascó population the maximal levels found in the study (table 3.1, fig. 3.2C). MT2 mRNA levels in scales showed a weak, but significant correlation with mercury content in liver and muscle, but not in kidney (table 3.3).

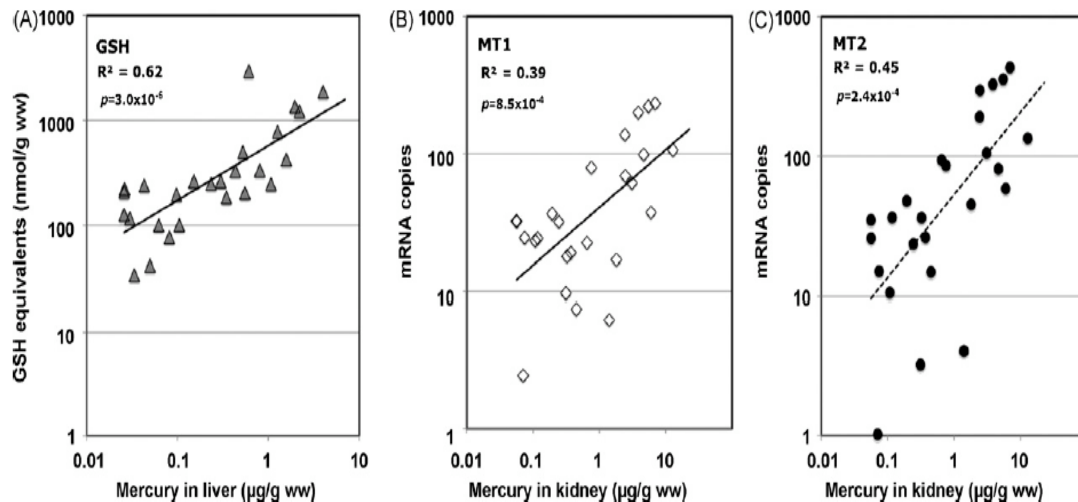

Fig 3.3 Double logarithmic correlation between: (A) GSH and mercury levels in liver and (B,C) MT1 and MT2 mRNA abundance and mercury concentration in Kidney. Regression lines, correlation coefficients and *p* values associated to the null hypothesis are shown for the three graphs.

Kidney has been gested as primary target in fish for inorganic mercury, presumably the main form discharged by chlor-alkali plants (Zalups, 2000; Sato and Kondoh, 2002; Neculita et al., 2005).

Our results indicate that kidney adjusts MT mRNA abundance according to the levels of mercury present, which is consistent with the proposed protective role of MT against mercury poisoning (Satoh et al., 1997; Zalups, 2000; Yoshida et al., 2004). Hepatic GSH levels are likely fulfilling a similar role (Maracine and Segner, 1998; Zalups, 2000), as the two molecules may have a complementary role protecting cells from mercury and other heavy metals (Ochi et al., 1988). This protective role provides a rationale for the use of these two biomarkers to monitor chronic exposure to mercury.

The use of scales as source for environmentally relevant biomarkers has a high potential interest as it does not require killing of the specimens. Scale cells are fundamental to the calcium equilibrium in the fish body, responding to a variety of

physiological and external effectors, such as hormones, different organic compounds and heavy metals, including mercury (Suzuki et al., 2004; Rotllant et al., 2005; Quirós et al., 2007b). MT2 mRNA induction in scales appears to be much weaker than in kidney; further investigation is needed to implement it as a suitable biomarker for protected or threatened fish populations.

3.3.4 Experimental induction of metallothionein mRNA levels in carp liver and kidney by mercury

The differential response of metallothionein genes in carp liver and kidney was further tested by experimental exposure to mercury in a controlled environment. Mercury was administrated intra-peritoneally to maximize transcriptional response (Gonzalez et al., 2005). Levels of MT1 and MT2 mRNA in liver were minimally affected by mercury injection, whereas their abundance increased 20-fold in kidney (table 3.4). These values can be compared to the differences we found between reference and impacted sites in natural carp populations from the low Ebro course (fig. 3.5 C and table 3.4). Metallothionein mRNA abundance in liver was high both in treated and control animals. In contrast, MT1 and MT2 mRNA levels in kidney was barely detectable in control animals and reached values similar to the ones observed in liver only after mercury treatment. These results suggest that MT1 and MT2 genes are constitutively transcribed in carp liver at relatively high levels and that these levels remain essentially unchanged upon mercury injection. In contrast, MT basal transcription in kidney appears to be very low and it becomes strongly activated as a response to external inputs. A similar pattern of response to metals in both tissues has been reported in zebrafish (Danio rerio; Gonzalez et al., 2005), which shows high basal transcription and low inducibility of MT genes in liver and low basal transcription but high inducibility in kidney.

Tissue	Gene	Group	mRNA abundance		REST analysis	
			Median	Range	Induction factor	p
Kidney	MT1	Control	3.6	1.1 – 14.5	18.06	<0.001
		Injected	82.8	36.5 – 130.9		
	MT2	Control	3.1	1.3 – 20.8	21.68	<0.001
		Injected	94.3	79.9 – 118.2		
Liver	MT1	Control	405.1	104.7 – 792.2	NS	0.34
		Injected	478.5	98.0 – 3573.4		
	MT2	Control	271.1	86.7 – 638.1	NS	0.063
		Injected	653.9	105.8 – 1923.6		

NS, non-significant variation.

Table 3.4 Expression levels and significance parameters fro MT1 and MT2 experimental induction in kidney and liver.

3.4. DISCUSSION

Our data shows physiological differences between four populations of carps from the low Ebro River. These effects correlated with the burden of mercury in these populations, which can be traced to continuous mercury discharges from the chlor-alkali factory at the Flix dam. Maximal levels of mercury in muscle as well as the highest biological impact did not occur at the discharge sites, but several kilometers downstream, at Ascó and Xerta sites. This circumstance may well reflect the dynamics of the river: the Flix dam reservoir (the discharge site) receives a continuous input of clean upstream water, which probably limits the impacted area to the surroundings of the factory, located right to the dam. Recent results with zebra mussel also show that the mercury impact at the Flix reservoir is essentially limited to the area surrounding the factory itself (Carrasco et al., 2008). In addition, mercury bioavailability may vary among different sampling sites, depending on the local activity of methyl-mercury producing bacteria (Scheuhammer et al., 2007). Mercury pollution is also reflected on river sediments, reaching the delta itself. Historical data shows that this contamination has been essentially constant for at least the last 10 years. Therefore, reported environmental data indicate that the observed effects are related to long term and continuous exposure to mercury rather than to recent sporadic short-term exposures.

Elevated levels of GSH in liver and of MT mRNA abundance in kidney and, to some extent, in scales, occurred at mercury concentrations at which no other stress response was detected. Therefore, we consider that these physiological responses were related to metabolic acclimation to the continuous presence of mercury, especially in kidney. A similar phenomenon has been observed in rainbow trout chronically acclimated to cadmium (Chowdhury et al., 2005). These data confirm the utility of these biomarkers to monitor environmental stress at levels at which no gross pathological alterations were observed.

Acknowledgments

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CHAPTER 4: Development and validation of molecular biomarkers in order to asses the effect in adults and larvae of Zebra mussel by heavy metals

Transcriptional stress genes to metal exposure in zebra mussel larvae and adults

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ABSTRACT

Development of stress markers for the invader freshwater zebra mussel (*Dreissena polymorpha*) is of great interest for both conservation and biomonitoring purposes. Gene expression profiles of several putative or already established gene expression stress markers (Metallothionein, Superoxide dismutase, Catalase, Glutathione S transferase, Glutathione peroxidase, Cytochrome c oxidase, the multixenobiotic resistance P-gp1, and heat shock proteins HSP70 and HSP90) were analyzed by quantitative Real-Time PCR in adults and pediveliger larvae after exposure to metals (Hg, Cu, Cd). A defined pattern of coordinated responses to metal exposure and, presumably, to oxidative stress was observed in gills and digestive gland from adults. A similar, albeit partial response was observed in larvae, indicating an early development of stress-related gene responses in zebra mussel. The tools developed in this study may be useful both for future control strategies and for the use of zebra mussel as sentinel species in water courses with stable populations.

Keywords: Transcriptional regulation, Metal toxicity, ANOVA, Ebro river, In vitro exposure, Chronic and acute toxicity, Molluscs

4.1. INTRODUCTION

The zebra mussel (*Dreissena polymorpha*) is a freshwater mussel native to the Caspian Sea. Infestations in the United States and several European countries resulted in adverse impacts on industrial and municipal infrastructures and on the ecology of affected water bodies (Mackie, 1991; Schloesser et al., 1996; Ricciardi, 2003; Sabater et al., 2008). *D. polymorpha* is a dioecious species with an r-selected reproductive strategy, consisting in external fertilization and planktonic larval stages. Its success colonizing new environments may be attributed to high fecundity, efficient larval dispersal, few natural controls and its ability to adhere to hard substrates using byssal threads (Ackerman et al., 1994).

Since zebra mussel first appeared in Spain in 2001, it has already colonized several of the major river basins, including the Ebro basin (Sabater et al., 2008). It has become a dangerous threat, by filter feeding competition and alteration of river sediments, to native mussels, such as Unio, Anodonta and Potomida. As these native mussels are threatened or endangered, current control strategies in Spanish water bodies are therefore limited to avoid spreading of zebra mussel by regulating boating and fishing activities (http://www.chebro.es/). In contrast, active eradication measures are severely limited to not to increase ecological pressure over native species. In addition, zebra mussel is nowadays the only freshwater mussel species that can be legally collected in Spanish rivers, and probably in those of many more countries, for environmental monitoring without limitations. For these reasons, the development of stress markers for zebra mussel is of great interest both for conservation and biomonitoring purposes (Guerlet et al., 2007; Contardo-Jara et al., 2009; Faria et al., 2010a). Different biochemical and histological biomarkers have been successfully implemented in zebra mussel in last years. These biomarkers have been applied to the analysis of both pollution impacts in water courses (Binelli et al., 2006; Minier et al., 2006; Guerlet et al., 2007; Osman et al., 2007; Contardo-Jara and Wiegand, 2008; Faria et al., 2010a), and toxicity risk assessment of specific contaminants, both isolated and in combination (Lecoeur et al., 2004; Singer et al., 2005; Ivanina et al., 2008; Faria et al., 2009, 2010b). However, the use of such small animal as the zebra mussel for biochemical analysis imposes some restrictions due to necessary limitations of sample. In this context, markers based on RNA quantification methods, such qRT-PCR, provide a suitable alternative, as they only require minute amounts of sample (Rotchell and Ostrander, 2003; Valasek and Repa, 2005; Piña et al., 2007). Using these methodologies, it is possible to analyze multiple endpoints on a single individual

(Kessabi et al., 2010) or to explore the differential response of a single gene in different tissues (Navarro et al., 2009). Biomarkers based on mRNA levels only reflect the initial steps of a cascade of events linking environmental insults to ecological impact (van der Oost et al., 2003); therefore, their results should be further supported by parallel biochemical, immunological and/or morphological/pathological data, when possible.

The main disadvantage on the use of mRNA markers for environmental monitoring is the requirement for the sequence of test and reference genes in the impacted species (Piña et al., 2007). This has been facilitated by recent efforts to sequence large collections of ETS (expressed tag sequences) and mRNA clones from different Dreissena species (Bultelle et al., 2002; Xu and Faisal, 2009), http:// srs.ebi.ac.uk). In this paper, we used the relatively well-known response of *D. polymorpha* to heavy metals (Cu, Cd, Hg) as stress model. The genes used for this analysis included several of the best-known markers for metal, oxidative and general stress responses. Metallothionein (MT) is the main metal-responsive gene in many systems, including bivalves (Naimo, 1995; Amiard et al., 2006; Choi et al., 2007, 2008; Zorita et al., 2007). Superoxide dismutase (SOD) is actively involved as a first defensive system against reactive oxygen species production dismutating $O2^-$ to H_2O_2. Catalase (CAT) reduces H_2O_2 to water, whereas glutathione peroxidase (GPx) detoxifies H_2O_2 or organic hydroperoxides produced, for example, by lipid peroxidation (Gutteridge and Halliwell, 2000; Contardo-Jara et al., 2010). Glutathione S-transferase gene (GST, (Doyen et al., 2008; Contardo-Jara et al., 2009) encodes one of the many phase II metabolizing enzymes known to catalyze the conjugation of glutathione with various electrophilic substances, and plays a role preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al., 1983). The mitochondrial enzyme Cytochrome c oxidase (COI) is involved in the respiration chain (Choi et al., 2007; Kim et al., 2007; Osman et al., 2007; Contardo-Jara and Wiegand, 2008; Doyen et al., 2008; Contardo-Jara et al., 2009) and its expression is altered by metal exposure in bivalve hemocytes (Matozzo et al., 2001). Protein chaperones HSP70 and HSP90, which are involved in the processing of misfolded proteins due to different kinds of stress (Snyder et al., 2001; Olsson et al., 2004; Piano et al., 2005; Singer et al., 2005; Choi et al., 2008; Ivanina et al., 2008), and the multixenobiotic resistance gene encoding for the transmembrane protein transporter P-gp1 that acts efluxing out of the cells many xenobiotics (Pain and Parant, 2003, 2007; Minier et al., 2006).

The two tissues selected for analysis exemplify different ways of exposure to pollutants in water, either through respiration/filtration (gills) or trough digestive processes (digestive glands). Finally, the selection of metals and exposure scheme intended to cover both acute (24 h) and medium-term toxicity (one week), and different toxic effects, like inactivation of protein sulfur groups by Hg (Zalups, 2000), metal imbalance for Cd and Hg (Suzuki et al., 2004), and oxidative stress for Cu (Naimo, 1995; Stohs and Bagchi, 1995; Canesi et al., 1999). The same treatments were also applied to larvae in the pediveliger stage, to analyze their patterns of response and compare to those found in adults. This stage represents a crucial point in the larval development of Dreissena, and occurs when free-swimming velliger larvae attain a size of about 200 mm. At this size, they become too heavy to remain afloat and settle on the bottom to find a hard surface to attach (Ackerman et al., 1994).

Our study aims the development and application of gene expression analysis to study the response of *D. polymorpha* to heavy metals. We think that these tools could be further implemented to other kinds of stress, facilitating the use of zebra mussel as sentinel species for pollution effects in freshwater bodies. In addition, a deeper knowledge of *D. polymorpha* stress responses may provide possible targets to facilitate its control without further endangering native bivalve species.

4.2. MATERIAL AND METHODS

4.2.1 Zebra mussel sampling, maintenance and treatment protocol

4.2.1.1 Adults

Zebra mussels 2 cm long (shell length) tied on rocks, were collected from Riba-Roja reservoir by a scuba diver at 3e5 m depth and transported to the lab. Riba-Roja reservoir is located in Ebro River, NE Spain and can be considered a reference site with low pollution levels (http://oph.chebro.es/DOCUMENTACION/Calidad/CalidadDeAguas.html). Mussels attached to rocks were rinsed and introduced into a glass aquaria at a density of 0.5 L per individual (approx.) and maintained under constant oxygenation > 90%, temperature (20°C) and photoperiod (14 h:10 h; light:dark). Animals were cultured in local field collected water, which was progressively replaced by artificial ASTM hard water (ASTM, 1995), and fed daily with a suspension 1:1 of algae

Scenedesmus subspicatus and Chlorella vulgaris (106 cells/mL per tank). The medium was renewed every other day for 10 days to allow the acclimatization of animals. After this period, 300 mussels with similar length (2 cm long) were selected for the experiments. They were gently cut off from rocks, placed on sheets of glass suspended in glass 30 L aquaria filled with of 20 L medium and maintained a further seven days in the same conditions described above but exposed to the different treatments. Only specimens able to re-attach their byssus on the sheet glass were used in the experiments. The test medium was changed daily and mussels were fed adding food only 2 h before water renewal. Adults were exposed to four different treatments: Control (CT), Cadmium (Cd; 20 mg/ L), Copper (Cu; 20 mg/L) and Mercury Chloride (Hg; 20 mg/L, all from Sigma-Aldrich, St. Louis, MO). Exposure levels were selected following previous studies showing biochemical biomarker responses at these concentrations (Canesi et al., 1999; Lecoeur et al., 2004; Binelli et al., 2006; Faria et al., 2009). Stock solutions of each metal (expressed as total ion concentrations) were prepared by adding analytical reagent grade salts to 1 L deionised water (Milli-Q; 18 MΩ cm^{-1} resistivity) and sonicated during 1 h. Nominal test concentrations were subsequently prepared by adding the whole content of the solutions to the 20 L tanks pre-filled with 19 L of ASTM hard water (Faria et al., 2009). After being exposed for 1 and 7 days, gills and digestive gland of five animals for each treatment were dissected, placed in a cryogenic vial with 1 mL RNAlater® (Sigma-Aldrich, St. Louis, MO), and stored at -80ºC for mRNA analysis. Soft tissue from another five animals was sampled for chemical analysis and stored at -20ºC.

4.2.1.2. Spawning and larvae culture

Spawning of zebra mussel was induced by exposing 200 adults to 10^{-3} M serotonin creatinine sulphate monohydrate (Sigma-Aldrich) for 15 min in 2 L ASTM hard water, rinsed and then transferred to 10 L tank filled with 9 L clean ASTM hard water. Males and females usually spawned within 15-30 min and 1-2 h, respectively. About 5 x 106 of fertilized eggs were cultured in a 100 L closed flow system with ASTM hard water under constant oxygenation (>90% saturation), temperature (20ºC) and photoperiod (14 h:10 h; light:dark) and fed daily with 1:1 of *Scenedesmus subspicatus* and *Chlorella vulgaris* (106 cells/mL) suspension. Once larvae reached the pediveligar stage (about 10 days postfertilization) at 20ºC, they were collected by filtration and used for experimental purposes. *D. polymorpha* larvae tests were performed by exposing 104 pediveliger (167-300 mm) larvae in 10 L (10 larvae/mL) test solutions using 10 L glass bottles at 20ºC in

the dark during 24 h. Test solutions of Cd (1 mg/L), Cu (1 mg/L) and Hg (0.8 mg/L) were prepared by adding appropriate amounts of a concentrated toxicant stock to each 10 L bottle. These exposure levels were half the estimated 48 h EC50 for embryo developmental effects (Faria et al., 2010b)

After exposure period, larvae from each exposure were collected by filtration, checked for survival, and further concentrated by gentle centrifugation (1000 rpm, 10 min). Pellets were resuspended in 5 mL and equally aliquoted to 1.5 mL Eppendorf tubes. After a quick spin centrifugation the supernatant was discharged and the pellet, containing larvae, was resuspended in RNAlater® (SigmaeAldrich) and stored at -80ºC.

4.2.2 RNA extraction and qRT-PCR analysis

Total RNA was isolated from the tissues using Trizol reagent® protocol (Invitrogene). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). RNA was treated with DNAseI to remove genomic DNA contamination. Quantities from 1 mg to 100 ng of DNAseI-treated RNA were retrotranscribed to cDNA using First Strand cDNA Synthesis Kid Roche® (Germany) and stored at -20ºC. Aliquots of 5.5 ng for Digestive Gland, 12 ng for Gills and 2.5 ng for larvae of original RNA preparations were used to quantify specific transcript in LightCycler® 480 Real-Time PCR System (Roche, Germany) using SYBR® Green Mix (Takara Bio INC) using the primer pairs listed in table 1. Primers for *D. polymorpha* HSP90 genes were derived from conserved DNA sequence regions in the three previously existing molluscs HSP90 sequences, *Mytilus galloprovincialis*, *Agropecten irradians* and *Clamys ferreri* (not shown). The sequence of amplified PCR products (amplicons) was confirmed by DNA sequencing in a 3730 DNA Analyzer (Applied Biosystems). Amplified sequences were compared to the corresponding references in GenBank (table 4.1) by the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). Cp values for stress-related target genes (TG) were compared to the corresponding values for a reference gene (ref) to obtain ΔCp values ($\Delta Cp = Cp_{ref} - Cp_{TG}$). The ribosomal protein S3 was selected as reference gene after examination of its variability among a large subset of samples (section 3.1). PCR efficiency values for reference

and tested genes were calculated as described (Pfaffl, 2001), and assumed to be close to 100% from these calculations. To facilitate reading of tables and graphs, mRNA abundance values were represented as mRNA copies of target gene per 1000 copies of the reference gene mRNA (% of reference gene, 1000 x $2^{\Delta Cp}$).

Gene name	Short name	Forward primer	Reverse primer	Amplicon size (bp)	Accession number
Ribosomal protein S3	S3	CAGTGTGAGTCCCTGAGATACAAG	AACTTCATGGACTTGGCTCTCTG	154	AJ517687
Elongation factor 1	EF1	CGAGAAGGAAGCCGCTGA	AAGCAATGTCAATGGTGATACCAC	104	AJ250733
β-Actin	BAct	CCTCACCCTCAAGTACCCCAT	TTGGCCTTTGGGTTGAGTG	153	AF082863
Metallothionein	MT	TGCGTTGAAACCGGTGATT	GACAGTTTTCTCCACATTTGCATG	139	U67347
Heat-shock Protein 70	HSP70	GCGTATGGACTTGATAAGAACCTCA	GAACCCTCGTCGATGGTCA	104	EF526096
Heat-shock Protein 90	HSP90	TTGATCATCAATACTTTCTATTC	ACACCAAACTGTCCAATCAT	101	GU433881[a]
Glutathione S-transferase	GST	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTAGTCCGC	152	EF194203
Superoxide dismutase	SOD	GACAGCATGGCTTCCATGTG	AGGAGCCCCGTGAGTTTTG	100	AY377970
Glutathione peroxidase	GPx	GAACGGCGTGGAGTTGATG	GAGGAAAATTCGGCACGAAA	71	DQ459994
Catalase	CAT	ATCAGCCTGCGACCAGAGAC	GTGTGGCTTCCATAGCCGTT	101	EF681763
Cytochrome c oxidase	COI	GACATTCAGGCCCTGCGATA	GATGTGCAGAACAAAGGGACC	151	AM749000
P-gycoprotein (Multixenobiotic resistance)	P-gp1	CACCTGGACGTTACCAAAGAAGATATA	TCACCAACCAGCGTCTCATATTT	104	AJ506742

[a] New sequence.

Table 4.1 Summary of genes, primer sequences (5′-3′) and amplicon size of qRT-PCR probes used in this work

4.2.3 Chemical analyses

Actual metal concentrations were measured in both acidified (pH < 1) 10 mL water samples collected at the beginning and the end of larvae experiments. In parallel, tissue levels of Cd, Cu and Hg were determined in whole mussel samples collected at 1 and 7 days of exposure as described in (Barata et al., 2005). Freeze-dried organisms were digested in concentrated nitric acid and hydrogen peroxide using Teflon bombs at 90°C overnight. Within each digestion series, appropriate blanks with no animals and samples of similar weight of a certified reference material of dogfish muscle (NRCDORM-2; LGC Promochen, Middlesex, UK) were digested during each analytical run and measured trace metal concentrations were within the certified range for the metal. Cooled digested samples were diluted to a standard volume with deionized water. Trace metal analyses in both water and aqueous tissue extracts were determined using a Perkin Elmer model Elan 6000 inductively coupled plasma mass spectrometer (ICP-MS) at the Scientific Technical Service of the University of Barcelona (Spain). Calibration standards and a reagent blank were analyzed with every ten samples to monitor signal drift. In every instance, the signal typically changed by 3e5% throughout an analytical run.

Rhenium was used as an internal standard to correct for any non-spectral interference. Detection and quantification limits (LOD, LOQ) were calculated from blank measurements and varied slightly across metals. LOD, LOQ values for water samples were 1.1, 1.2 mg/L for Cu and <0.5 mg/L for Cd and Hg. LOD, LOQ values for digested tissue samples were 0.91, 1.71 mg/g d.w. for Cu; < 0.18; 0.3 d.w. for Cd, 0.09, 0.12 mg/ g d.w. for Hg.

4.2.4 Statistic tests

Statistics tests were performed using the SPSS 17 (SPSS Inc., Chicago, IL) package. All statistical calculations were performed using ΔCp values, as this parameter followed normal distributions, as assessed by the Kolmogorove-Smirnov test.

4.3. RESULTS

4.3.1 PCR amplification results and determination of reference genes

Cloning and sequencing of conventional PCR products showed that all primer pairs shown in table 1 amplified fragments with the correct length and whose sequence corresponded to their intended gene products. Amplification efficiencies were calculated by qRTPCR as close to 100% in all cases. Three putative reference genes (Ribosomal protein S3, Translation elongation factor EF1 and ß- Actin) were tested for mRNA abundance and stability in the three RNA sources used in this work, gills, digestive gland, and larvae. Table 4.2 shows average Cp values and their standard deviations from all samples tested in this work (each sample tested in triplicate). The minimal variation corresponded to the ribosomal protein S3 mRNA, which showed SD values below 1 Cp unit in all three tissues; therefore, this gene was selected as reference gene in all subsequent experiments.

RNA source	Gene	Cp Values[a] (Average ± SD)	*n*
Larvae	S3	**20.26 ± 0.98**	66
	EF1	18.56 ± 1.23	66
	β-Actin	19.74 ± 1.18	66
Gills	S3	**15.59 ± 0.62**	116
	EF1	15.88 ± 1.72	108
	β-Actin	13.96 ± 1.28	113
Digestive Gland	S3	**17.98 ± 0.63**	108
	EF1	17.44 ± 1.33	118
	β-Actin	16.53 ± 1.20	109

[a] HKG with the lowest variability in **bold.**

Table 4.2 mRNA abundance variability for different housekeeping genes in zebra mussel tissues

4.3.2 Quantitative analysis of mRNA abundance of stress genes in zebra mussel adults and larvae

Exposure to Cu, Cd or Hg resulted in significant changes in mRNA abundance for a limited subset of the analyzed zebra mussel genes (fig. 4.1). MT mRNA levels increased significantly upon incubation with Cd or Hg, in gills, irrespectively to the length of the treatment, whereas the corresponding levels of animals treated with Cu were indistinguishable from controls (fig. 4.1). A similar pattern of response was observed in larvae (only 24-h exposure) and in digestive gland, although the latter seemed irresponsive to any treatment during the first 24 h. HSP70 mRNA levels also increased in the presence of heavy metals, especially in gills (50-100 fold) after 24 h of exposure to Cu or Hg, but this response faded after 7 days of exposure. Digestive gland showed a milder, but sustained response to Cu or Cd treatments (5-10 fold at most). In larvae, only Hg elicited a mild (1.5 fold), albeit significant response (fig. 4.1). These data indicate that Cu seems to be a poor MT inducer in zebra mussel, whereas gills seem to respond better at the short term and digestive gland at the medium term. Larvae showed a mild, but consistent response to Cd and Hg, but not to Cu.

Changes in HSP90, COI, CAT and GST mRNA levels were in general mild and restricted to particular subsets of samples (fig. 4.1). COI mRNA levels significantly increased at the first day of treatment with all the three metals assayed, but only for gills, whereas HSP90 showed a similarly transient response, also restricted to gills, but only in the group treated with Cu. CAT and GST showed a decrease, rather than an increase, on their mRNA levels in treated samples relative to

controls, but only for some groups of samples, and only in animals maintained in the lab for seven days.

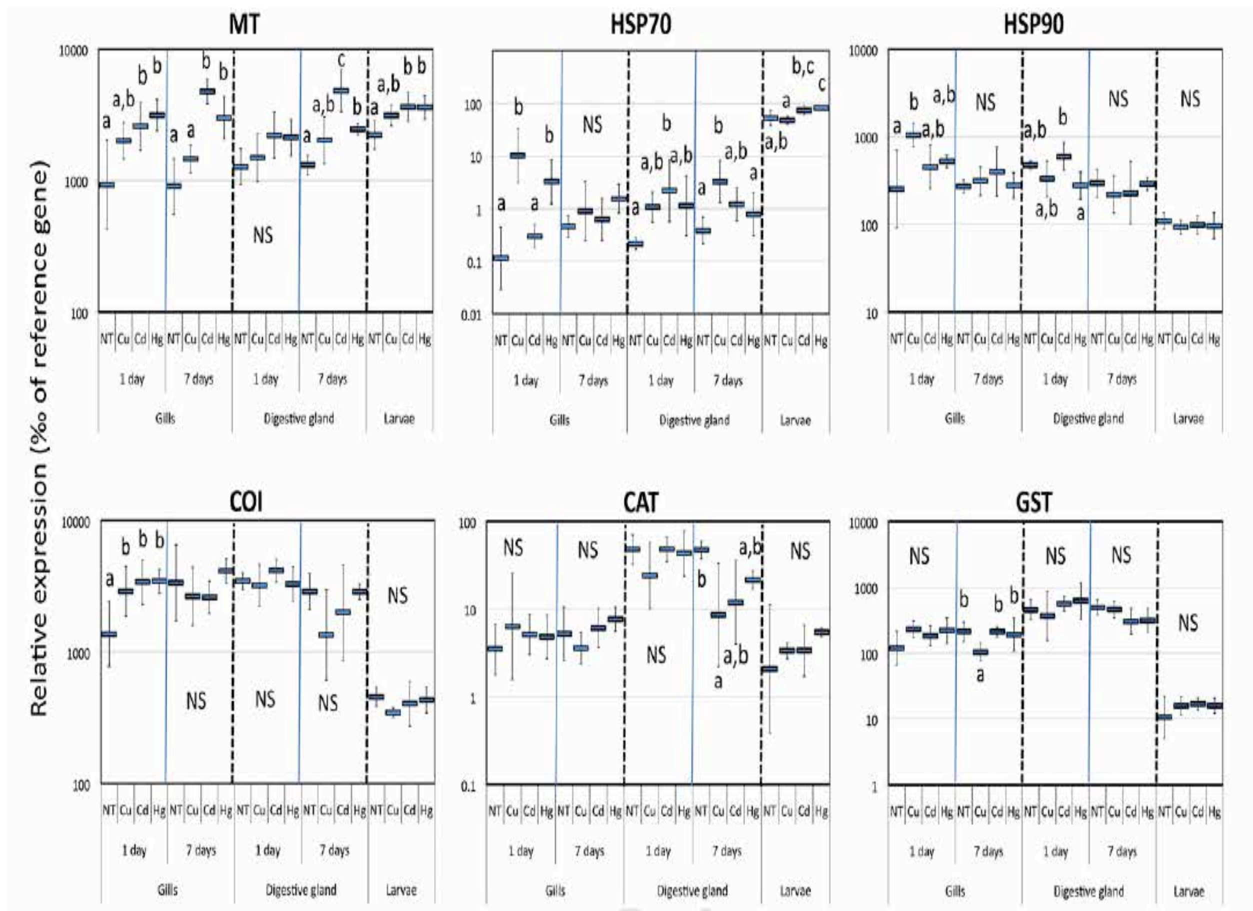

Fig 4.1 Relative mRNA abundance values (in ‰ of the reference gene) of MT, HSP70, HSP90, COI, CAT and GST in larvae, gills and digestive gland of animals control and treated with Cu, Cd and Hg for 1 or 7 days (only 1 day treatment for larvae). Letters indicate homologous subsets of data calculated for each tissue and treatment time (Tukey's test).

Metal accumulation in adult tissues was similar for all three metals. At day 0, Cu was already present in the animals at around 20 mg/g w.w, whereas Cd and Hg concentrations were barely detectable (table 4.3). By day 7, the maximal concentration corresponded to Hg (almost 130 µg/g), whereas Cu and Cd reached a similar lower concentration, around 90 µg/g (table 4.3). This pattern of accumulation may account for the observed changes in gene expression in digestive gland, but it is at variance with the attenuation of the stress response observed in gills after 7 days of exposure. The results suggest that the different responses elicited by the three metals were more related to their relative toxicity than to their accumulation potential. Measured metal levels in water collected from larvae tests were within 10% of nominal levels (not shown), although no appropriate accumulation tests could be performed, due to the characteristics of the sample.

	Cu	Cd	Hg
Day 0	19.3 ± 1.7	0.9 ± 0.1	0.3 ± 0.05
Day 1	39.4 ± 0.9	19.7 ± 3.4	16.3 ± 0.1
Day 7	90.3 ± 10.1	93.3 ± 8.8	129.9 ± 10.3

Data in μg/g d.w.

Table 4.3 Metal accumulation in zebra mussel after 1 and 7 days of exposition.

4.3.3 Coordinate expression of stress-related genes in *D. polymorpha*

Bivariate correlation analysis of mRNA abundance levels of different genes in adult tissues and larvae defined different clusters of co-regulated genes in both systems (tables 4.4 and 4.5). Levels of mRNA from oxidative metabolism-related genes (GST, SOD, CAT, COI, and GPx) showed a very high correlation in digestive gland (table 4.4). This cluster also included HSP90 and P-gp1, in principle not directly related to oxidative metabolism. Similar, although smaller clusters of putatively co-regulated genes appeared also in gills (including GST, SOD and CAT genes, table 4.4), and in larvae (COI, SOD, GST, CAT and GPx, table 4.5). Expression levels of MT, HSP70, HSP90 and COI appeared also mutually correlated in gills, a finding consistent with their relatively similar response to metal treatments (fig. 4.1, table 4.4). The correlation between MT and HSP70 expression is also significant, although weaker, in digestive gland and in larvae (tables 4.4 and 4.5).

Our experimental setup allows comparison of mRNA levels of a given gene in gills and in digestive gland of a single individual (fig. 4.2). MT and P-gp1 appeared to be strongly co-regulated in both tissues (fig. 4.2, table 4.4). MT co-regulation in gills and in digestive gland can be easily explained as resulting from the observed increase of its mRNA in response to the presence of Cd or Hg (see fig. 4.1). However, this explanation does not apply to P-gp1 expression, as this gene did not show any response to the treatments. The plot in fig. 4.2 suggests that P-gp1 expression in both tissues was more related to the time the mussels were kept in the lab (dark versus light grey symbols) than to any of the treatments, although the interpretation of this effect is presently not clear. P-gp1 expression in digestive gland appeared linked to the oxidative stress response, whereas gill P-gp1 expression appeared more correlated to SOD and P-gp1 expression in digestive gland than to the expression in gills of any other gene included in the study (table 4.4).

Tissue	Gene	Gills									Digestive gland								
		MT	HSP70	HSP90	COI	GST	SOD	CAT	GPx	P-gp1	MT	HSP70	HSP90	COI	GST	SOD	CAT	GPx	P-gp1
Gills	MT	1.000																	
	HSP70	0.429	1.000																
	HSP90	0.371	**0.663**	1.000															
	COI	**0.449**	**0.472**	0.335	1.000														
	GST	0.376	0.200	0.080	0.216	1.000													
	SOD	0.365	0.055	0.223	0.216	**0.583**	1.000												
	CAT	0.371	0.221	0.072	**0.438**	**0.595**	**0.504**	1.000											
	GPx	0.201	0.046	0.051	0.310	0.076	0.106	0.005	1.000										
	P-gp1	0.292	0.247	0.274	0.058	0.144	−0.023	0.057	−0.101	1.000									
Digestive gland	MT	**0.578**	0.117	0.186	0.056	0.078	0.185	0.038	0.062	0.002	1.000								
	HSP70	0.390	0.254	0.192	0.239	0.036	−0.032	0.196	−0.099	0.385	**0.413**	1.000							
	HSP90	−0.018	−0.342	0.106	−0.055	0.162	0.290	0.188	−0.118	0.101	−0.073	0.047	1.000						
	COI	0.072	−0.066	0.304	0.121	0.371	**0.470**	0.290	−0.072	0.210	0.055	−0.067	**0.773**	1.000					
	GST	−0.161	−0.132	0.058	−0.221	0.254	0.226	−0.264	−0.003	0.237	0.082	0.006	0.183	0.260	1.000				
	SOD	0.077	0.041	0.312	0.001	0.374	**0.451**	0.178	−0.127	**0.420**	0.074	0.050	**0.718**	**0.890**	**0.448**	1.000			
	CAT	−0.157	−0.146	0.188	−0.111	0.229	**0.444**	−0.085	−0.100	0.228	−0.002	−0.172	**0.598**	**0.794**	**0.589**	**0.862**	1.000		
	GPx	−0.104	−0.094	0.166	−0.178	0.081	0.250	0.193	−0.086	0.068	−0.116	0.138	**0.624**	**0.540**	0.117	**0.482**	**0.422**	1.000	
	P-gp1	0.206	−0.011	0.181	0.194	0.204	0.084	0.224	−0.170	**0.550**	0.138	0.212	0.377	**0.479**	−0.026	**0.514**	0.318	0.021	1.000

[a] Pearson's correlation. Bold indicate correlations with p values <0.01; shadowed boxes indicate correlations with p values <0.001.

Table 4.4 Correlation matrix between mRNA abundance values in adults gills and digestive gland

Gene	MT	HSP70	HSP90	COI	GST	SOD	CAT	GPx	P-gp1
MT	1.000								
HSP70	0.549	1.000							
HSP90	0.232	0.014	1.000						
COI	−0.383	0.144	−0.165	1.000					
GST	0.379	0.258	−0.140	−0.032	1.000				
SOD	−0.397	−0.018	−0.108	**0.675**	−0.118	1.000			
CAT	0.151	0.484	−0.354	0.194	**0.845**	0.168	1.000		
GPx	−0.440	−0.103	0.008	**0.691**	0.033	**0.743**	0.147	1.000	
P-gp1	−0.317	0.062	0.397	0.422	−0.360	0.444	−0.107	0.270	1.000

[a] Pearson's correlation. Bold indicate correlations with p values <0.01; shadowed boxes indicate correlations with p values <0.001.

Table 4.5 Correlation matrix between mRNA abundance values in larvae[a]

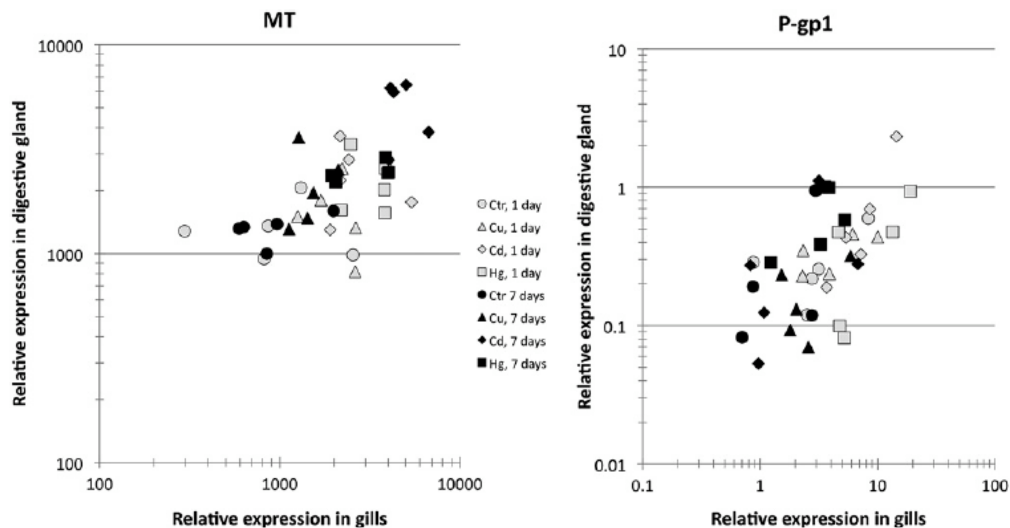

Fig 4.2 Comparison of individual expression of MT and p-gp in gills and digestive gland. Values are expressed as relative mRNA abundance values (‰ of the reference gene). Control (dots) and the different treatments (Cu, triangles; Cd, diamonds: Hg, squares) are indicated by shape; filling distinguish between samples treated 1 day (pale grey) or 7 days (solid symbols)

4.4. DISCUSSION

Exposure to heavy metals resulted in only moderate changes in mRNA levels of most of the analyzed stress-related genes in *D. polymorpha*. Only two genes, MT and HSP70, showed a distinct response in the three tissues analyzed (gills, digestive gland and larvae). Two more genes, HSP90 and COI, presumably related to the oxidative stress response, did also increment their mRNA levels upon metal exposure, but this effect was transient and restricted to gills. We propose that the

observed variations in CAT and GST mRNA abundance may reflect physiological differences between the different individuals rather than being related to the treatments.

The observed increase on MT mRNA abundance upon exposure of heavy metals has been previously reported in many organisms, including zebra mussel (de Lafontaine et al., 2000; Amiard et al., 2006; Zorita et al., 2007; Faria et al., 2009). Our results indicate that Cd is a much stronger MT inducer than Cu, an effect already observed in many systems, including zebra mussel and other molluscs (Lecoeur et al., 2004; Dondero et al., 2005; Zorita et al., 2007; Ivankovic et al., 2010). Mercury, which by some accounts is a poor MT inducer in many systems (Zalups and Koropatnick, 2000; Lavado et al., 2006; Quirós et al., 2007), appeared as a relatively strong inducer in zebra mussel, as previously suggested (Dondero et al., 2006; Marie et al., 2006). Gills seemed to respond transiently and more readily than digestive gland to metals, perhaps due to their direct exposure to contaminated water or due to the fact that had less mRNA of the studied genes and hence less buffering capacity against metals. Larvae followed a similar pattern of MT induction as adult tissues, although the absolute magnitude of the induction was considerably lower. All these results are consistent with a classical mechanism of response to metals through metal-responsive binding factors (MTF) (Haq et al., 2003; Dondero et al., 2005).

Whereas the response of MT to metals met aprioristic expectations, the strong increase of HSP70 upon the presence of metals was somewhat surprising. The effect was particularly strong in gills (where HSP90 was also induced) after the first day of exposure but faded completely after a week. The response was milder and more persistent in the digestive gland, and also weak, but significant, in larvae treated with Hg. HSP70 expression is known to increase in Dreissena upon the presence of some transition metals, like Cu, Pd, Pt and Rh, but this has been linked to a response to oxidative stress (Clayton et al., 2000; Singer et al., 2005; Ivanina et al., 2009). Both HSP70 and MT promoters from different organisms include antioxidant response elements (ARE), (Haq et al., 2003; Jones et al., 2007), which may provide a mechanistic explanation for the observed correlation on mRNA levels between the two genes. In addition, HSP70 and HSP90 are protein chaperones that are induced in conditions of cellular stress, including heat shock, exposure to organic and inorganic pollutants, and aging in bivalves and in many other species (Snyder et al., 2001; Olsson et al., 2004; Ivanina et al., 2008; Contardo-Jara et al., 2009). The relatively weak correlation between HSP70 and MT expression in both gills and digestive gland suggests that the observed increase on HSP70 (and HSP90

in gills) may also be related to a general toxic effect. Adults exposed to Cu showed the greatest mRNA HSP70 abundance levels in gills at day 1 and in digestive glands at day 7. Under this point of view, the results suggest that Cu is more toxic than Cd or Hg in gills and, probably, in digestive gland, despite its moderate effect in MT expression levels. The finding that HSP70 mRNA abundance also increased in larvae treated with Hg may also be explained by this general toxicity effect.

A large cluster of oxidative metabolism genes (GST, SOD, CAT, COI, and GPx) showed a tight correlation in their mRNA levels in our analysis, particularly in the digestive gland. These genes are under the control of AREs in many systems (Jones et al., 2007), and their coordinate expression may reflect variations in the redox status of the animals. Variations on mRNA abundance of these genes were largely unaffected by the presence of metals, suggesting a rather weak oxidative stress in zebra mussel upon metal exposure. Oxidative stress response to metals is relatively weak in bivalves (Canesi et al., 1999; Geret et al., 2002, 2003; Gomez- Mendikute and Cajaraville, 2003; Amiard et al., 2006), including Dreissena (Faria et al., 2009), a characteristics that may be associated to the multiple routes of metal accumulation, speciation and sequestration in subcellular compartments present in mollusks (Canesi et al., 1999). P-gp1 and HSP90 also participate in the oxidative-stress cluster of co-regulated gene in the digestive gland. Induction of these genes has been associated to different types of stress and to apoptosis (Minier et al., 2006; Pain and Parant, 2007; Ivanina and Sokolova, 2008). Therefore, we consider likely that the observed expression pattern was associated to metabolic differences among individuals, although the exact nature of the factors responsible for this coordinated regulation is currently unknown. It is most interesting to observe that mRNA abundance of GST and CAT, on one side, and COI, GPX and SOD, on the other, mutually correlated in larvae, suggesting that the putative factors co-regulating these genes (presumably, through ARE or similar elements) are already present at the larval stage.

The use of quantitative RNA biomarkers allowed us to explore different pathways of stress response in individual mussels and in relatively small pools of larvae, as well as to compare these responses in two tissues in the same individual. We consider that this approach opens the possibility to analyze the regulatory networks implicating the stress-related genes in zebra mussel. The combination of ANOVA and bivariate correlation analysis can define clusters of genes likely to be regulated by a common factor and to associate the regulatory mechanisms subjacent to each type of stress, an approach already tested in well-known systems, like yeast of zebrafish (Estruch, 2000; Wang et al., 2010). These tools may be further used for

risk assessment using zebra mussel as a model for other freshwater mussels. They may also facilitate the development and testing of new biocides aiding to its control in areas at which zebra mussel constitutes an ecological or economical problem. In this regard, the analysis of the larval response is particularly interesting, as it is probably the most sensitive stage of the whole life cycle of *D. polymorpha*, and the one that differs most from endangered native freshwater bivalves, as suggested by (Faria et al., 2010b).

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CHAPTER 5: Development and Characterization of a new molecular marker for endocrine disruption

Retinoid x Receptor gene expression study in adult *Dreissena polymorph:* Laboratory and Field approach

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Manuscript

ABSTRACT

A partial sequence encoding a putative Retinod X Receptor (RXR) has been identified in the zebra mussel *Dreissena polymorpha*. The sequence encompasses 79 amino acids, from the C-terminal end of the DNA binding domain to the N-terminal portion of the ligand binding domain. The protein sequence shows more than 75% identity to known RXR sequences from other mollusks, both bivalves (*Mytillus galloprovincialis)* and gastropoda *(Haliotis diversicolor*, *Limnaea stagnalis*, *Thais clavigera)*. Comparison with the know tertiary structure of the human RXR revealed that most sequence variations concentrate in a 10 amino acid loop placed between the two functional domains but not involved in their folding. The cloned fragment is expressed in eggs, veliger larvae (up to 48h post fertilization), and several adult tissues (gills, digestive gland, and gonads). Analysis of mRNA levels of this putative RXR in natural populations revealed significant variations along the annual life cycle, with a maximal levels just before the spawning season and minimal ones just after it, but no significant correlation with other parameters, like pollution levels. Experimental exposure of feral animals in the laboratory showed increased mRNA levels after a chronic (7 days) incubation with tributyltin (TBT), a suspected RXR disrupter. We conclude that the cloned sequence corresponds to a RXR homolog, probably belonging to a new subfamily, and functionally related to development and/or to annual life cycle functions

Key Words: Retinoid X Receptor, *Dreissena polymorpha*, Tributyltin, Larvae, Annual cycle

5.1 INTRODUCTION

Tributyltin, or TBT is a tri-substituted organotin that it has been used as antifouling in paints due to its biocides properties. It has been used since the 60's and it has been released into the aquatic systems via leaking form boat paints producing a worldwide pollution the aquatic environments. Around the 70's it was observed a repeated disturbance in aquaculture farms of *Crassostrea gigas* in the Atlantic Coast of France and it was found that TBT contamination was responsible of these anomalies (Alzieu, C. and Heral M. 1984). Since then, studies of TBT contamination has been focused in bivalves because they are important species in sea food resources with a high potential for TBT accumulation, due to their condition of filter feeders and they lack of enzymatic activities able to degrade TBT. It has been probed the high toxicity of TBT in organisms (Fent, K. 1998), and its effect in gastropods in particular, affecting reproduction and producing larvae malformation and imposex, a well documented example of endocrine disruption (Alzieu, C. 2000; Horiguchi, T. 2006).

The retinoid x receptors (RXR) are proteins belonging to the nuclear receptor superfamily of ligand activated transcription factors. These factors modulate its activatory potential by binding to a specific ligand, which in the case of RXR is the 9-cis Retioinc acid. In vertebrates, the retinoic acids regulate genes involved in many biological processes, including cell proliferation, differentiation and apoptosis during development. In invertebrates, retinoic acids are less known, studies of Nishikawa, et al (2004) demonstrated that RxR sequence of a gastropod (*Thais clavigera*) is similar to human and other vertebrates and invertebrates. (RXR) binds TBT with similar affinity as its cognate natural ligand, and this suggests that RXR may play an important role in the development of imposex in gastropods (Horiguchi, T. et alt. 2007; Lima, D., et alt 2011).

In this work we cloned, for the first time, a partial cDNA clone encompassing a putative Retinoid x Receptor gene for the invasive specie *Dreissena polymorpha*. We studied the variation of RxR mRNA levels in different tissues, exposed animals and field populations in order to better understand the physiological role of this new gene.

5.2. MATERIAL and METHODS

5.2.1 Animal collection, Field sampling

Adult zebra mussels with similar shell length were collected from Riba Roja, place considered as a reference site with low pollution levels, and Meander, a reported historical polluted site downstream from the Flix Chlor-alkali factory in the low course of the Ebro River (NE Spain; Eljarrat, E., et al 2008; Navarro, A., et alt 2009; Olivares, A et al 2010). Sampling took place in different development stages: January (in maturation process), May (mature animals), June (spawning season) and September (gonadal regression) to perform an annual cycle study (fig 5.1).

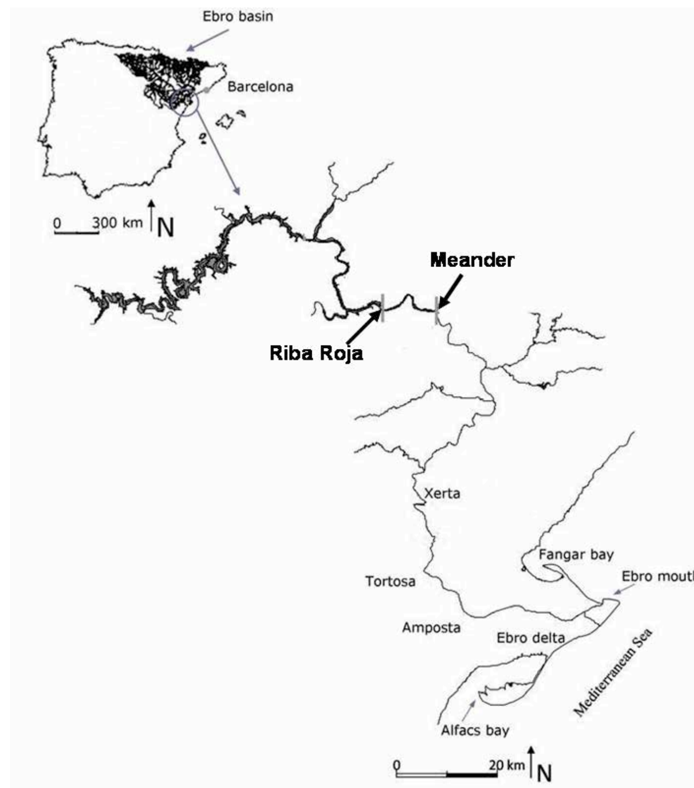

Fig 5.1 Map showing the location of the two sampling sites for the annual cycle study of RXR expression. The sampling points where located in Riba Roja reservoir (Upstream Flix) and Meander (Downstream) in the low Ebro River.

5.2.2. Animal Treatment

The animals used for TBT exposition were collected in Riba Roja. Once in the laboratory, they were rinsed with clean water, introduced into glass aquaria at density of 0.5L of ASTM water per individual, and maintained under constant

oxygenation (>90%), temperature (20°C) and photoperiod (12h:12h; light:dark). The animals were fed a suspension of 1:1 of algae (*Scenedesmus subspiatus* and *Chlorellavulgaris*), and the medium was renewed every two days during 10 days for acclimatization. After this period, animals with similar size (2 cm long) were selected for the experiment. The animals were placed on sheets of glass in aquaria of 30L, with 20L of medium and maintained further seven days in the same conditions mentioned above. The test medium was changed every day and fed only 2 hours before the water renewal. Adult *D. polymorpha* were exposed to three different treatments: Control (Ctr), Acetone (Act) and Trybutiltin (TBT) at 1ppb concentration. After the exposition of 1 or 7 days, five animals for each treatment were dissected to collect the digestive gland and the gonad. The tissues were placed in a cryogenic tube with 1mL of RNAlater® (Sigma-Aldrich, St Louis, MO.) and stored at -80°C until they were used for RNA analysis.

5.2.3 Spawning induction and exposure of larvae

Spawning of the zebra mussels was induced by exposure to 10^{-3}M serotonin creatinine sulphate monohydrate for 15 min. The mussels were then transferred to clean ASTM hard water. Spawning occurred within 15–30 min in males and within 1–3 h in females. A composite of about 10^6 eggs from at least 5 females was pooled in 200mL ASTM hard water and fertilized with 1mL of sperm from at least 3 males. Only gametes obtained from clean ASTM hard water (no serotonin) were used (Faria et al., 2010).

Eggs (1 h after being fertilized) and larvae of 2h, 24h and 48h for measuring gene transcription profiles of RxR time course. On the other hand, 48 hours larvae were exposed during 24h to 0.6 ppb of TBT. Samples were concentrated by gentle centrifugation (1000 rpm, 10 min). Pellets were re-suspended in 5mL and equally aliquoted in 1.5 mL eppendorf tubes. After a brief centrifugation the supernatant was discharged and the pellet, containing larvae, was re-suspended in RNAlater® (Sigma–Aldrich) and stored at −80°C.

5.2.4 Design sequence of Retinoid x Receptor (RXR) for *Dreissena polymorpha*

D. polymorpha total cDNA was amplified by PCR using standard protocol of PCR procedures of Ecostart (Ecogen) and degenerated primers to amplify the conserved region of the Receptor X Receptor. Oligos were designed after aligning known

sequences of different mollusk species: *Mytilus galloprovincialis* (EF644351), *Lymnaea stagnalis* (AY846875), *Biomphalaria glabrata* (AY048663), *Thais clavigera* (AY704160), *Nucella lapillus* (a isoform, EU024473) and *Nucella lapillus* (b isoform, EU024474). (ClustalIX software, at EMBL-EBI, Cambridge,UK). The final primer sequences were (ZM_RxR deg_ FW: 5′ TGTCAGTWCTGYCGYTAYATGAA 3′; ZM_RxR deg_RE: 5′ TGDGDATKCKYTTGGCCCABTC 3′).

The amplified product was purified with the PCR Clean Up Gel Extraction NuceloSpin® Extract II (Macherey-Nagel KG, Düren, Germany) and inserted into a plasmid pTZ57R/T (Inst/Aclone PCR Product Cloning Kit®, Fermentas, Burlington, ON, Canada).

DNA sequencing was performed in a 3730 DNA Analyzer (Applied Biosystems). The sequences obtained were compared to previous reported sequences of homologous gene for similar species using BLAST at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi),

5.2.5 RNA extraction and qRT-PCR analysis

Total RNA from the collected tissues was isolated using Trizol Reagent® (Invitrogen) protocol. The concentration of RNA was measured by spectrophotometric abstortion at 260mm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE), and the quality was checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNAseI to remove DNA genomic contamination. One to 0.1 µg of this treated RNA were retro-transcribed to cDNA using First Strand cDNA Synthesis Kit Roche® (Roche, Germany) and stored at -20°C. Aliquot of 5.5 ng of the original RNA preparation from each tissue were used to quantify specific transcripts in a LightCycler® 480 Real Time PCR System (Roche, Germany), using SYBR® Green I Master (Roche, Germany) chemistry. The primers used were: S3 (reference gene) S3_Fw 5′ CAG TGT GAG TCC CTG AGA TAC AAG 3′ and S3_Re 5′AAC TTC ATG GAC TTG GCT CTC TG 3′; RxR gene: RxR_Fw 5′GGT GGA CAA TGA CAA CGT CAA C 3′ and RxR_Re 5′ ATG TTT GTG ACT GCG TCT TTC TG 3′.

Relative gene expression values were calculated according to the equation below using the second derivative maximum of the amplification curve (Ct) values from the triplicates measured in the analysis, as previously described (Pfaffl, 2001)

$$\frac{mRNA_{TG}}{mRNA_{ref}} = \frac{E_{ref}^{Ct_{ref}}}{E_{TG}^{Ct_{TG}}} \cdot 1{,}000$$

In which TG and HKG indicated the gene of interest and the reference gene respectively. The reference gene (S3) was chosen by testing different suitable genes via BestKeeper program (Pfaffl et al. 2004). The sequences of amplified PCR products were confirmed by DNA sequencing in an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems).

5.2.6 Statistical analysis

The statistics tests were performed using the SPSS17 (SPSS Inc., 2002) package. All statistical calculations were performed using ΔCt values (Ct_{S3}- Ct_{TG}). This parameter followed a normal distribution, as assessed Kolmogorov-Smirnov test. Values are presented as means ± SEM (standard error of means). Statistical comparison of mean values were made using One Way Analysis of variance (ANOVA)

5.3. RESULTS

5.3.1 Retinoid X Receptor sequence

Amplification of cDNA fragment obtained by PCR using the degenerated primers yielded a 237 bp product. The corresponding protein sequence was up to 78% identical to other mollusk RXR sequences deposited in GeneBank. The alignment shows three sections with high sequence homology among all RXR sequences (including vertebrates, fig. 5.2). The most N-terminal section (labeled "A" in fig. 5.2) corresponds to the C-terminal portion of the DBD, whereas the two C-terminal ones ("B" and "C") constitute most of the RXR LBD. Note that Boxes B and C are fused in all mollusk (including *D. polymorpha*) sequences, whereas vertebrates genes include a variable intervening peptide. The newly found *D. polymorpha* sequence differs from the rest (both mollusks and vertebrates) in another short peptide between boxes A and B, which corresponds to the transition (sometimes called "hinch") between the DBD and the LBD.

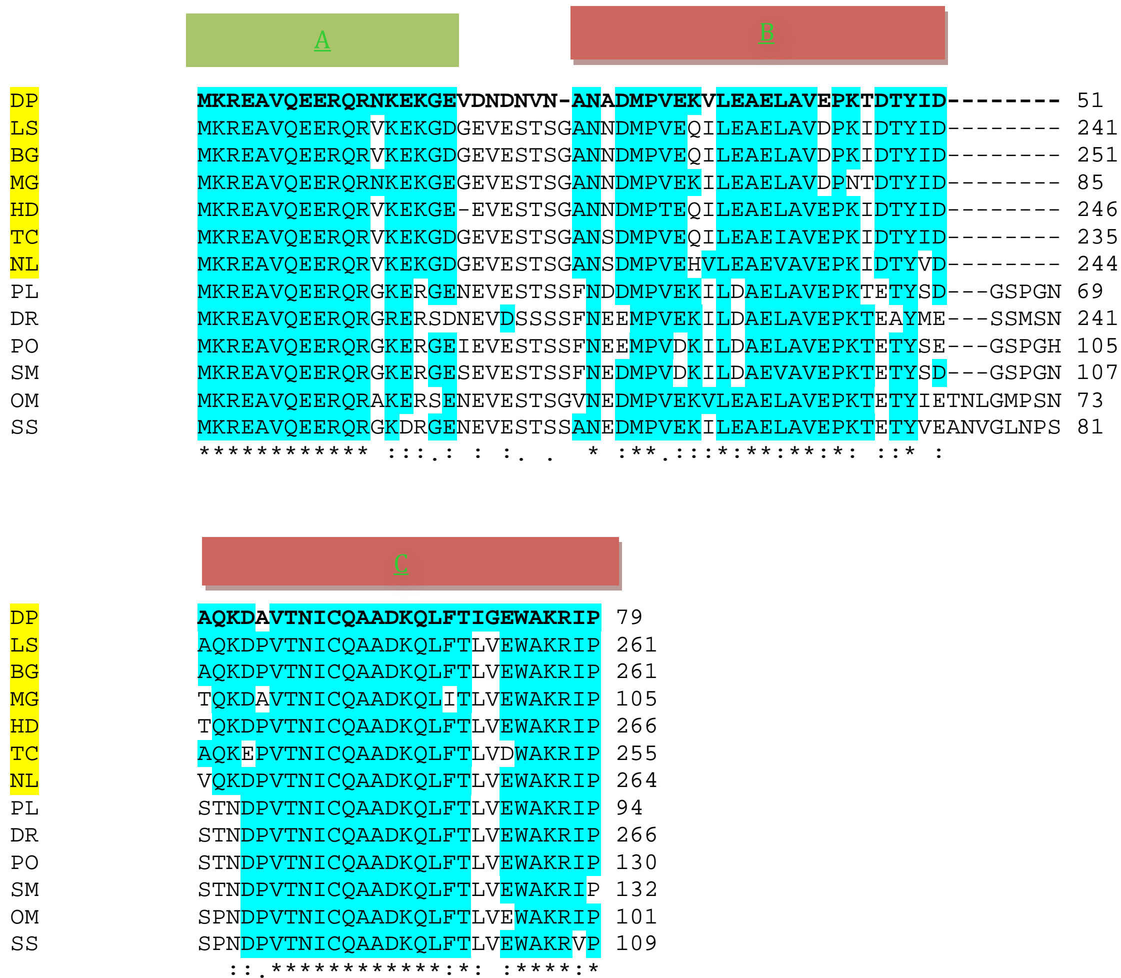

Fig 5.2 Comparison of deduced amino-acid sequence of the *D.polymorpha* RxR with the protein sequences reported in the GeneBank for similar sequences of protein fragments. The alignment were performed using the RxR sequence of the following species: M.g (*Mytilus galloprovincialis*, ABU89804), L.s (*Lymnaea stagnalis*, AAW34268), H.d (*Haliotis divericolor*, ADK60866) N.l (*Nucella lapillus*, ABS70715), T.c (*Thais clavigera*, AAU12572), P.l (*Poeciliopsis lucida*, ABW06847), D.r (*Danio rerio*, AAC59720), P.o (*Paralichthys olivaceus*, BAB71758), S.m (*Scophthalmus maximus*, AAQ05028), O.m (*Oncorhynchus mykiss*, CAI94598) and S.s (*Sus scrofa*, AAD19578). Mollusk sequences are labeled in yellow; amino acid residues identical to the D. polymorpha sequence (on top) are labeled in blue

A phylogenetic analysis of different RxR gene sequences homologous to the clones *D. polymorpha* fragment (fig 5.3) placed this sequence together with *Mytillus galloprovincialis* (ABU89804) and the gastropods *L.stagnalis* (AAW34268), *H.diversicilor* (ADK60866) and *B.glabrata* (AAL86461) in a Mollusca branch. Two species of gastropods, *N. lapillus* (ABS70715) and *T.clavigera* (AAU12572), clustered in a different branch separated from the rest of Mollusca and from the Vertebrata branch (*P. lucida* (ABW06847), *P. olivaceus* (BAB7175), *S. maximus* (AAQ05028), *H. sapiens* (AAH63827) and *S. Scrofa* (AAD19578) diverged to separate branches from the Mollusca. Sequences form the microcrustacean

D.magna (DQ530508) and the Cnidaria, *T. cytisohora* (AF091121) were loosely cluster together and relatively close to the Vertebrata branch.

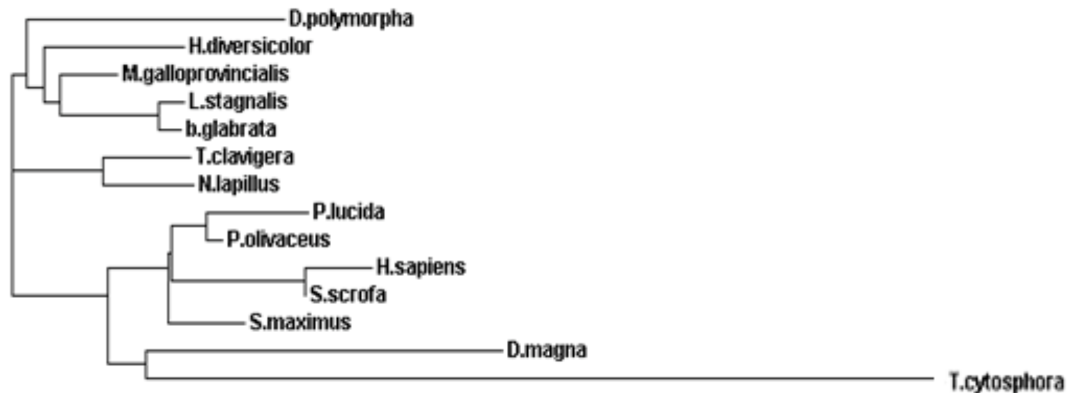

Fig 5.3 Phylogenic consensus tree of RxR sequences for the RxR sequence of D.polymorpha and the sequences of RxR for the species listed in Gene Bank. Tree calculated using the Pubmed software (http://www.ncbi.nlm.nih.gov/blast/treeview)

The branching depicted the magnitude of changes suffered at amino acid level. Interestingly, with the phylogenic results, we hypothesized that RxR genes from Vertebrata were more similar to Crustacea and Cnidaria than to Mollusca suggesting that RxR may diverge in the ligand binding, but further studies need to be done.

The sequence obtained with the degenerated primers was used to design the primers that were used for qRT-PCR analysis. The fragment produced by the qRT-PCR was re-sequenced and proved 100% identical to the original cloned fragment.

5.3.2 Gene expression of the new sequence in zebra mussel

Amplicons of qRT-PCR products obtained using the primers listed above were of the expected size and the sequence corresponded to their intended gene product. The gene S3 was used as a reference gene, as it Cp values did not differ in more than 1 cycle among samples, as showed in the previous study (chapter 4, see also table 5.1)

5.3.2.1 RxR Expression in different tissues and larvae

RXR mRNA levels were measured in gills, digestive gland and testes from adult zebra mussel as well as eggs and post fertilized larvae at different stages of development (2h, 24h and 48h) (fig 5.4). RXR mRNA was more abundant in adult gills than in digestive glands, whereas testes showed intermediate values. Eggs and larvae showed a higher RxR mRNA levels that adults, without significant variations among the different developmental stages (fig. 5,4)

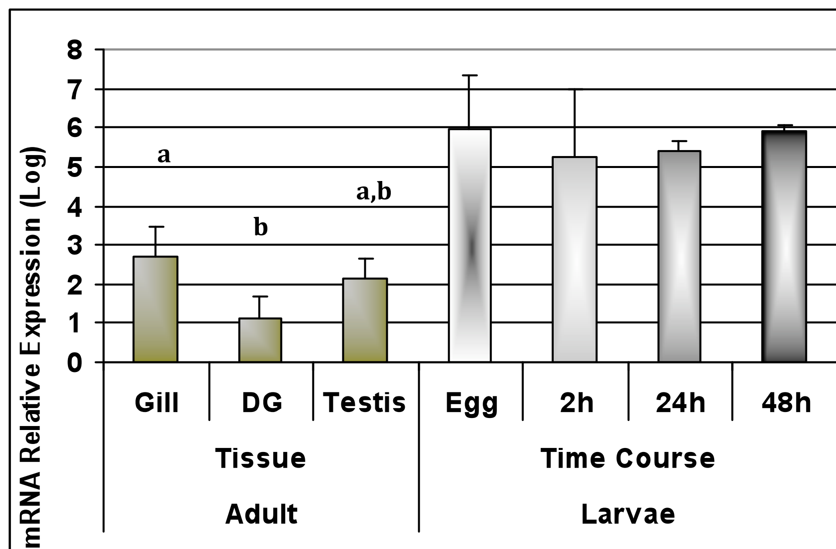

Fig 5.4 Relative mRNA expression for putative *Dreissena polymorpha* RxR in adult gill, digestive gland (DG) (n= 5 in each tissue) and testis, and eggs and 3 stages of larvae (2h, 24h and 48h; n=30000 eppendorf, 3 eppendorf each treatment). The low case letters grouped the values significantly different after ANOVA analysis (Tukey's <0.05)

Comparison of Cp values is listed in table 5.1, where it can be seen that Cp values of RxR gene that differ the most were gills in adults and larvae of 48h post fertilization being the lower values of all tissues. On the other hand, the higher Cp values for S3 were found in eggs and larva of 2h post-fertilization. These differences of Cps can explain the high induction found in the samples of larvaes even no significant variations among larvae was found.

	RxR Cp's	S3 Cp's
Adults		
Gills	22.2± 0.76	15.09±0.38
Digestive Gland	26.7±0.65	17.95±0.31
Testis	26.27±1.5	17.72±0.42
Larvea		
Eggs	25.09±0.8	21.07±0.66
2h	24.23±0.19	20.88±0.69
24h	23.56±0.94	18.85±1
48h	21.38±0.38	17.34±0.38

Table 5.1 List of Cp's average of the genes RxR and the housekeeping S3 for the three tissues and the larvae and eggs measured during the study

5.3.2.2 RxR expression after TBT exposure

RxR seems to be unresponsive in gonad after an exposition of 1ppb of TBT in adults zebra mussel for 1 or 7 days. Same result was obtained in digestive gland after one day of exposure. However, an induction of RxR occurred in digestive gland after 7 days of exposition with a fold induction of 2.05 (fig 5.5).

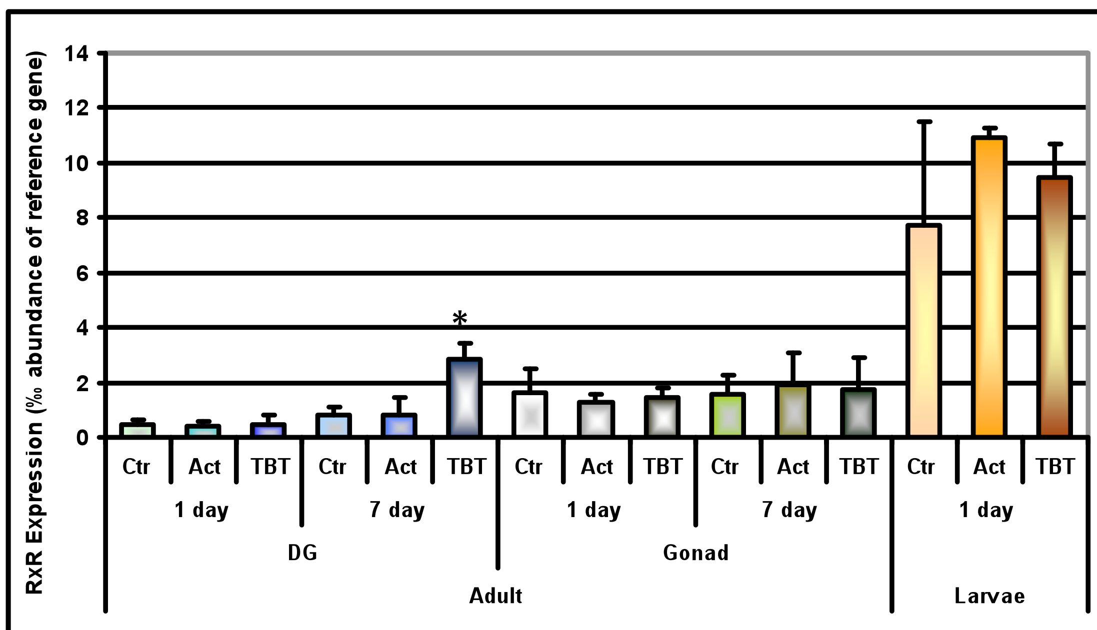

Fig 5.5 Relative expression of RxR levels showed as ‰ of S3 ribosomal mRNA abundance in (n= 5 in adults; n= 4, total larvae 41300) *Dreissena polymorpha* adults and larvae. Asterisk indicate significant difference (<0.05 Tukey) from control and treatments.

On the other hand, although the relative expression of RxR was 8.4 higher in larvae than in gonad or digestive gland, 48h old larvae exposed during 24 hours to TBT did not show any significant variations in RxR mRNA levels.

5.3.2.3 RxR Expression in Annual Cycle

RxR gene expression was measured by qRT-PCR in a half a body of adult zebra mussel sampled in January, May, June and September form Riba Roja and Meander sites (fig 5.6). The data obtained from the annual cycle of *Dreissena polymorpha* showed significant variations in RxR mRNA levels among the different maturation stages. The two sites showed similar averaged RXR mRNA levels and temporal patterns. The highest values were found in January, and June, when the animals were in the maturation process (January) and spawning time (June). The levels found in May present a significant drop, coinciding with the spawning season of the zebra mussel. mRNA levels of RxR showed a slow recovery on its expression just before the spawning season (fig. 5.6).

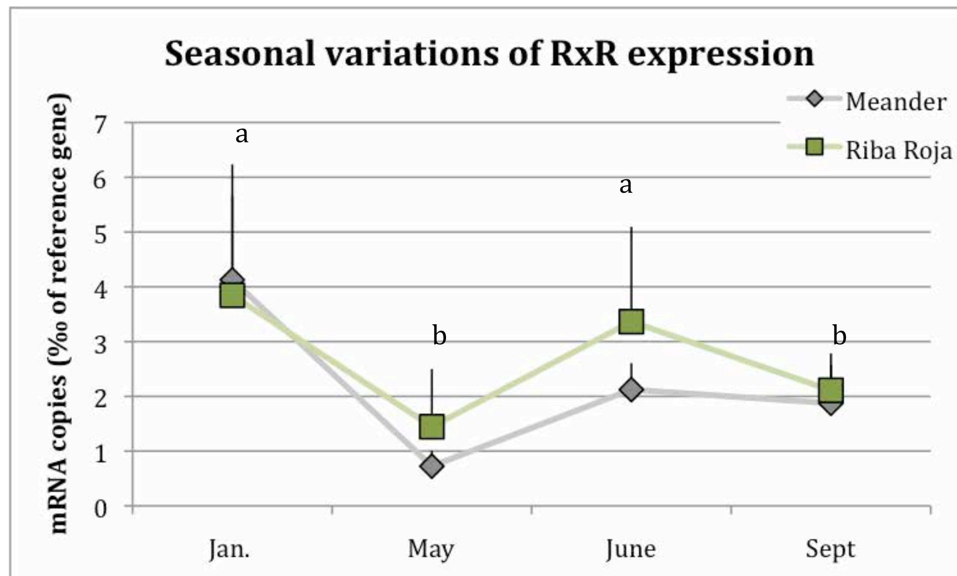

Fig 5.6: Relative expression of RxR gene during annual cycle listed in logarithm values of two sites in Ebro River: Meander (M) and Riba Roja (RR). Low case letters indicated significantly different sets of data after statistical analysis (Tukey <0.05)

5.4. DISCUSSION

In the present work a partial fragment of Retinoid X Receptor (RxR) was cloned for the first time in *Dreissena polymorpha*. RxR is a multifunctional nuclear hormone receptor, found in vertebrates and invertebrates, whose primary function is to regulate several different processes during differentiation and development (Evans, 1988). In vertebrates, RxR has been identified as important factor necessary for an effective binding to DNA of several nuclear hormones by forming heterodimers (Germain et alt 2006). There is considerably less information about RXR function in invertebrates, although it has been suggested that it plays an important role inducing imposex in gastropods (Horiguchi T et alt 2006; Castro 2007; Lima, D et alt 2011).

The putative RxR fragment cloned from zebra mussel showed high similarity to known RxR sequences from the Mollusca phylum, such as *Mytillus galloprovincialis (74% identity)* and the gastropod *Thais clavigera* (75%). Alignment of the putative RxR protein sequence for zebra mussel with another 11 sequences showed an amino acid gap only present in mollusk RXR sequences (fig 5.2), whereas a phylogenic analysis showed that the new sequence clustered with the rest of Mollusca sequences and separated to Vertebrata, Cnidaria and Crustacea sequences.

Analysis of RXR mRNA levels a by qRT-PCR revealed a strong expression during embryogenesis development, compared to comparing with adult tissue levels, although no differences were observed among the different development stages (eggs, 2h, 24h and 48h). It has been shown that RxR plays an important role in the modulation of expression of several genes involved in cellular differentiation and growth (Cahu, CL., et al 2009). Studies of RxR in *Daphnia magna* (Wang, YH, et al 2007) showed also a high expression levels in the early stages of larvae development, suggesting its importance to the embryology development. In adults, maximal expression was observed in gills and the minimal in the digestive gland (fig 5.4).

After a TBT exposure in adults and larvae zebra mussel we observed that RXR mRNA increased in zebra mussel digestive gland, but not in gonad, after a long (7 days) exposure to TBT. Similarly, Lima, D et al in 2011 found no differences in RxR transcription in the gastropod *N. lapillus* upon exposure to TBT, even during the early stage of imposex. We suggest that in zebra mussel the production of RxR is

done by the digestive gland. *D. polymorpha* exposed to TBT for 7 days showed induction of oxidative stress genes such as CAT, and GPx (Annex 1). Similar results have been reported in *Aurelia spp* exposed to TBT (Schroth, W, 2004). In the same study of TBT effect in zebra mussel it was observed, by microarray and qRT-PCR, a response of the multixenobiotic resistance gene, with an induction in the digestive gland after 7 days of treatment (Annex1).

No effect was detected in samples after 24h of TBT exposition for any of the genes analyzed. This data suggests that the TBT effects we observed only occur at long exposures and therefore they do not belong to the primary response to TBT. TBT accumulates rapidly in *Mytilus edulis* gills, followed by a gradual decrease until reaching a stable minimum, lower value after 24h of exposure (Laughlin RB, 1986), Most of the studies on TBT effects in mollusks have been done following chronic, long term exposition schemes (even months, Horiguchi, T. et alt, 2007; Lima, D. et alt 2011). These data is consistent with the lack of response we observed both in adults and larvae after short exposures. Therefore, the primary responses of bivalves (and *Dreissena*) to TBT remain to be elucidated.

The study of RxR mRNA abundance during an annual cycle of zebra mussel showed variations linked to the developmental stage, with maximal levels in the maturation stage (February) and during spawning season (June) a sharp drop in the spawning season (May) and slow recovery of levels in June and September (fig 5.6). This temporal pattern was essentially identical in the two studied populations, separated by only 10 km, but with pollution records very different. The Riba-roja site lies upstream from the Flix chlor-alkali factory, and it is considered a clean site, whereas the Meander site, downstream the factory, it is considered as a historically polluted site (Bosch, C. et al. 2009;Olivares, A., et al 2010). This indicates that the observed variations on mRNA levels were due to changes in the life cycle of zebra mussel, without significant influences of the pollution in the water.

As a summary, we found that changes in mRNA levels of RXR could be used as a good indicator of maturation stage of the zebra mussel populations. Although more studies need to be done for the full understanding of TBT effect in *Dreissena polymorpha*, our results show that zebra mussel can be used as a potential sentinel specie to monitor TBT pollution in aquatic systems. This is particularly interesting in the low Ebro River, which harbor a series of well established zebra mussel populations, whose control is considered a strategic target for the regulatory authorities.

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Chapter 6: Study of ABC membrane transportation proteins family in Larvae and adult Zebra mussel and its role in detoxification.

ABCB and ABCC transporter homologs are expressed and active in larvae and adults of zebra mussel and induced by chemical stress

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ABSTRACT

Multixenobiotic resistance (MXR) of aquatic invertebrates has so far been associated with cellular efflux activity mediated by P-glycoprotein (ABCB1) and MRP (multidrug resistance protein; ABCC) type ABC (ATP binding cassette) transporters. Expression and activity of an ABCB1 homolog has been shown in eggs and larvae of the zebra mussel *Dreissena polymorpha*. Here we report identification of a partial cDNA sequence of an abcc/ABCC homolog from zebra mussel that is transcribed and active as a cellular efflux pump in embryos and gill tissue of adult mussels. Transcript expression levels were comparatively low in eggs and sharply increased after fertilization, then maintaining high expression levels in 1 and 2 dpf (days post fertilization) larvae. MK571, a known inhibitor of mammalian ABCC transporters, blocks efflux of calcein-am in larvae and gill tissue as indicated by elevated calcein fluorescence; this indicates the presence of active ABCC protein in cells of the larvae and gills. Dacthal and mercury used as chemical stressors both induced expression of abcb1 and abcc mRNAs in larvae; accordingly, assays with calcein-am and ABCB1 inhibitor reversin 205 and ABCC inhibitor MK571 indicated enhanced ABCB1 and ABCC efflux activities. Responses to chemicals were different in gills, where abcb1 transcript abundances were enhanced in dacthal and mercury treatments, whereas abcc mRNA was only increased with mercury. ABCB1 and ABCC activities did not in all cases show increases that were according to mRNA levels; thus, ABCC activity was significantly higher with dacthal, whereas ABCB1 activity was unchanged with mercury. Our data indicate that ABCB1 and ABCC transporters are expressed and active in larvae and adult stages of zebra mussel. Expression of both genes is induced as cellular stress response, but regulation appears to differ in larvae and tissue of adult stages.

Keywords: multixenobiotic resistance, MXR, ABCC, ABCB1, environment tissue barrier, gene expression, efflux activity, zebra mussel

6.1 INTRODUCTION

Aquatic organisms are constantly exposed to complex mixtures of structurally diverse chemicals dissolved in the water and must avoid their potential toxic impact. The cellular multixenobiotic resistance (MXR) system represents a broad-scale defense mechanism protecting cells and organisms against both endogenous and environmental toxicants, including also anthropogenic chemicals (Epel et al., 2008). MXR is mediated by membrane transport proteins from the ABC (ATP binding cassette) protein family, which recognize a wide variety of potential xenobiotics as substrates, pumping them out of the cell in an energy dependent, ATP-driven process.

ABC transporters constitute a large protein family, sub-categorized into sub-families ABCA-ABCH with a variety of functions (Dean, 2005). The transporters with MXR related functions are P-glycoprotein (P-gp, MDR1, ABCB1[1]) belonging to the ABCB subfamily, multidrug resistance associated proteins 1-5 (MRP1-5, ABCC1-5) from the ABCC subfamily, and breast cancer resistance protein (BCRP, ABCG2) from the ABCG subfamily (Leslie et al., 2005). The earlier studies associated MXR of aquatic invertebrates with ABCB1 homologs (Epel, 1998; Kurelec, 1992), and more recently with ABCC subfamily homologs, such as in embryos of sea urchin and gill tissue of marine mussels (Hamdoun et al., 2004; Luckenbach and Epel, 2008).

Induction of expression and activity of an ABCB1 homolog by chemical and physical stressors has previously been shown in bivalves, indicating responsiveness of this gene as a general stress response (Eufemia and Epel, 2000; Lüdeking and Köhler, 2002; Lüdeking and Köhler, 2004). Various studies have provided evidence for cellular protection against environmental toxicants by MXR transporters in tissues (Britvic and Kurelec, 1999; Contardo-Jara et al., 2008; Smital and Kurelec, 1997; Smital et al., 2003) and larvae of *D. polymorpha* (Faria et al., 2011) and MXR could be associated with an ABCB1 homolog (Faria et al., 2011; Tutundjian and Minier, 2007). In addition, there was indication for ABCC type efflux activity in *Dreissena* larvae as accumulation of calcein-am in the cells was increased in the presence of MK571, an inhibitor of ABCC transporters (Faria et al., 2011).

We here present a study where we identified a partial abcc type homolog from *D. polymorpha*, quantified constitutive transcript levels of abcb1 and abcc homologs in larval stages and adult tissue and upon impact of chemical stressors and assayed

[1] Our nomenclature for gene/protein names is: abcc/ABCC for bivalve and *ABCC*/ABCC for human

ABCB1 and ABCC type efflux activities with proxy dye calcein-am and specific pharmacologic inhibitors.

We show that both abcb1/ABCB1 and abcc/ABCC type transporters are constitutively expressed and active in cells of larvae and tissue of adult mussels and expression of both transporters is induced by chemicals.

6.2 MATERIAL and METHODS

6.2.1 Chemicals

Reversin 205, MK571, calcein-am (Ca-AM), mercury as $HgCl_2$, dacthal and serotonine creatinine sulphate monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone and DMSO (analytical grade) were obtained from Merck (Darmstadt, Germany).

6.2.2 Origin and maintenance of adult zebra mussels, egg production and maintenance of larvae

Sexually mature zebra mussels (*Dreissena polymorpha*) were collected in shallow water (0.5 - 1 m depth) in the Mequinenza reservoir / Ebro River (NE, Spain) from May to August 2010. Within 3 h of collection, animals were transported in local water in aerated 10 L plastic containers to the lab. The animals were then rinsed and placed in glass aquaria in a density of 0.5 L per individual and maintained in ASTM hard water (ASTM, 1999) at >90% oxygen saturation at 20°C and at a 14h:10h/light:dark photoperiod.

Prior to spawning mussels were acclimated for at least 1 d. Spawning of mussels was induced by exposure of animals to 10^{-3} M serotonin creatinine sulphate monohydrate for 15 min. For obtaining gametes, at least three males and at least five females were then placed in separate beakers with 200 mL of clean ASTM hard water. Spawning occurred within 15 to 30 min in males and within 1 to 3 h in females. About 10^6 eggs from the pool of females were transferred to 200 mL ASTM hard water and fertilized with the sperm contained in 1 mL of the water from the beaker with the males. Only gametes that were released by mussels into the serotonin-free water were used for fertilization (Faria et al., 2011). Larvae were cultured in 9 L ASTM hard water in 10 L glass bottles under constant oxygenation (> 90%

saturation), temperature (20˚C) and photoperiod (14 h:10 h/light: dark) without food.

6.2.3 Identification of a partial abcc-like cDNA sequence from D. polymorpha

For designing primers for RT-PCR of a partial cDNA of an unknown *D. polymorpha* abcc transporter conserved sequence stretches of molluscan abcc transporter cDNAs were identified from a clustal alignment of abcc sequences from *Cyphoma gibbosum* (EU487192), *Mytilus californianus* (EF521415) and *Tritonia hamnerorum* (abcc1 - EU487194; abcc2 - EU487195). The primer pair used was ACR GAR ATT GGW GAA AAG GG (forward) and TDG TBA CNC AYG GMV TYV ABT (reverse).

RT-PCR was performed using a MiniCycler™ (MJ Research) with total RNA isolated from mussel gills as described below. PCR conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec and 55°C and 72°C for 30 sec each, and a final step at 72°C for 10 min.

The PCR product was cloned into vector pTZ57R/T from the InsTAclone™ PCR Cloning Kit (Fermentas) and propagated using X Blue competent cells. Sequencing of DNA was performed on a 3730 DNA Analyzer (Applied Biosystems). Homology of the obtained putative abcc transporter cDNA with abcc transporter sequences from other organisms was confirmed using NCBI (National Center for Biotechnology Information, Bethesda, MD, US) blastx and the sequence was deposited on GenBank (accession no. HM448029). For phylogenetic analyses of the sequence alignments were performed with a range of ABCC and ABCB1 sequences from various organisms using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Based on the alignment percent sequence identities were determined and a phylogenetic tree was generated using BioEdit v. 7.0 software (Hall, 1999).

6.2.4 RNA extraction and qRT-PCR analysis

Total RNA was isolated from eggs/larvae and gill tissue of adult animals using Trizol reagent® (Invitrogen™). The RNA concentration was measured by spectrophotometric absorption at 260nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technilogies, Delaware, DE) and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). Quantities from 1 µg to

100 ng of DNAse I-treated RNA (Ambion®) were retro-transcribed to cDNA using First Strand cDNA Synthesis Kit (Roche Applied Science®) and stored at -20°C. The amounts of cDNA used for quantitative real-time PCR (qRT-PCR) corresponded to 10 ng and 12 ng of the original RNA preparation for larvae and for gills, respectively. qRT-PCR was performed with a LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master (Roche Applied Science®). Primers were designed with Primer Express software and the sequences were MRP_fw "GTA TCA ACC TGT CCG GTG GG", MRP_re "TTG TTG TAC ACC GCC CTG G"; S3_fw "CAG TGT GAG TCC CTG AGA TAC AAG", S3_re "AAC TTC ATG GAC TTG GCT CTC TG". Sequences of amplicons were confirmed by sequencing using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and by comparing them to the corresponding references in GenBank (table 10.1) using the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Relative mRNA abundance values were calculated from the second derivative maximum of their respective amplification curve (Cp, values from triplicate assays). Cp values obtained for the target genes (TG), abcc and abcb1, were compared to the corresponding values of the reference gene S3 (Navarro et al., 2011) to obtain ΔCp values ($\Delta Cp = Cp_{ref} - Cp_{TG}$). PCR efficiency values for reference and target genes were calculated using the equation described by (Pfaffl, 2001) and assumed to be close to 100% from these calculations. To facilitate reading graphs, the mRNA abundances were represented as mRNA copies of target gene per 1000 copies of the reference gene (% of reference gene, $1000 \times 2^{\Delta Cp}$).

Constitutive expression levels of the abcc homolog were determined in freshly fertilized eggs, in embryos 2 h post fertilization and in 1 d and 2 d trochophora larvae. Induction of abcb and abcc gene expression by chemicals was quantified in 1 d trochophora larvae and in gills of adult mussels upon 24 h chemical exposures.

6.2.5 Dye efflux assay for determining MXR transporter activities in mussel eggs/larvae and in gills

For determining MXR transporter activities in eggs/larvae and in gill tissue of zebra mussel, dye accumulation assays were performed. Assays with eggs/larvae were conducted as described by Faria et al. (2011); assays with gills were according to Luckenbach and Epel (2008) with modifications. The assays are based on the property of certain fluorescent dyes to act as substrates of ABC efflux transporters. The dyes are kept out of cells if efflux transporters are active and increasingly

accumulate inside the cells if transporter activities are disrupted by transporter inhibiting chemicals. As proxy for efflux activity we used Ca-AM, a substrate of ABCB1 and ABCC transporters. Ca-AM is not fluorescent but forms fluorescent calcein once it reaches the cytosol where it is cleaved by cellular esterases. Low calcein fluorescence levels of cells/tissues indicate high efflux transporter activity and enhanced fluorescence occurs with low efflux activity. For disrupting ABCB1 and ABCC like efflux activities in mussel eggs / larvae and in gill tissue we used the model inhibitors of mammalian ABCB1 and ABCC transporters, reversin 205 and MK571, respectively.

Larvae: MXR activity assays were performed with freshly fertilized eggs and 1 d and 2 d trochophora larvae. In addition, MXR activity was examined in 1 d trochophora larvae that had been subjected to chemical treatments (see below). About 6000 eggs/larvae were incubated in 50 ml glass vials in 40 ml of ASTM hard water with 1 μM Ca-AM (stock dissolved in DMSO) at 20ºC in the dark with gentle shaking for 90 min. In parallel to vials with Ca-AM only (control) and Ca-AM and DMSO (solvent control; DMSO at 1ml/L) treatments with 5 μM of model inhibitors MK571 and reversin 205, respectively, were run. The inhibitor concentrations used corresponded approximately to reported IC50 values (inhibitory concentration causing 50% of the maximal fluorescence) of the compounds in the Ca-AM assay (Faria et al., 2011). All treatments were set up in quintuplicates. Following exposures, larvae were filtered using a 20 μm nylon mesh, washed and re-suspended in 5 mL of clean ASTM hard water. The fluorescence of approximately 1000 larvae was then measured in a microplate fluorescence reader (Synergy 2, BioTek, USA) using excitation/emission wavelengths of 480/530 nm. Measurements of each replicate were run by triplicate and were corrected for background fluorescent levels of ASTM water. Accumulated dye is reported as fluorescence units per larva. The exact number of larvae in each replicate was counted using a 1 mL Sedgewick rafter chamber (Pyser-Sgi, UK) with 10 x magnification (Nikon Abbe Labphot – 2 bright field microscope, Japan). Data are presented as fold changes of fluorescence levels relative to respective DMSO controls.

Adults: MXR activity assays were conducted with gill tissue of freshly dissected mussels that had been subjected to treatments with model ABCB1 and ABCC inducers, dacthal or $HgCl_2$, or were from respective controls or solvent controls (see below). Gills from each mussel were divided into four equally-sized pieces and incubated individually for 90 min in 1 ml ASTM hard water at 20ºC with 0.5 μM calcein-am with or without 10 μM of model inhibitors reversin 205 and MK571. Initial

preliminary Ca-AM assays with mussel gills showed that 10 µM approximately correspond to the IC50 for reversin 205 and IC25 for MK571 (Fig. S2). The final concentration of DMSO used as solvent was 1µL/mL in all treatments. Seven replicates per treatment were run. After incubations, gill pieces were washed in ASTM hard water to remove medium from the tissue, tissue pieces were weighed with a microbalance and sonicated in 0.5 mL ASTM hard water. Homogenates were centrifuged at 3000 g for 10 min and the fluorescence of the supernatants was measured. Fluorescence values are reported as fluorescence units per mg fresh gill tissue weight. Data are presented as fold changes in fluorescence levels relative to respective DMSO controls.

6.2.6 Chemical exposures and sample preparations for studies of induction of transporter gene expression and transporter protein activities

Larvae: For studying effects of chemicals on induction of transporter gene expression and efflux transporter activities in 1 d old trochophora larvae about 10^5 of fertilized eggs were exposed to known model inducers of abcc´s/ABCC´s (inorganic mercury, Hg) and abcb1/ABCB1 (dacthal). Five treatments in 9 L ASTM hard water each were set up: control (C), solvent control (SC, 0.1 mL acetone /L), dacthal at 0.5 mg/L (Dac 0.5) and at 2 mg/L (Dac 2) and $HgCl_2$ at 1 µg/L (Hg 1). Experimental doses were selected from previous studies (Faria et al., 2011; Tutundjian and Minier, 2007) and did not impair embryo development during exposures. In dacthal treatments acetone was used as solvent carrier at 0.1 mL/L. After a 1 d exposure period, half of the larvae was collected and concentrated by filtration into 50 mL centrifuge tubes and then used for MXR activity assays (see above). For determining gene transcript abundance profiles (see above) the remaining larvae were further concentrated by gentle centrifugation (500 gs, 10 min), pellets were then re-suspended in 5 mL of ASTM water and equally aliquoted in 1.5 mL Eppendorf tubes. After a quick centrifugation the supernatant was discarded and the pellet with the larvae was re-suspended in RNAlater® (Sigma–Aldrich) and stored at -80°C. Five replicates per age class were used.

Adults: After at least 7 d of acclimation in the lab after sampling, 100 animals with similar valve lengths (approx. 2 cm) were selected for the experiments. Twenty animals were placed in glass aquaria filled with 10 L ASTM water with the same conditions as described above but with chemicals added. The following five treatments were set up: Control (C), solvent control (SC, 0.1 mL acetone /L), dacthal at 1 mg/L (Dac 1) and at 5 mg/L (Dac 5) and $HgCl_2$ at 20µg/L (Hg 20). Previous

studies have shown that the selected doses of dacthal and mercury induce MXR gene expression or transporter protein activity without causing measurable detrimental effects in zebra mussels (Navarro et al., 2011; Tutundjian and Minier, 2007). After 24 h exposure to chemicals, gills of 10 animals were dissected on ice and placed in Eppendorf tubes with 1 mL RNAlater that were stored at -80ºC until mRNA expression analyses (see above). Gills of the remaining animals were used to determine MXR transporter activities (see above).

6.2.7 Statistical Analysis

Gene expression levels: For comparisons of abcb1 and abcc transcript levels, expressed as ΔCp values, across egg / larval stages and across chemical treatments one way ANOVA followed by Tukey's post-hoc test were used. Beforehand, normal distribution of ΔCp values was confirmed with the Kolmogorov- Smirnov test (data not shown).

MXR activity assays: Fold increase changes in fluorescence levels of larvae and adult gills exposed to MK571 and reversin 205 across controls, solvent controls, dacthal and mercury treatments were compared also using one way ANOVA followed by Tukey's post-hoc test. Prior to analyses, fluorescence values were log transformed to improve ANOVA assumptions of normality and variance homoscedasticity (Zar, 1996). Statistic tests were performed using the SPSS 10.0 package (SPSS Inc., Chicago, IL).

6.3 RESULTS and DISCUSSION

6.3.1 Identification of a partial abcc cDNA sequence from zebra mussel

We cloned a 634 bp cDNA sequence from *D. polymorpha* (NCBI access. no. HM448029) that, based on comparisons with ABC transporter sequences, we identified as partial abcc (MRP) homolog, which is the first ABCC protein sequence identified from *D. polymorpha*. The structure of full ABC proteins, such as ABCB1 and ABCC transporters, comprises two subunits, each with a membrane spanning domain and a nucleotide binding domain (NBD). The NBDs contain typical and highly conserved motifs, such as Walker A, Walker B and ABC signatures (Ambudkar et al.,

2006). Alignments of the respective aminoacid sequence with ABCC homologs from other organisms indicate that our identified sequence encompasses a NBD fragment as determined with the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011) including ABC signature and Walker B motif (fig. 6.1A). Alignments of the *D. polymorpha* partial ABCC aminoacid sequence with respective NBD sequences from subunit 1 and 2 of human ABCC1 showed 86 % identity with NBD1 and 36 % with NBD2 sequences (fig. 1B), indicating that the identified sequence encodes partial NBD1 of a *D. polymorpha* ABCC transporter. Percent identities with NBD1 sequences of ABCC transporters based on alignments of the sequences from a range of organisms are 56 to 75 %. (table 6.1., fig. A2.2). In comparison, percent identities with corresponding sequences of ABCB1 / P-glycoprotein homologs are only 17 to 24 % (table 6.1, refer to fig. A2.2 for alignments) as has been found for mollusk ABCC and ABCB1 sequences earlier (Luckenbach and Epel, 2008).

A

```
                                          ABC signature        Walker B
Partial ABCC [D. polymorpha]       EIGEKGINLSGGQKQRVSLARAVYNNADVYLLDDPLSAVDSHVGKHIFNKVVGPKG       % identity
ABCC1, NBD-1 [H. sapiens]     759  ......V.................S...I..F.........A.......EN.I....  815    86%
ABCC1, NBD-2 [H. sapiens]    1418  .CA.G.E...V..R.L.C....LLRKTKILV..EATA...LETDDL.QSTI---RT 1474    36%
```

B

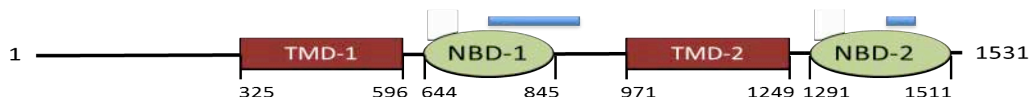

Fig 6.1. A) Graphic representation of the topology of human ABCC1 with transmembrane domains (TMD-1 and TMD-2, dark grey squares) and nucleotide binding domains (NBD-1 and NBD-2; light grey ovals). The two grey bars indicate the positions of the *D. polymorpha* ABCC-like sequence in the alignment with human ABCC1. Numbers represent the amino acid positions of TMDs and NBDs of human ABCC1 (Data from SwissProt). B) Alignment of the partial ABCC-like sequence from *Dreissena polyphorma* with NBD 1 and 2 from human ABCC1 (NCBI code AAB46616). Amino acid positions of human ABCC1 sequence stretches that align with the *D. polymorpha* ABCC sequence and percent identities of the aligned sequences are indicated. The conserved motifs ABC signature and Walker B domain are highlighted. From the human ABCC1 sequences only amino acids diverging from the *D. polymorpha* ABCC sequence are given; short dashes represent gaps in the alignment

The *Dreissena* ABCC sequence cannot be associated with a specific vertebrate ABCC subtype, but as can be seen in the phylogentic tree (fig. 6.2) it shows comparatively close relationship with a cluster of human ABCC1, ABCC2 and ABCC3 – which are the genuine multispecific chemical efflux transporters of the ABCC subfamily and related *Dreissena* sequence belongs to a gene encoding for a MXR mediating ABCC type efflux pump.

Species	NCBI access. no.	Phylum	Description	% identity
ABCC / MRP				
Cyphoma gibbosum	EU487192	Mollusca	ABCC1 1	75%
Rattus norvegicus	AAO44983	Chordata	ABCC1 var. A	64%
Homo sapiens	AAC05808	Chordata	ABCC1	60%
Pongo abelii	XP002834000	Chordata	MRP 1-like	67%
Ornithorhynchus anatinus	XP001517193	Chordata	MRP 1-like	68%
Drosophila melanogaster	NP885704	Arthropoda	MRP 1-like, isof. M	64%
Tritonia hamnerorum	ACA53361	Mollusca	ABCC 1	56%
Danio rerio	ADX66438	Chordata	ABCC2	56%
Gallus gallus	XP420102	Chordata	ABCC3	58%
ABCB1 / P-gp				
Dreissena polymopha	CAD44840	Mollusca	Putative MXR protein	17%
Mytillus galloprovincialis	AB036618	Mollusca	MDR protein	24%
Bos taurus	XP002704105	Chordata	ABCB4	17%
Xenopus laevis	NP001081394	Chordata	ABCB1	22%
Oryctolagus cuniculus	XP002713882	Chordata	ABCB4 isoform 3	17%

Table 6.1 Percent identities of the newly identified partial *Dreissena polymorpha* ABCC like sequence with ATP binding domain 1 (ADB1) of ABCC´s and ABCB1 sequences from various species from different phyla. The *D. polymorpha* sequence aligns with both ABD1 and ABD2, but similarity is greater with ABD1 indicating that the sequence encodes for part of this domain of an ABCC (fig. 6.1). Percent identities were obtained with NCBI Blastx.

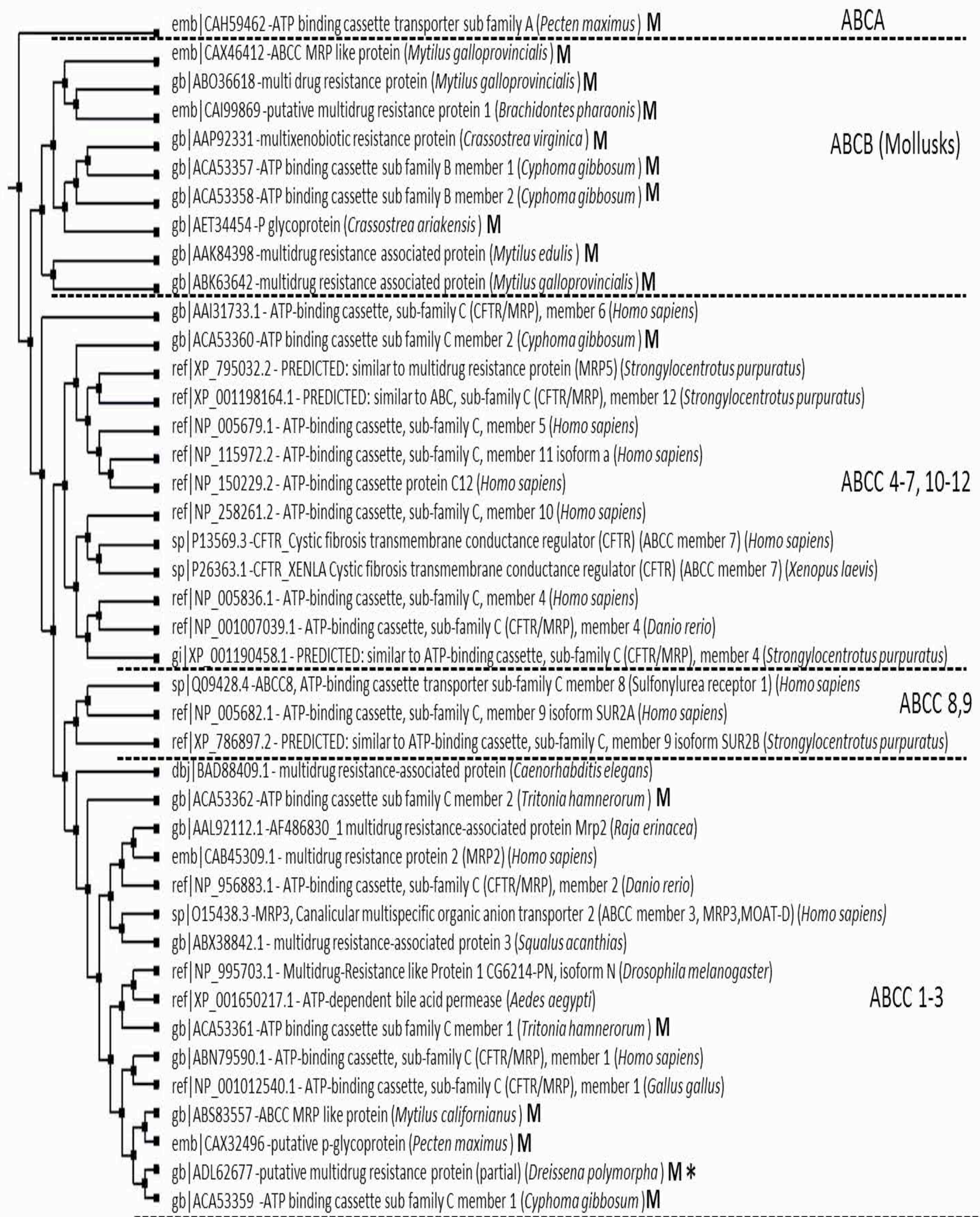

Fig 6.2: Phylogenetic tree including the *D. polymorpha* ABCC (marked with an asterisk), other known molluskan ABC sequences (marked with an "M"), and ABCC´s from different taxa that include all known representatives of human ABCC subtypes (1-12). Horizontal dashed lines mark clusters, named after the human ABCC subtypes they include. The ABCA and ABCB clusters (only molluskan sequences) are also marked. Gene definitions are from the GenBank original description, without considering their adequacy to the actual phylogenetic position of the sequence in the tree. Clustering only considered the protein region equivalent to the one sequenced in the *D. polymorpha* ABCC gene.

Compared to ABCC´s from other mollusks, the *D. polymorpha* ABCC sequence is closer to the homolog from the more distantly related marine snail *Cyphoma gibbosum* than to homologs from other bivalves, *Mytilus californianus and Pecten maximus* (fig. 6.2), indicating that the *Dreissena* ABCC sequence is a different paralog than the *Mytilus* and *Pecten* sequences.

6.3.2 Abcc transcript levels and ABCC function in D. polymorpha eggs and larvae

Levels of mRNA for the obtained abcc homolog were relatively low in eggs but they increased about three-fold during the first 2 h post fertilization (hpf), subsequently remaining essentially unchanged until 2 d post fertilization (dpf) (fig 6.3A). This expression pattern is reminiscent to transcript levels found for the abcb1 homolog, which was barely detectable in eggs with levels increasing ten-fold upon fertilization (Annex 2; fig A2.1).

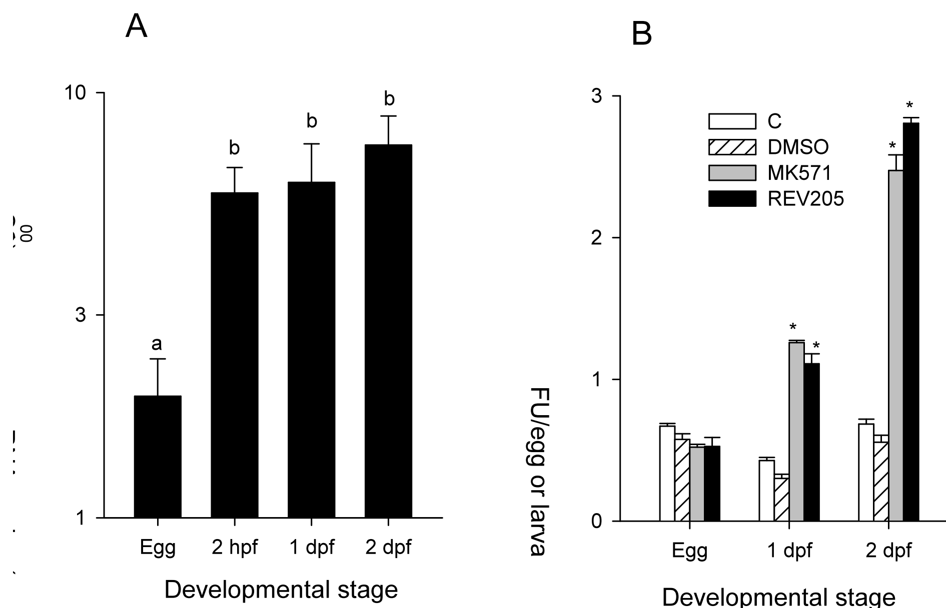

Fig 6.3. Relative mRNA abundances of the abcc homolog in eggs and larvae after 2 h, 1d and 2 d post fertilization of *D. polymorpha* (A) and MXR transporter activities in controls, DMSO controls and under exposure to MK571 and REV205 (B). Data is depicted as mean ±SE (N = 4-5). Different letters indicate significant (P<0.05) differences among groups following ANOVA and Tukey's post-hoc tests. Y axis in graph A is in log scale.

So far, it is not sure whether available antibodies are specific for ABC transporters in *D. polymorpha*. We therefore used data from efflux assays with specific dye substrates and inhibitors as indication for the expression level of active transporter proteins in mussel tissue and embryos/larvae. Efflux assays with Ca-AM as transporter substrate and different transporter inhibitors enable to distinguish between "ABCB1 type" and "ABCC type" efflux activities (Luckenbach et al., 2008). These activity types may result from the sum of activities of several transporter homologs rather than activities of single transporter proteins. However, as outlined below, abcb1 and abcc transcript levels in larvae correlated well with "ABCB1 type" and "ABCC type" efflux activities; this can be seen as indication that the identified abcb1 and abcc sequences encode important components of the "ABCB1 type" and "ABCC type" efflux activities in *D. polymorpha*.

In concordance with transcript levels, functional protein activity assays also indicated low transporter activity in eggs, but higher activities in advanced stages. Thus, there was no significant ($P<0.05$) increase of calcein fluorescence levels in the eggs with the inhibitors present, showing the absence of inhibitable efflux transporter activity in the eggs (fig 6.3B). In contrast, both inhibitors caused enhanced accumulation of calcein in 1 dpf and 2 dpf larvae as indicated by significantly increased fluorescence levels (fig 6.3B). Previously, enhanced efflux transporter activity in 1 dpf and 2 dpf *Dreissena* larvae compared to eggs was shown by using cyclosporine A, a broad-scale inhibitor of mammalian ABCB1 and ABCC transporters, with the Ca-AM assay (Faria et al., 2011). Our finding that inhibitors that presumably are more specific for ABCB1 (reversin 205) and ABCC (MK571) type efflux activities affect Ca-AM efflux provides evidence for both efflux types in *D. polymorpha* larvae. Assuming that efflux activity is a measure for abundance of functional protein our data indicate that in parallel to enhanced transcript abundances during development ABCB1 and ABCC protein abundances also increase. ABCB1 type efflux has been shown for bivalve embryos before (Faria et al., 2011; McFadzen et al., 2000); in analogy to sea urchin embryos that have both ABCB1 and ABCC type efflux (Hamdoun et al., 2004) ABCC´s, in addition to ABCB1, appear to be expressed and active also in bivalve embryos/larvae .

In difference to bivalves, such as *D. polymorpha*, sea urchin embryos show initiation of efflux transporter activity upon fertilization, but not of transporter mRNA expression (Hamdoun et al., 2004). Thus, in sea urchin transporter protein is already present in eggs and in the process of restructuring the cell upon fertilization becomes activated, whereas in bivalves fertilization appears to initiate transporter gene transcription and *de novo* protein synthesis. This difference in initiating ABC efflux

transporter activity in the freshly fertilized embryo - *de novo* synthesis of protein vs. activation of protein that is already present - indicates two strategies of organisms to quickly establish the MXR system in the developing embryo. The sea urchin egg is comparatively large (100-150 µm diameter) and rich in resources, which enable the embryo to survive adverse conditions. Activation of transporter protein that is already present is likely a quicker process than protein *de novo* synthesis and transport of the protein to the outer cell membranes of the large/ embryo. On the other hand, production of bivalve eggs that are less amply equipped with resources – and therefore smaller and the embryo less resistant to adverse environmental conditions - requires less resources of the maternal organism. Since the eggs are smaller [70 µm diameter for eggs of *D. polymorpha* (Stoeckel et al., 2004)] the process of protein *de novo* synthesis and transport to the outer cellular membranes for establishing MXR of the cell "upon demand" (e.g., upon fertilization of an egg resulting in a developing embryo that needs protection) is nevertheless sufficiently rapid.

6.3.2.1 Effects of chemical exposures on transporter transcript and activity levels

It has been shown earlier that the MXR system in *D. polymorpha* is induced by various environmental factors, including chemicals.

For obtaining an insight into how the *D. polymorpha* abcb1 and abcc genes are involved in cellular stress response to chemical exposure we investigated the inducibility of the genes and protein activity in larvae and adults by dacthal and mercury. Dacthal, a chlorinated aromatic structure, is used as herbicide. It has been found to induce abcb1 expression in *D. polymorpha* (Tutundjian and Minier, 2007) and there is evidence that it is transported through ABCB1-mediated efflux in bivalves (Cornwall et al., 1995). Mercury induces expression of mammalian abcc1 (Kim et al., 2005) and ABCC transporters remove mercury from cells and confer resistance against mercury toxicity (Aleo et al., 2005; Bošnjak et al., 2009; Kim et al., 2005). In larvae, we observed dose-dependent, statistically significant 2- to 9.2-fold increases in transcript levels of abcc and abcb1 homologs upon exposures to 0.5 and 2 µM dacthal (fig 6.4A, B). Also exposure of larvae to 1 µM mercury resulted in increases of transcript levels of both transporter homologs that were up to 30-fold (fig 6.4A, B).

The changes in transporter gene expression in gills of adult mussels from chemical treatments were generally less pronounced than in larvae. Abcb1 and abcc transcript abundances increased 1.4- to 3.4-fold in gills from dacthal-treated animals (fig. 6.4 C,D). Increases in transcript levels in dacthal treatments were only statistically significant for abcb1, but not for the abcc homolog. In gills from mercury-exposed animals abcb1 and abcc transcripts increased significantly (P<0.05), being 2.2- and 11.3-fold, respectively, higher than in controls (fig. 6.4C, D).

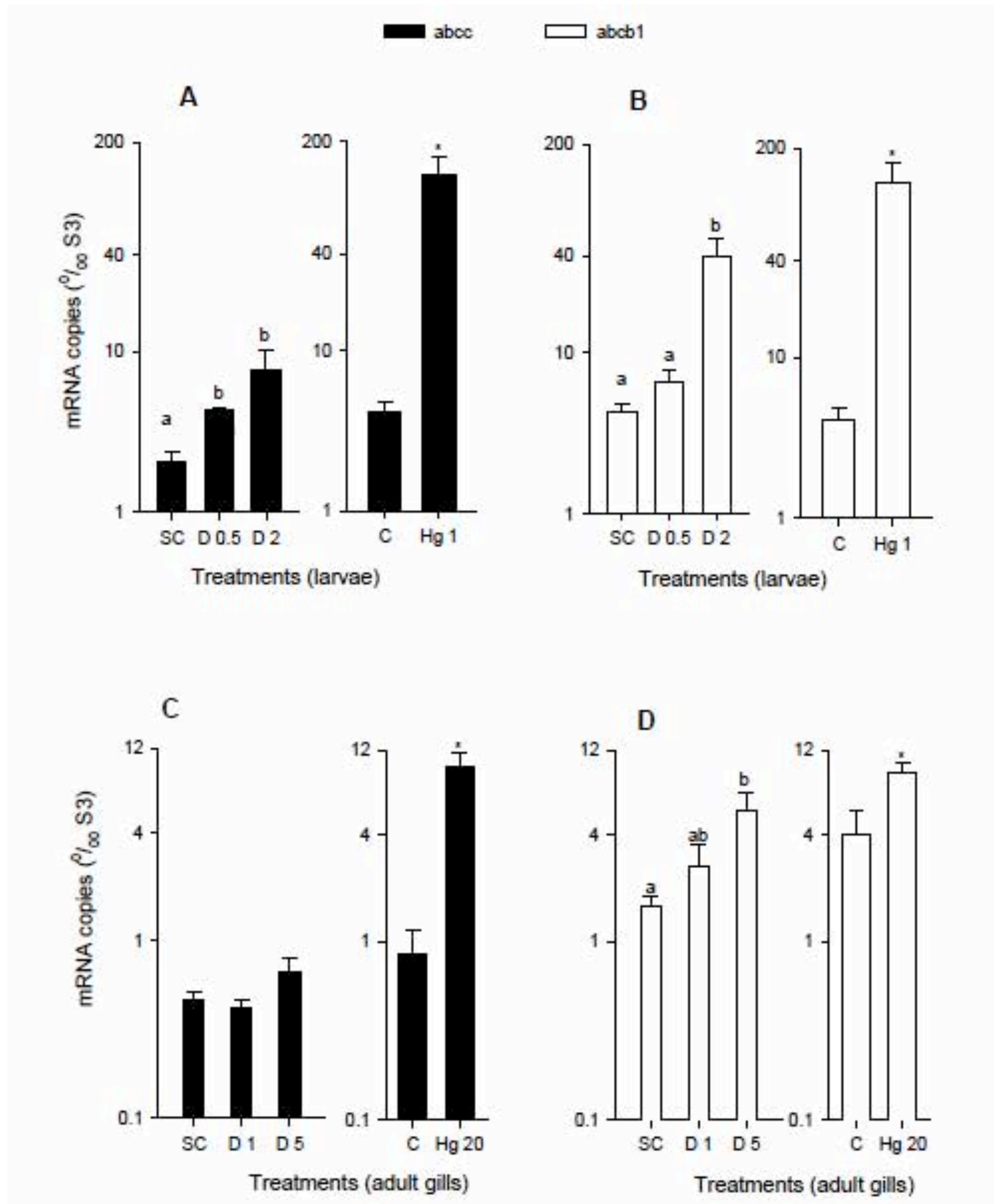

Fig 6.4. Relative abundances (Mean ± SE, N =7 for larvae, N =11 for adult tissue) of abcc and abcb1 mRNA in whole larvae (A, B) and in gills of adult individuals (C, D) of *D. polymorpha* exposed to dacthal and mercury, respectively, for 1 d. The applied concentrations were for dacthal: 0.5 mg/L, 2.0 mg/L for larvae and 1.0 mg/L, 5.0 mg/L for adult mussels; and for mercury: 1 µg/L for larvae and 20 µg/l for adults. Different letters or asterisks indicate significant (P<0.05) differences among groups following ANOVA and Tukey's post-hoc tests or Student's t tests. Dacthal and mercury treatments and their respective solvent controls and controls are depicted separately since they were from different experiments.

The Ca-AM efflux assays that we performed to obtain a measure for functional transporter protein levels also indicated enhanced ABCC and ABCB1 in larvae and adult animals upon chemical treatments. For the Ca-AM assays, larvae or gill tissue pieces from individuals from each chemical treatment were divided and part of the larvae/gill pieces were then exposed either to Ca-AM only or Ca-AM and one of the inhibitors. Ratios of calcein fluorescence changes by inhibitors were then calculated for each chemical treatment and control. The concentration of 5 µM that was applied for MK571 and reversin 205 in the Ca-AM assays was close to the concentrations that in chemically untreated larvae caused 50% of their maximal effect (i.e., the maximally measured calcein fluorescence in cells), expressed as IC50 (concentration of 50 % inhibition) that were 8.2 µM for MK571 and 4.2 µM for reversin 205 (Faria et al., 2011). Preliminary Ca-AM assays with mussel gills also showed that 10 µM approximately correspond to the IC50 for reversin 205 and IC25 for MK571 (Annex 2; fig. A2.3). Therefore, as 5 or 10 µM of each inhibitor will cause an effect on calcein accumulation within the dynamic range of the dose-response curve for inhibitor concentration and calcein fluorescence, changes in transporter activity will become visible as a change in ratio of calcein fluorescence with and without inhibitor. As can be seen in fig. 6.5 the fold changes in fluorescence by inhibitors were generally smaller in larvae / gill tissue from the chemical treatments than in the controls. For instance, the ratio in fluorescence of Ca-AM + MK571 vs. Ca-AM only was 1.7 in larvae that had been exposed to 2 mg/L dacthal, whereas this ratio was 2.8 in larvae from the solvent control (fig. 6.5A). A similar difference between fluorescence ratios for embryos treated with dacthal compared to solvent control was seen with reversin 205, inhibitor of ABCB1 (fig. 6.5B). Thus, the inhibitory potency of the inhibitors was generally lower in the Ca-AM assays when larvae / adult mussels had previously been exposed to chemicals, which indicates higher efflux activity due to increased transporter protein titers. The decrease in inhibitory potency occurred for both inhibitors, indicating higher titers/activities of both the ABCB1 and ABCC homologs. Enhanced transporter ABCB1 and ABCC protein titers in tissues from mussels exposed to chemicals are also indicated by higher basal efflux activities (fig. A2.4).

In larvae, also mercury treatment resulted in lower inhibitory potencies of both MK571 and reversin 205 in the Ca-AM assays. As with larvae, decreases in the ratios of Ca-AM + inhibitor vs. Ca-AM only were also found for gill tissue from dacthal treated adult mussels (fig. 6.5 C, D). In tissues from mercury treated animals the fold increase in fluorescence was only decreased with MK571, but not with reversin 205 indicating a change in ABCC titer/activity but not in ABCB1.

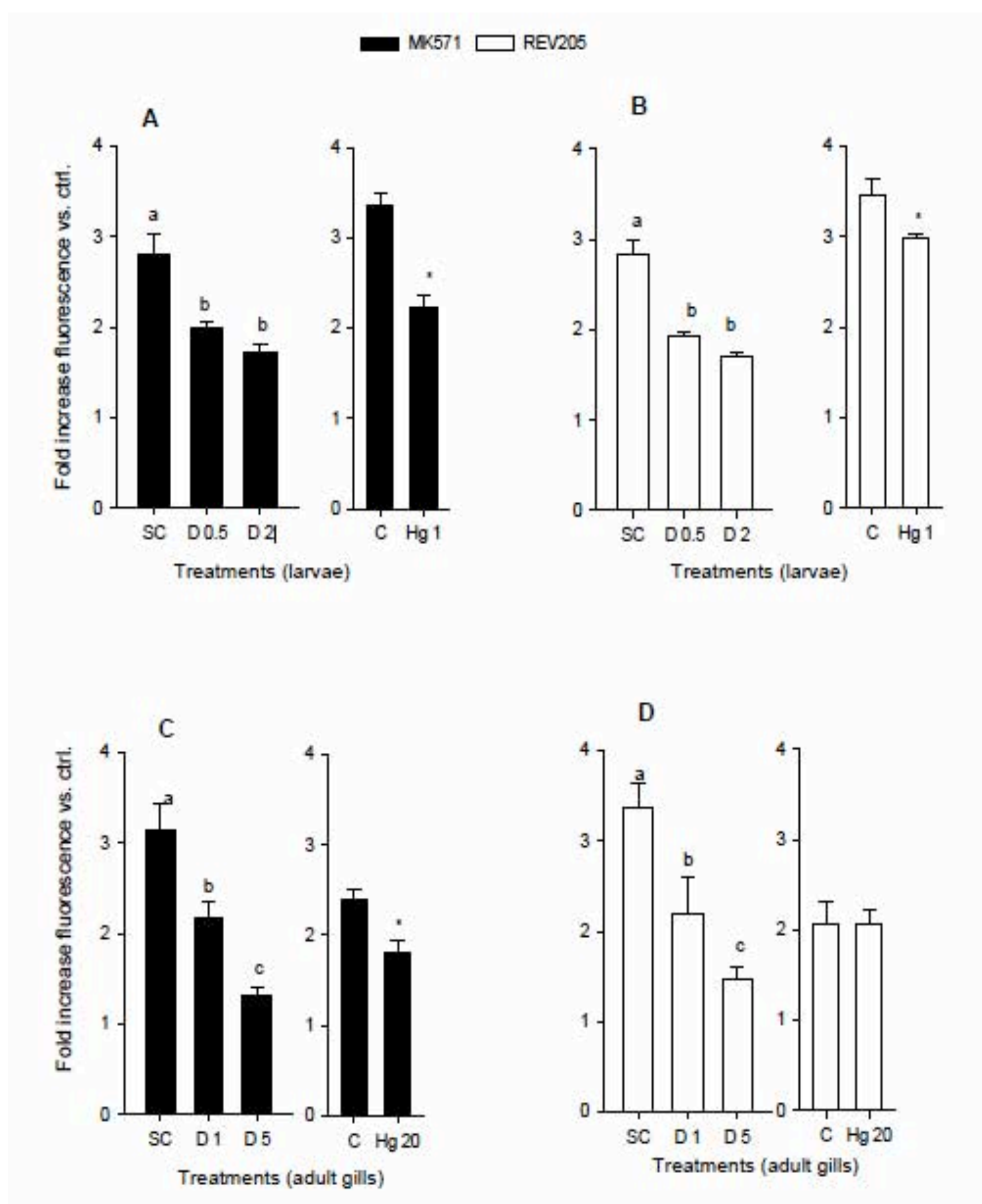

Fig 6.5. Fold changes in calcein fluorescence levels (Mean ± SE, N=7) caused by MK571, inhibitor of ABCCs, and REV 205, inhibitor of P-glycoprotein/ABCB1 in whole larvae (A, B) and in gills of adult individuals (C, D) upon exposure to dacthal and mercury. Different letters or asterisks indicate significant (P<0.05) differences among groups following ANOVA and Tukey's post-hoc tests or Student's t tests. Dacthal and mercury treatments and their respective solvent controls and controls are depicted separately since they were from different experiments

In larvae, occurrence of lower potencies of transporter inhibitors in the Ca-AM assays corresponded with enhanced abcb1 and abcc transcript levels (fig. 6.4A, B; fig. 6.5A, B). Thus, with induction of abcb1 and abcc transcript expression upon exposure of

larvae to chemicals protein titers – as indicated by transporter activity levels – are also higher.

Interestingly, this concordance of changes in transcript levels and in transporter activities was not in all cases observed in gill tissue of adult mussels. Thus, whereas abcc transcript levels barely changed with dacthal (fig. 6.4C) the calcein assays indicated a substantial increase in ABCC efflux activity in these tissue samples (fig. 6.5C). Furthermore, there was a significant ($P<0.05$) increase in abcb1 transcript levels upon mercury exposure (fig. 6.4D), but the sensitivity of efflux activity to ABCC inhibitor MK571 was unchanged (fig. 6.5D).

These findings could indicate that the single components mediating the ABCB or ABCC type efflux activities are differentially regulated. Thus, for instance changes in transcript levels of the abcc homolog that we measured may differ from homologs that also contribute to the ABCC type efflux. Further, the data may indicate that interrelation of gene activity and protein titer in adult *D. polymorpha* is complex and does not necessarily correlate. It has been described that *ABCB1* mRNA levels that were increased upon induction of the gene in human cells is only under certain conditions followed by an increase in ABCB1 protein titer (Yague et al., 2003).

Furthermore, our data show that transporter gene regulation upon chemical exposures differs in larval and adult stages of *D. polymorpha*. The MXR transporter activity in *D. polymorpha* has been proposed as useful endpoint in biomarker studies for detecting pollution (Parant and Pain, 2001), however, difficulties of data interpretation due to the complexity of genetic regulation of the MXR system and dependence of MXR activity on various environmental parameters apart from chemical stress have been discussed (Pain et al., 2007).In the context of the application of MXR activity in *D. polymorpha* for biomarker studies the following aspects of our data are important:

- We identified the partial sequence of an abcc homolog that based on its similarity to Abcc 1 – 3 is a candidate to encode for a MXR related MRP transporter.
- ABCC transporters in addition to ABCB1 appear to mediate MXR efflux in larvae and adult stages of this organism. Thus, the overall MXR efflux activity is a result of joint action of various transporter types and could be highly complex.
- Abcb1 and the abcc homolog were both upregulated by chemical stress, however, as shown with differing responses of abcb1 and abcc in adult tissue

upon dacthal exposure, appear to be regulated through different pathways. Assuming that efflux activity data are a measure of protein titer our data also indicate that the response of transporter expression observed on the level of transcription may not necessarily occur also on the protein level and vice versa. Thus, changes of transcription cannot be concluded from efflux activity; on the other hand, changes in transcript abundances may not have an effect on function. However, this needs to be further studied, because the "ABCB1 type" or "ABCC type" efflux activities in *D. polymorpha* gill tissue and larvae may additionally be mediated by other homologs than the ones encoded by the partial abcb1 and abcc sequences we studied.

Acknowledgements

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CHAPTER 7: Development of microarray for non- model specie: Zebra mussel

Seasonal variations in gene expression profiles in natural populations of zebramussel (*Dreissena polymorpha*) from the Ebro River (NE Spain)

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Manuscript

Abstract

The zebramussel *Dreissena polymorpha* is a freshwater bivalve native from Caspian Sea that has invaded freshwater bodies worldwide during the XX century, generating major economical and environmental problems. To analyze the impact of seasonal and environmental variations on the physiology and metabolism of zebramussel, we developed a custom microarray using 4057 publicly availabe DNA sequences from *Dreissena* and other related genera. Transcriptome profiles were analyszed using half-body samples captured in Riba Roja (low Ebro River), a location considered relatively free of chemical pollution, at three different stages of the vital cycle. The results showed a clear separation on transcriptome profiles between samples collected in February and June (pre- and spawning, respectively) and samples collected during gonad resorption (September). A total of 745 transcripts changed significantly their mRNA levels during these three months (ANOVA $p<0.01$, fc ±1.5). These changes could be considered as response to variations of temperature, reproductive stages, as well as changes in food availability. Genes involved in oxidative stress and glucose metabolism were overrepresented in September, whereas genes related to reproductive functions were overrrepresented in the spawning and pre-spawning period. The data also shows sexual differences for 29 transcripts, particularly at the spawning season (June). The use of custom microarray for seasonal studies will help in the better understanding of new organism behaviour, to identify developmental stages at which the organisms is more vulnerable. However, our still limited current knowledgement of bivalves (and molusks in general) genome and Molecular Biology prevents the fully comprehension of the observed stress responses.

Keywords: Microarray, seasonal cycle, zebra mussel, gene expression, Ebro River, sex differenciation

7.1. Introduction

Environmental factors may influence the internal processes of organisms and the distribution of populations and species. Physiological strategies that allow organisms to thrive in hostile habitats are poorly understood, particularly for invasive species. Zebra mussel (*Dreissena polymorpha*), an invasive bivalve specie from the Caspian Sea, has spreaded all over the world in freshwater systems, from European rivers to freshwater bodies in the East Coast of the USA. In Ebro River, located in NE of Spain, it was first deteced in 2001 in Riba-Roja dam and spread from there to the rest of the river basin (Strayer, D.L 1991; Durán, C and Anadón, A. 2008). Like other bivalves, zebra mussel can be used in biomonitoring programs as they are sessile filter feeders, which facilitates studies of pollutant bioaccumulation and determine its effects (Morcillo, Y. et al 1998; Regoli, L. et al 2000).

Analysis of changes on gene expression represents a potentially powerful tool to characterize immediate cell responses to stressors, and may constitute an early warning of the effect of contaminants, and represent an useful complement to existing monitoring methods to study the effects of pollutant on bivalves at the biochemical level (Faria, M. et al 2009; Faria, M et al 2010). Among existing gene expression quantifying techniques, microarray analysis allows a simultaneous quantification of a large number of genes, helping to define the mode of action of pollutants in different species. However, microarray analysis requires a substantial knowledge of the species genome and Molecular Biology, two aspects that are particularly lacking for aquatic organisms of ecological relevance (Denslow, N.D., et al 2007; Piña, B. and Barata, C, 2011). Bivalves possess relatively large genomes, with C values ranging from 0.65 to 5.4 pg (Venier, P., et al. 2006), whereas the number of gene sequences currently deposited into gene databanks is still scarce and limited to a small number of species, like *Mytilus galloprovinciallis*, *Ruditapes philippinarum*, and *Crassostrea gigas*, (Saavedra, C. and Bachère, E. 2006, Milan, M. et al 2011, Piña, B. and Barata, C, 2011). Public DNA sequence repositories contain a limited, but substantial amount of gene sequences from *Dreissena rostiformes* (some 3,000 sequences see below) and a limited number of sequences for *D. polymorpha*. We used this existing information to design and validate a microarray intended to explore gene expression variability in *D. polymorpha* during the yearly life cycle. To our knowledge, this is first microarray analysis of the seasonal variations of adults zebramussels from a natural, well established population at molecular level.

7.2. Material and methods

7.2.1 MicroArray design

The array for *Dreissena polymorpha* was designed using the *eArray* tool from Agilent (https://earray.chem.agilent.com/earray/). A total of 4057 sequences were compiled from the GeneBank using the EBI website (European Bioinformatic Institute, http://srs.ebi.ac.uk). From these sequences, 3253 belongs to *Dreissena rostriforme*, 705 to *D. polymorpha* and the rest to other bivalve species.

An 8x15 Array (a slide with 8 arrays with 15000 probes) was designed using primers of 60 nucleotides. Three probes for each sequence were designed following a position criteria zone (5', center, and 3'), using the eArray software from Agilent. Three extra probes were also designed from each gene, based in the criteria of non self-complementation and low redundancy. After eliminating redundant probes, the array included from four to six probes for each gene sequenced (array design deposited at GEO, GPL15152).

7.2.2 Animal sampling

7.2.2.1 Seasonal Study

Adult zebra mussels (n=10) of similar shell length were collected from Riba Roja reservoir once a month during a year. The animals were dissected with a symmetrical longitudinal cut to have the same representative tissues in the sample. Half a body of each sample were placed in a cryogenic vial with a 1 mL of RNA later® (Sigma-Aldrich, St. Louis, MO). Once in the lab the samples were stored at -80ºC until their use. For the seasonal study, 3 different months were chosen to get a better picture of the changes of the annual cycle for *Dreissena polymorpha:* February, period when gonads are being developed; June, the spawning season and September, when the gonad is being re-absorbed. The experimental design included 2 samples from February (male and female) and 4 samples from June and September (2 males and 2 females). For tissue comparison, some animals collected in June were dissected into different tissues, namely gills, digestive gland, gonad (male and female), and half a whole body and treated as above.

7.2.2.2 *Tissues preparation*

For tissue comparison, some animals collected in June were dissected into different tissues, namely gills, digestive gland, gonad (male and female), and half a whole body.

For studying the gene expression patterns in *D. Polymorpha* larvae, about 105 of fertilized eggs
(1 h of age) were cultured in a 10 L ASTM hard water under constant oxygenation (>90% air saturation), temperature (20 ◦C) and photoperiod (14 h: 10 h; light:dark) without food. 24 h larvae were collected and concentrated by filtration into 50mL centrifuge tubes and then further concentrated by gentle centrifugation (1000 rpm, 10 min). Pellets were resuspended in 5mL and equally aliquoted in1.5mLeppendorf tubes. After a brief centrifugation the supernatant was discharged and the pellet, containing larvae, was resuspended in RNAlater® (Sigma–Aldrich) and stored at −80 ◦C.

7.2.3 RNA extraction

Total RNA was isolated from the samples using Trizol reagent® protocol (Invitrogene). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Delaware, DE) and it was treated with DNAseI to remove genomic DNA contamination. RNA quality was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The samples that did not show degradation were used in the process of microarray analysis.

Zebra mussel transcriptome analyses were performed in a custom cDNA microarray designed in our laboratory using the eArray platform, previously described in the article 7 of this chapter.

7.2.4 Sample preparation for microarray analysis

The preparations of the samples were performed according the Agilent One-Color Microarray –Based Gene Expression Analysis protocol using Cyanine 3 (Cy3) dye. A total 500ng of total RNA for each sample was used as start material. After the amplification, labeling and purification procedure, samples were evaluated for yield and effectiveness of Cy3 dye incorporation using the NanoDrop spectrophotometer.

The samples that presented a specific activity ≥9.0 pmol Cy3/µg cRNA and a quantity of 1.65 µg Cy3 labeled were used for the analysis. Fragmentation of the cDNA was performed in the recommended blocking agent and volume of 2x GE Hybridization Blocking Buffer (Agilent, Gene Expression Hybridization Kit). A final volume of 40µl containing the fragmented cDNA was added to the 8x15 arrays and the hybridization proceeded for 17h at 65°C.

Microarrays slides were washed according the supplemental Agilent protocol ("One-color Microarray-Based Gene expression analysis"). The slides were kept on dark until scanner using Agilent G2565CA and signals were extracted using the Agilent Feature Extraction Software v9.5. The quality of the Microarray data was evaluated manually using the QualityControl report that provides the Agilent Software

7.2.5 Data Analysis

Raw expression data was imported into JMP Genomic v5 (SAS, Cary, NC) where the raw intensity data were normalized using Loess normalization with a smooth factor of 0.2. Then, data for each array were re-valuated for a quality control by distribution analysis, correlation analysis and principal components to make sure that the normalization was effective to all samples. Differentially expressed transcripts were identified using One Way analysis of variance (ANOVA) with a False Discovery Rate of 5% (FDR) (Benjamini and Hochberg, 1995). Raw microarray data have been deposited into the NCBI Gene Expression Omnibus (GEO) database (series GSE35352 for tissues and GSE35353 for seasonal; platform GLP15152)

Gene expression data were subjected to hierarchal clustering. The distance calculations were done using the program Cluster (Eisen et al 1998) and visualized using the Java TreeView program (Saldanha, 2004). Clustering was based on centred correlation and completed linkage. Additional analyisis of microarray data were performed in Mev software (Saedd, A.I. et al 2006)

7.2.6 Microarray validation

Total RNA was extracted and measured from the tissues (gills, gonad, half a body and larvae) as well as the half body of the seasonal study, as mentioned in section 2.3. RNA was treated with DNAse I to remove genomic DNA contamination. Quantities of 1µg were retrotranscribed to cDNA using First Strand cDNA Synthesis

Kid Roche® (Germany) and stored at -20ºC. Aliquotes of 10ng were used to quantify specific transcripts in Lightcycler® 480 Real Time PCR System (Roche, Germany) using Lightcycler 480 SYBR Green I Master® (Roche, Germany). The primers used in qRT-PCR were described in previous study (chapter 4) and were designed from existing *D. polymorpha* sequences. Relative abundance values of all genes were calculated from the second derivative of their respective amplification curve, Cp values calculated by technical triplicates. The Cp values of target genes were compared to the corresponding reference gene (Pfaffl, 2001).

All statistics for qRT-PCR were performed using the SPSS 17 (SPSS Inc, 2002) package. The calculations were performed using ΔCp values ($Cp_{housekeeping} - Cp_{target}$) as this parameter follows a normal distribution, assessed by Kolmogorov –Smirnov test. Statistical comparison of mean values was done using One Way analysis of variance (ANOVA).

7.3. Results

7.3.1 Microarray expression in different Tissues

More than 90% of the probes present in the microarray showed fluorescence values significantly different from background in at least one of the hybridizations performed in this work. Microarray analysis identified a total of 202 probes differentially expressed among the analyzed tissues (gills, gonad and half a body) of adult zebra mussel and larvae after ANOVA, (FDR 5%), 188 after removing duplicate probes ($p<0.01$, Fold change 1.5). Hierarchical correlation clustering divided samples in two clusters, one constituted by testes, ovaries and half-body samples, and a second with gill samples (fig 7.1). This is consistent with the predominance of gonad in the overall body mass in the pre-spawning and spawning periods. Using the half body sample as a reference, gills samples showed the maximal number of probes (spots) with significant differences on gene expression (94 probes overrepresented and 48 probes underrepresented), whereas gonad samples showed 15 probes over-represented and 48 under-represented. From these results, half-body samples were choosen for subsequent analyses of seasonal variation.

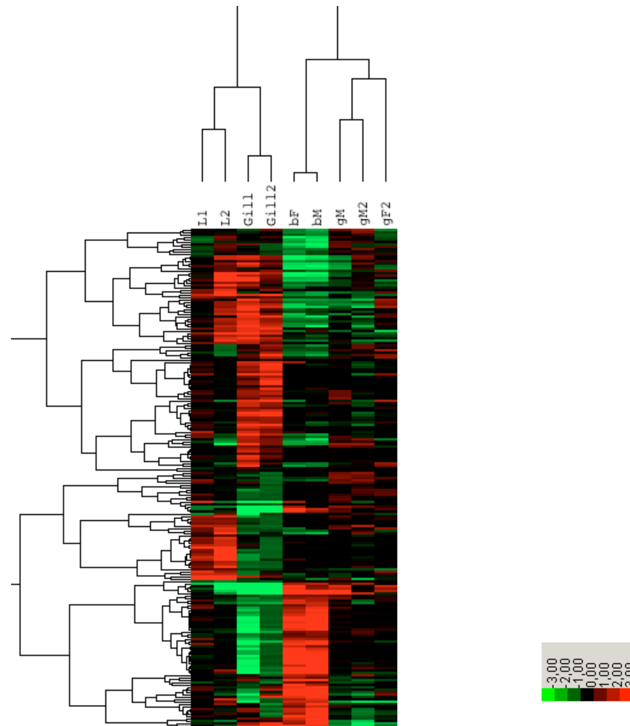

Fig. 7.1 Hierarchical clustering for zebra mussel tissues and larvae microarray samples and its duplicates. The distance calculations were made using Cluster (Eisen et al. 1998) and visualized using Java TreeView (Saldanha, 2004). The color present in the cluster represents the intensity of the dye signal with red being the high probe intensity and green the low probe intensity.

7.3.2 Seasonal analysis for Riba Roja area

Annual variations on the transcriptome of *Dreissena polymorpha* during an annual cycle were analyzed by sampling the same population on February, June and September. Microscopic examinations confirmed that animals were at the predicted maturation stages, pre-spawning, spawning and resorption, respectively (data not shown, Araujo, R. et al. 2010. Male and female individuals were sampled in all three campaings

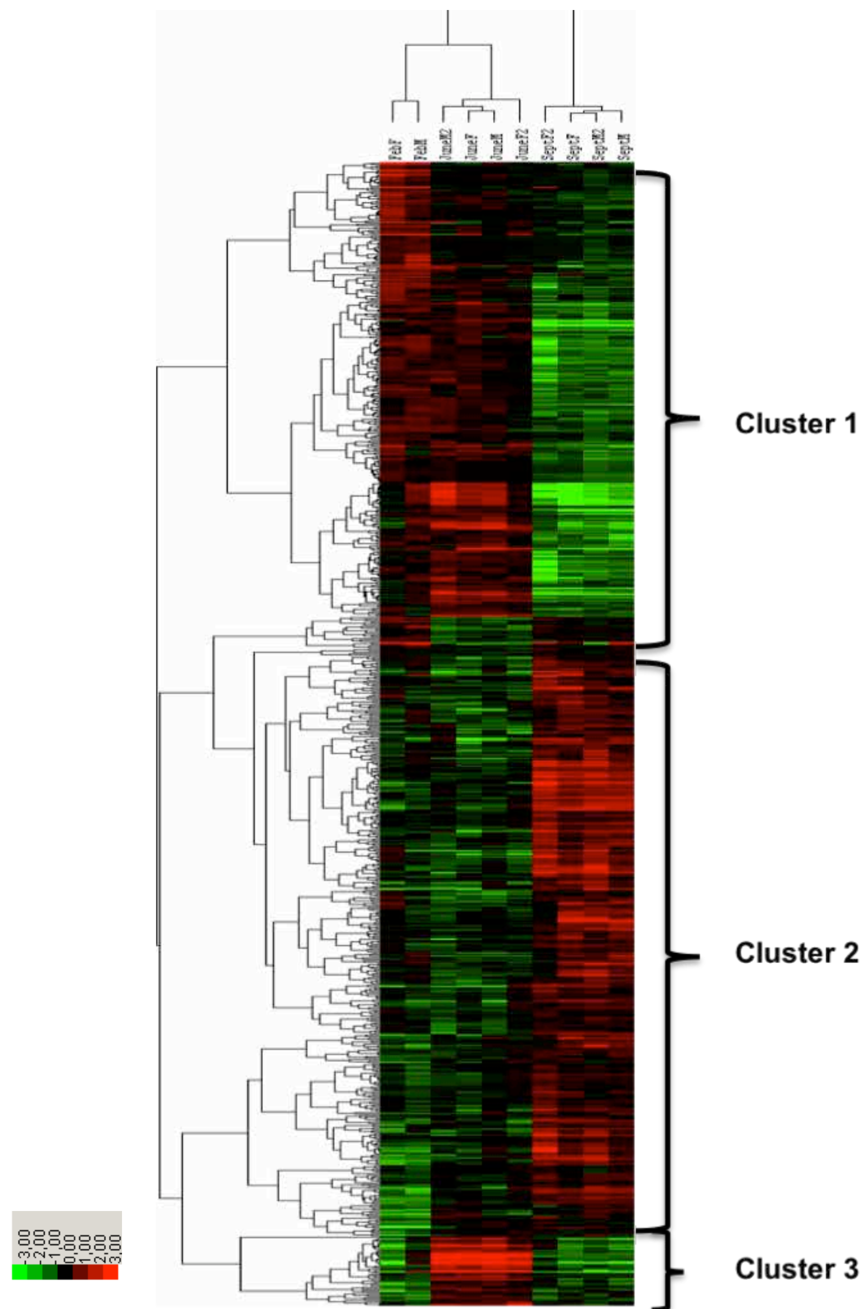

Fig 7.2 . Gene cluster analysis (p<0.01; fold change> ±1.5) for February, June and September microarrays. The color represents the intensity of the signal dye channel with red being the high probe intensity and in green being the low probe intensity.

A total of 1479 probes showed significant seasonal changes (ANOVA p<0.01; fold change ±1.5), corresponding to 745 unique sequences. Hierarchical clustering analysis revealed two clusters of the samples, the first one including samples from September (gonadal resorption) and the second one grouping the samples form June and February (pre-spawning and spawning periods) (Araujo, R. et al. 2010; fig 7.2). The significant genes were grouped in 3 different clusters, divided by the overexpression of genes (fig 7.2). Cluster 1 with a total of 293 probes corresponded to the genes whose transcription increased in Febraury and June in

the pre-spawning and spawinig season, cluster 2 with 406 genes overexpressen in September and cluster 3 during the spawning season with a total of 46 probes (fig 7.3)

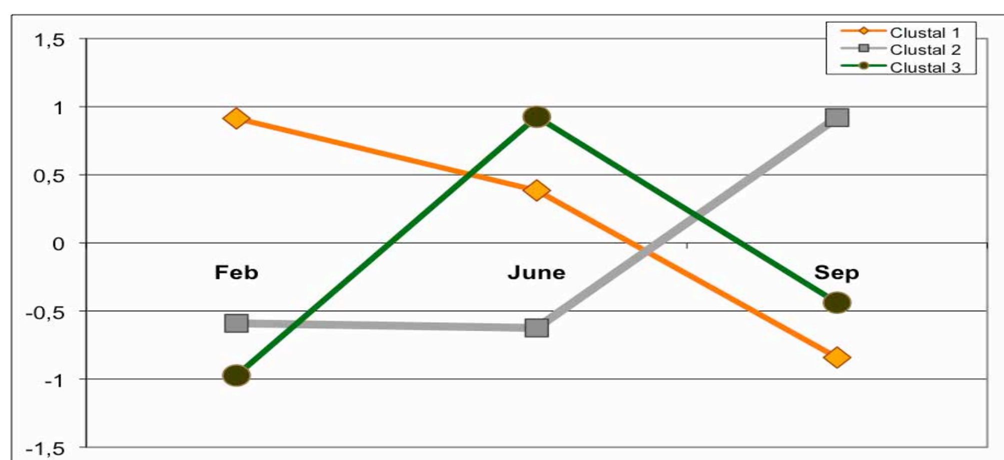

Fig 7.3. Transcriptional profiles of genes classified into the clusters obtained after the hierarchical clustering analysis. Data are shown as logarithmic values.

The most significant genes of each cluster are listed in the table 7.1 (ANOVA $p<0.01$, fold change of 1.5; completed list can be seen in Annex 3). Probes significant expressen were analyzed by Blast at NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to find the putative gene name of each probe. Cluster 1 showed an up regulation of genes that belongs to the oxidative stress pathway such as metallothionein, heat shock protein and SOD, and some representative of reproductive pathway like chordin. Cluster 2 showed genes of oxidative stress with genes like HSP70 and 90 and MT, also showed over expression of genes from the protein recycle pathway like ubiquitin and zinc finger. Cluster 3 had the representation of the reproductive pathways with genes such as chordin and putative spermatogenesis-associated proteins.

	Gene	Acc. Num.	*p* Value
Cluster 1	DMC1 mRNA [Dreissena polymorpha]	AF508222	<0.001
	Chordin-like 2 [Monodelphis domestica]	AM230059	0.003
	Cu/Zn SOD [Venerupis sp]	AY377969	0.002
	Cytochrome c oxidase subunit I [Dreissena polymorpha]	EY434941	0.004
	Glutathione S-transferase [Danio rerio]	EY434057	0.002
	Heat shock protein 70 [Meretrix meretrix]	EY436967	0.008
	Kielin/chordin-like protein-like [Xenopus tropicalis]	AM230055	0.002
	NADH dehydrogenase subunit 5 [Utterbackia peninsularis]	EY434995	0.002
	Ubiquitin-protein ligase, putative [Candida dubliniensis]	AM230100	0.001
	Metallothionein mRNA [Dreissena polymorpha	U67347	0.006

Cluster 2	Retinoid X receptor mRNA [Mytilus galloprovincialis]	EF644351.1	<0.001
	ABC transporter, ATP-binding protein [Bacillus thuringiensis	EY436647	<0.001
	Cytochrome P450 30 [Mercenaria mercenaria]	EY434718	<0.001
	Ferritin subunit [Meretrix meretrix]	EY436700	<0.001
	Heat shock protein 70 mRNA [Dreissena polymorpha]	EF526096	0.02
	Heat shock protein 90 [Laternula elliptica]	EY435944	<0.001
	Metallothionein [Dreissena polymorpha]	EY434440	0.006
	Metallothionein mRNA [Dreissena polymorpha]	U67347	0.006
	Similar to ABC transmembrane transporter [Ciona intestinalis]	EY436141	0.02
	Ubiquitin/actin fusion protein 3 [Lotharella globosa]	AM503944	0.005
	Zinc finger protein 484 isoform 1 [Xenopus tropicalis]	EY434679	0.005
Cluster 3	Chordin-like 2 [Monodelphis domestica]	AM230412	<0.001
	Cytochrome c oxidase subunit I [Dreissena polymorpha]	EY434603	0.001
	Glycoprotein [rabies virus]	EY434547	0.009
	Metal dependent phosphohydrolase [Petrotoga mobilis]	EY436573	0.008
	Similar to sperm associated antigen 17 [Ciona intestinalis]	EY433929	0.002
	Similar to spermatogenesis associated 6 [Canis familiaris]	EY434681	<0.001

Table 7.1. List of the most representative probes expressend in the different gene cluster found after a hierarchical clustering analysis.

The result obtained with microarray analysis were validated by qRT-PCR using additional samples of half a body from each season, and comparing the results. Genes from oxidative stress pathway previously studied and validate for *D. polymoprha* in Chapter 4 like MT, HSP70, HSP90, GST and a reproductive gene sequenced for the first time in zebra mussel, RxR (Chapter 5) were measured for the validation (fig 7.4).

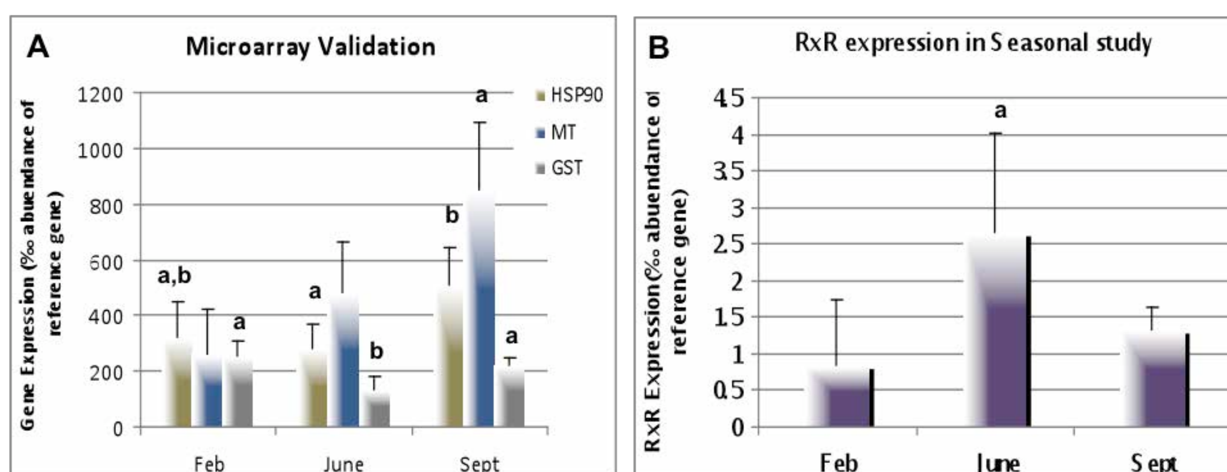

Fig 7.4 Gene expression (in ‰abundance of tge reference gene) of A) HSP90, MT and GST and B) Retinoid X receptor, both graphics for the three months of the study in order to validate the results obtained by microarray.

Genes such as MT and HSP90 presented higher levels in September, whereas GST was higher in February, validating the results obtained by the microarray. Because no reproductive gene from zebra mussel up-regulated in the microarray is sequenced and chracterized we used the RxR, a gene characterized by our group (chapter 5). RxR presented higher levels in June as it was expected after microarray analysis.

7.3.3 Sex differentiation

The same samples were re-analyzed to detect putative differences between females and males (fig 7.5). This analysis revealed a clear separation between males and females, especially in February and June samples.

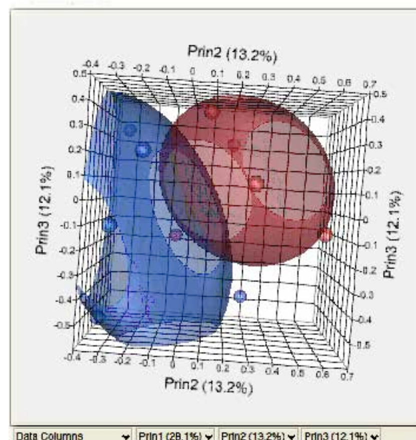

Fig 7.5. The scatterplot show the distribution of the samples in the 3 axis of principal components. Red samples are females and blue ones represent males. It can be seen that in the middle stay the samples of both sexes that gets similar behavior.

A hierarchical cluster analysis with the unique significant probes showed a clear differenciation between males and females during the three months studied after a *t- test* analysis ($p < 0.01$ and fc ±1.5) for the uniques probes (fig 7.6). In total, 41 transcripts were differently expressed ($p < 0.01$, fold change ±1.5), 29 after removing redundant probes, in male and female samples. From them, 14 transcripts were overrepresented in males and 15 overrepresented in females.

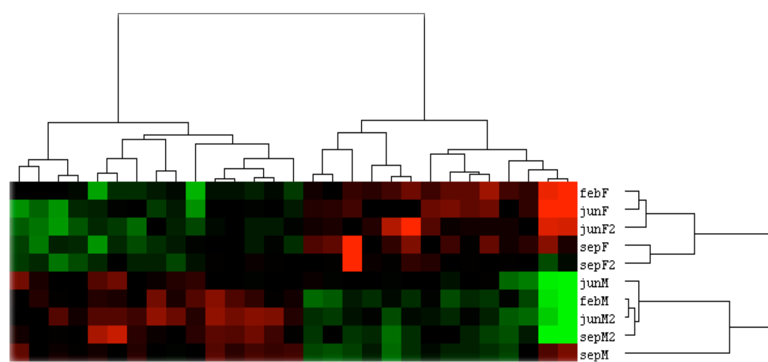

Fig 7.6 . Gene cluster analysis (p<0.01; fold change> ±1.5) for males and females. The color represents the intensity of the signal dye channel with red being the high probe intensity and in green being the low probe intensity. In the cluster are pointed three of the genes most representatives up-regulated in females. The names of genes showed are putative genes after a Blast analysis

7.4. Discussion

The analysis of gene expression by microarray techniques provides a large amount of information that can be related to different modes of action of pollutants (Denslow, N.D et al 2007). However, its application in aquatic organisms is still in the early stages, and only for a limited number of species (Reyero-Garcia, N. et al 2008). Although bivalves have been widely used in environmental monitoring due to its ecological and economical importance, there are still relatively few bivalve genomes sequenced, even partially. Consequently, only few DNA microarray studies from bivalves have been reported, all of them on species with high economic importance as food resources: *Crassostrea virgnica* (American oyster), *C. gigas* (Oyster from the Pacific) (Jenny et al. 2007), and *Mytilus galloprovincialis* (Venier, P., et al 2003). The fresh water mussel *Dreissena polymorpha*, an invasive species causing severe ecologic and economic damage, is gaining importance from the ecotoxicological point of view due to its spread around the world. However, not much information at molecular level is known from this species; to our knowledge the only microarray data of zebra mussels is a small (716 spots) array focused on adhesion proteins (Xu, W. and Faisal, M. 2009). Our approach of designing a microarray with all available sequences from related species aimed to obtain a general view of gene regulation after environmental stress or pollutant exposure.

Several xenobiotics can cause oxidative stress, enhancing ROS and generating cell damage in the organisms. The study of oxidative stress is often evaluated by changes enzymatic activity of different enzymes like SOD, gluthatione metabolism enzymes, etc, (Loprasert, S., et al 1996; Inoue, Y et al 1999) and metabolites or oxidation products (oxidized and reduces glutathion, oxidized lipids and proteins, etc.) However, antioxidant defense mechanisms are subjected to seasonal

variations, fluctuating during the year depending on the nutrients, temperatures, reproductive cycle and other factors. In aquatic systems, these variations have a strong effect in physiological status of invertebrates affecting growth, reproduction and immune system, but not much information is known about these variations. Some studies of reproduction mechanisms and seasonal variations have been performed in biochemical and physiological point of view in *Mytilus edulis and galloprovincialis* respectively (Livingstone, D.R 1981; Lubet, P. et al 1986), but few studies have been done at the molecular level.

The study of the seasonal transcriptome changes in the invasive bivalve specie *Dreissena polymopha* showed a clear differenciation between the resorption period (September) compared to the pre-spawning and spawning seasons (February and June, fig 7.2) . This result correlates to the reproductive cycle of zebra mussel, in which gonadas are being developed in late winter/early spring, maturate in late spring, spawn in early summer, and are re-bsorbed in autumm (Borcherding, J. 1991; McMahon, R.F. 1996)

Transcripts overreperesented in June (and partially, February) samples included genes related to the reproductive cycle, as putative spermatogenesis-associated proteins or chordin-like proteins, related to embryo development. September samples showed overexpression of transcripts related to the oxidatives stress response, ferritin, heat shock protein and metallothionein. In addition, overrepresented transcrips included genes related to protein degradation pathways, like zinc finger-encoding proteins, and ubiquitin. These genes may be associated to the re-absortion of the gonadal tissue that occurs during the fall.

The study of transcriptomic seasonal variations in the invasive specie zebra mussel, using a custom microarray, revealed relevant information about the behavior and the response of this species to environmental changes in natural populations. However, the limited information at molecular level of this bivalve makes difficult some of the valuations in the results, and further studies need to be done in order to increase the comprehension of the results.

Acknowledgements

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Florida, FL) for their assistance on the training for microarray data analysis using JMP® software.

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CHAPTER 8: General discussion and final conclusions.

8.1 General discussion

The biological impact of aquatic pollution has been steadily increasing during the past decades due to the intentional or non-intentional release of new manufactured chemicals into the environment. This is especially significant in the Ebro River, which suffers a considerable ecological impact from different industrial activities located predominantly in the last third of its course. The mercury-cell, chlor-alkali factory in Flix, 120 km from the river's mouth, represents a particular issue for concern, as it continuous operation since the beginning of the 20th century resulted in the accumulation of high amounts of heavily polluted sediments in the adjacent riverbed. These sediments include elevated quantities of organochlorine compounds as well as mercury and other mining residues. Several studies showed the accumulation of metals and organic xenobiotics in aquatic organisms captured downstream Flix (Terrado, M. et al 2006; Olivares, A. et al 2010), although the impact of these pollutants in local populations of aquatic organisms has been evaluated only recently (Lavado, R. et al 2004; Quirós, L. et al 2008). These results underscored their potential risk for human health.

This PhD thesis aims to two major objectives: the development and validation of new biomarkers to evaluate the impact of xenobiotics in aquatic organisms such as fish and mussels, and the confirmation of these biomarkers in natural populations living in exposed and unexposed environments.

8.1.1 Validation of molecular markers in fish

Quantitative mRNA biomarkers are useful tools to detect early warning responses to pollutants. Changes of mRNA levels of specific genes are considered as the first step of the response of living organisms to pollutant exposure.

Chapter 3 focuses in the use of molecular biomarkers to detect the effect of contamination in fish. This chapter presents the study of the biological impact by heavy metals, present in high concentrations in sediments and biota from the lower part of Ebro River (Ramos, L. et al 1999; Carrasco, L., et al 2011). The study showed maximal levels of mercury in muscle, kidney and liver of carps captured down stream from Flix (fig 8.1), being kidney the tissue with the highest mercury concentration. These results correlate with the concentrations measured in the sediments of the river determined by a previous study (Carrasco, L. et al 2008). The lowest concentration of mercury corresponded to carps captured upstream the

factory, thus confirming that it represents the major source of mercury pollution in the low Ebro River.

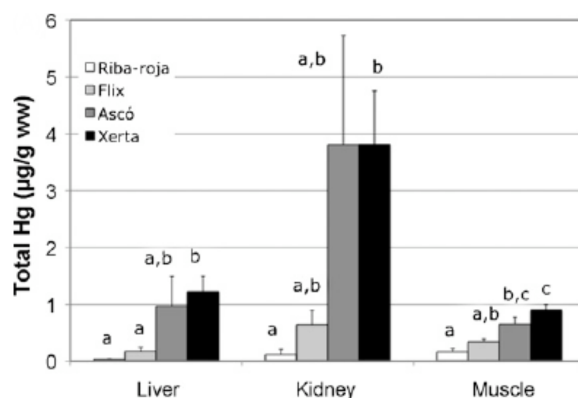

Fig 8.1. Representation of average values of mercury levels in liver, kidney and muscle (µg/g wet weight) of Common carp in different sampling sites: Riba-roja (empy bars), Flix (pale grey), Ascó (dark grey) and Xerta (solid bar). Different lower case letters indicate statistically different groups of samples (Tukey's test)

Metallothionein (MT) plays an important role in the metabolism and detoxification of heavy metals and its induction is considered a marker for the presence of metals in the environment (Price-Haughey, J. et al 1986; Woo, S. et al 2006). In this chapter 1 we analyze the variation of mRNA levels of two isoforms of metallothioneins (MT1 and MT2) in liver, kidney and scales of different carp populations.

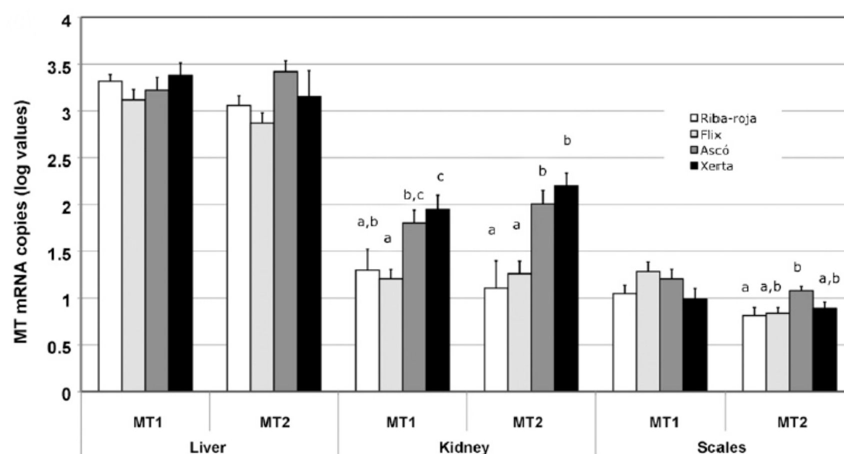

Fig 8.2 Representation average values of mRNA levels of two isoforms of metallothionein, MT1 and MT2 in liver, kidney and scales. The values are expressed in copies (mRNA copies per 1000 copies of ß-actin mRNA, logarithmic values). The lower case letters indicate statistically different groups of samples (Tukey's test)

Liver showed the maximal levels of metallothioneins for both isoforms, but no significant differences were found between populations, nor any correlation exists between MT mRNA levels and mercury content. In contrast, kidney showed lower mRNA levels for both isoforms of metallothioneins, MT1 and MT2, in non-exposed

carp populations, but these levels increased significantly in samples collected down stream Flix (fig 8.2). These results indicate that kidney is particularly affected by mercury contamination.

The response of kidney MT mRNA levels to mercury exposure was tested in the laboratory. After a single intraperitoneally injection of 20 µg/kg of mercury, kidney mRNA levels for both MT isoforms showed a strong increased (20 fold), whereas liver levels remained unchanged (fig 8.3).

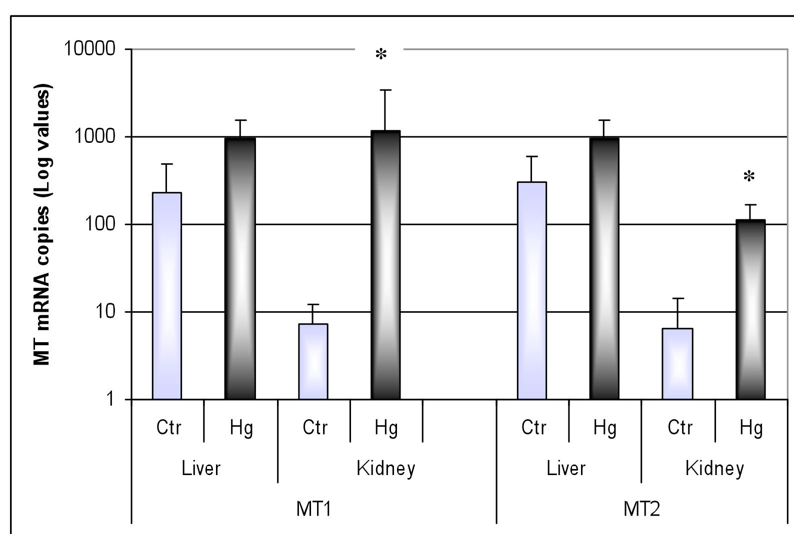

Fig 8.3 Representation average values of mRNA levels of two isoforms of metallothionein, MT1 and MT2 in liver, kidney after Hg injection. The values are expressed in copies (mRNA copies per 1000 copies of ß-actin mRNA, logarithmic values). The asterisk indicate statistically different groups of samples (Tukey's test)

In an attempt to develop a non-invasive monitoring method of biological impact on pollutants in water systems, mRNA levels of MT were also measured in scales of the same carp populations. Scales showed the lowest mRNA levels of all analyzed tissues, making their evaluation more difficult than the analysis of liver or kidney samples. We observed a significant increase of MT2 mRNA levels in scales from the Ascó population, consistent with the results from kidney (fig 8.2). Despite the low level of response of MT2 mRNA levels in scales, the results suggest that scales respond to the presence of chemicals in the environment. Therefore, scale analysis is a potential alternative method for monitoring of water quality, avoiding unnecessary animal killing.

These results demonstrate that scales are not a simple passive element of protection in fish skin, but they are metabolically active and able to respond to

changes in the environment, including the presence of pollutants. They contain different bone cells similar to the mammalian and avian membrane bone osteoclasts and osteoblasts, which respond to a variety of physiological and external effectors, including hormones and pollutants (Elwing, et al. 1990). The use of scales as target tissue has several advantages, as they are easy to collect without killing the animal and make time course analysis possible ([a]Quirós, L. et al 2007), at least for some fish species. The typical low amount of RNA obtained from scales is nevertheless sufficient for the modern mRNA quantification techniques, whose sensitivity allow the use of small animals or animal parts. Fish scales have been used in other aquatic monitoring studies, such as the analysis of endocrine disruption by the measurement of estrogen receptors in Sea bream and Mozambique tilapia scales (Pinto, P.I.S, et al 2009).

Markers based on mRNA quantitation techniques present many advantages for the studies of contamination effects. A same set of instruments and reagents can be applied to many stressors in different tissues, as showed in chapter 3. In ecotoxicology studies, for example, liver of fish is considered an important target tissue due to its role in the detoxification process in organisms and its relative large size in many species allows the use of chemical and biochemical analyses. However, liver may not always be the best choice for analyzing certain toxic effects, as exemplarized by our analysis of mercury pollution in carps or a previous study in *Barbus graellsii* ([b]Quirós, L. et al. 2007). Whereas our results indicate kidney as the relevant tissue to measure MT mRNA levels for metal contamination studies, its small size in many fish species required the use of molecular biomarkers. These assays allow the use ofever smaller tissues, such as scales, to develop a non-invasive technique on contamination effect studies. Fish scales are active metabolic tissues that show early response to exposure to metals (Navarro, A. et al 2009), dioxin-like compounds ([a] Quiros, L. et al 2007) and estrogens (Pinto, P.L. et al 2009).

Bioasays based on mRNA quantitation technique allow also the study of marine ecosystems, as shown for the monitoring the effect of metals and organic pollutants in *Aphanius fasciatus* (Kessabi, K et al 2010), or in the study of new species from tropical ecosystems like *Parachromis dovii* and *Poecilia gilii* from Costa Rica (Navarro, A. et al (Submited).

8.1.2 Study of molecular biomarkers in new invasive specie.

Ebro River suffers an invasion of a small bivalve native from Caspian Sea, the zebra mussel (*Dreissena polymorpha*). First detected in 2001, it has become a dangerous threat, both because its capacity to clug pipes and other water conducts and for representing a serious competitor for native mussels, such as Unio, Anodonta and Potomida. On the other hand, bivalves are considered good sentinel species in aquatic systems, because they are sessile organisms with filter feeding strategies that allow the accumulation of the substances present in their habitat. In this thesis (chapters from 4 to 7) we developed a series of tests and techniques to introduce the use of this invasive species in the monitoring of the freshwater ecosystems as potential sentinel species in the low Ebro River.

8.1.2.1 Heavy metal effect in adult and larvae zebra mussel

The main problem for the application of gene expression analyses to a new species is the lack of information at the genetic level. The response to xenobiotics is poorly understood in non-model species, like *Dreissena polymorpha*, and the regulatory pathways affected by any specific contaminant are largely unknown. Therefore, the first step to get information on the stress responses at the molecular level was the development of a battery of molecular markers.

Chapter 4 shows changes on gene expression profiles for several putative stress markers (Metallothionein, Superoxide dismutase, Catalase, Glutathione S transferase, Glutathione peroxidase, Cytochrome c oxidase, the multixenobiotic resistance P-gp1, and heat shock proteins HSP70 and HSP90) in adults and pediveliger larvae after exposure to metals (Hg, Cu, Cd). These markers were chosen to measure the vulnerability of this specie to heavy metals as well as to detect its detoxification pathways. MT is a well-known biomarker to detect metals contamination, whereas GST, GPX, SOD, CAT, HSP70 and COI are considered part of different oxidative stress pathways. During this work, a partial sequence of zebra mussel HSP90 gene was obtained for the first time.

Analysis of the results from this battery of markers showed different patterns of response among tissues. Gills showed a faster and stronger response to metal exposure (24 h after exposure) than digestive gland (7 days after exposure), maybe because they are directly exposed to the polluted water. MT mRNA levels

increased after metal exposure in all tissues analyzed, whereas HSP70 mRNA levels increased strongly in gills. Hg and Cd were the strongest inducers of MT in zebra mussel, contrasting with the known low induction capacity of Hg in other systems, including fish ([b]Quirós, L et al 2007). Exposure to heavy metals exposure also induced mild but persistent changes in mRNA levels of oxidative stress-related genes, particularly in digestive gland after a long (seven days) exposure, Larvae analysis showed overall low mRNA levels but similar response patterns as in adult tissues.

A correlation analysis indicated coordinated regulation of GST and CAT on one hand and GPX, COI and SOD on the other, both in adult tissues and in larvae. These results suggest that the correlation factors of oxidative stress genes are already present at larvae stages of *D. polymorpha.*

8.1.2.2 Development and validation of new markers

The lack of information on gene DNA sequences limits the use of mRNA quantitation techniques in non-model species, like zebra mussel. In case of *D. polymorpha*, about 5,000 DNA sequences are currently deposited in public databanks, although many of them may be redundant, corresponding to repeated entries of a similar or identical sequences or unassembled fragments of larger genes. In this thesis we identified and validated two new genes with possible environmental relevance: the retinoid x receptor (RxR), important in the endocrine disruptor evaluation, and ABC transporters of membrane for xenobiotics (ABCC, chapter 5 and chapter 6 respectively). This was the first description of RxR and ABCC membrane transporters DNA sequences in *D. polymorpha*.

The methodology for the identification and cloning of these new genes started by the design of appropriated primers. Their sequence was derived from published sequences of the same genes in related organisms (bivalves, mollusks...). After amplification of the targeted fragments from zebrafish RNA samples, the obtained DNA fragment was cloned and sequenced. From these sequences, new sets of qRT-PCR suitable primers were designed and used for quantitative analysis of the corresponding mRNAs in samples from laboratory experiments.

Chapter 5 describes the partial sequencing and characterization of the expression of the retinoid X receptor, a nuclear hormone receptor whose primarily function is the regulation of several process during differentiation and development (Evans, 1988).

RxR may play an important role in the induction of imposex in gastropods by TBT (Castro,L.F.C 2007; Lima, D. et al 2011) and it has been used as a molecular marker for endocrine disruption in aquatic systems.

Eggs and larvae presented higher levels of RxR mRNA than gill, digestive gland and testis from adult zebra mussel (fig 8.4). These levels were constant throughout all the initial developing stages of zebra mussel (eggs, 2h, 24h and 48h post-fertilization). In adult tissues, gills presented the highest expression of RxR followed by digestive gland and testis (fig 8.4)

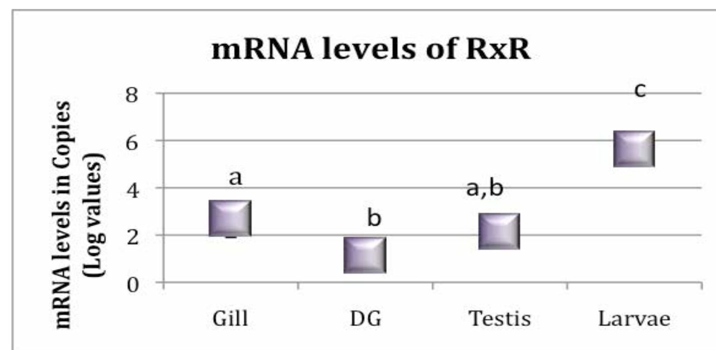

Fig 8.4 Average values of mRNA levels for retinoid x receptor gene in different tissues of zebra mussels (gills, digestive gland (DG), testis and larvae of). The values are expressed in copies (mRNA copies per 1000 copies of S3 mRNA, logarithmic values). The lower case letters indicate statistically different groups of samples (Tukey's test)

Gonad and digestive gland of adult zebra mussel were used to study the potential use of RxR mRNA as marker for endocrine disruption. Variations of RxR gene were only observed in the digestive gland after 7 days of exposure to 1ppb of TBT, a well-known endocrine disruptor in bivalves. TBT also induced mRNA levels of oxidative pathway-related genes, like CAT or GPx, in digestive gland and gonad, although only after 7 days of treatment (fig. 8.5), suggesting that this effect is a secondary reaction to the presence of the compound. Larvae did not show any response on mRNA abundance of any gene after 24h TBT treatment; longer treatments were not possible in this system given the short developmental stage of zebra mussel.

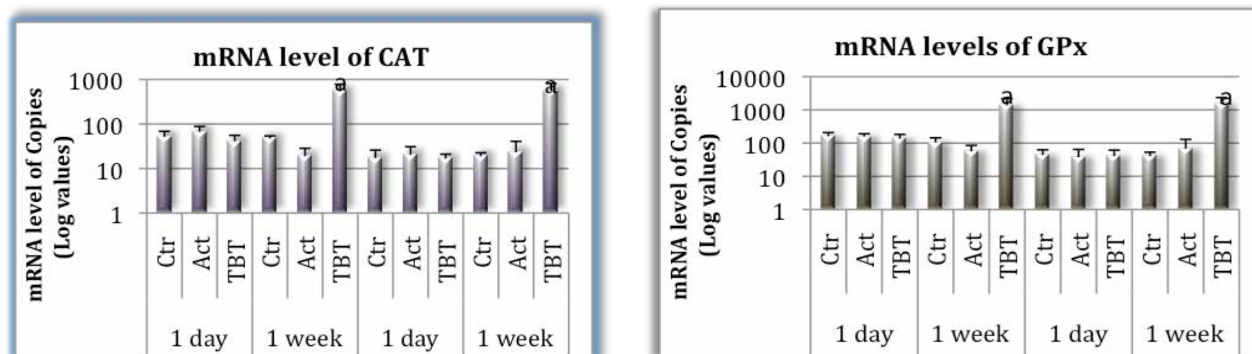

Fig 8.5 Average values of mRNA levels for two oxidative stress genes: Catalase (CAT) and Glutathione peroxidase (GPx) in digestive gland (DG) and gonad. The values are expressed in copies (mRNA copies per 1000 copies of S3 mRNA, logarithmic values). The lower case letters indicate statistically different groups of samples (Tukey's test)

The effects of TBT in *Dreissena polymorpha* digestive gland transcriptome were analyzed using custom microarrays. The results (Annex 1) show a clear a differentiation among control samples and treated samples (ANOVA test, $p<0.05$; $Fc\pm1.5$). TBT up regulated 139 transcripts, including MxR, a membrane protein whose role is to transport xenobiotics out of the cell, and some genes related to oxidative stress pathway. The results obtained by microarray technique were validated by qRT-PCR, which also show increased mRNA levels for MxR and some genes related to the oxidative stress.

RxR is known to play an important role in biological process like differentiation and reproduction. Besides the study of RxR as biomarker for endocrine disruption, RxR mRNA levels were also studied in zebra mussel natural populations. This study used half a body of adult zebra mussel from Riba Roja and Meandre (in Ebro River) during 4 different months to see annual variations. Meandre is considered a hot spot due to its proximity to the chlor-alkali factory whereas Riba-Roja is considered a clean area up-stream the factory.

Maximal RxR mRNA levels were found in January, the period at which zebra mussels builds the godan tissue for reproduction, and drop to their lowest values in May and June, the spawning season. At the end of the reproductive season, in September, levels of RxR started to recover. This expression pattern was followed by the two populations under study, suggesting that the putative pollution affecting the meander population had no influence on the reproductive cycle of zebra mussel.

The field results suggested that changes in mRNA levels of the new sequence could be used as a good indicator of maturation stage of the populations. However, further studies should be done for better understand TBT (and other substances) effects in zebra mussel and to use this species as potential sentinel for invertebrate endocrine disruption.

Chapter 6 describes and presents the characterization of two members of the ABC membrane transporters, the P-glycoprotein (P-gp), also known as MXR from the ABCB family, and MRP1, the first member of the ABCC family described in zebra mussel.

In this chapter is described the partial sequence of the zebra fish multidrug receptor protein (MRP), a member of ABCC transporter family. During early development, MRP mRNA levels followed a pattern similar to the one observed for the P-gp gene, with very low levels in eggs and a sharp increase short after fertilization. The observed activation of membrane transporters at early larvae stages suggests a fast initiation of protective mechanisms to tolerate the presence of chemicals during their planktonic stages. MRP and P-gp mRNA levels increased in larvae after 24h exposure to dacthal (2mg/L) and Hg (1mg/L) 3 and 10 fold, respectively, a result consistent with the proposed protective role of the multidrug transporters in larvae. We observed similar results in a cooperation study of P-gp characterization, where mRNA levels were barely detectable in eggs and increased by a factor of 10 after the first day of fertilization. A moderate decrease was noticed for the following days, although the values remained significantly higher than in eggs. Analyses of the P-gp associated transport activity showed that the efflux rate was low in eggs increasing significantly in 1- to 3-day trochophora (Faria, M. et al 2010).

Adults showed a similar, albeit milder response to both treatments. The results suggest that both families of transporters, ABCB and ABCC, may show a co-regulated response to the presence of stressors, like pesticides and metals (Buss, D.S. and Callaghan, A. 2007; Achard, M., et al. 2004). There are few reports showing the involvement of ABCB and ABCC activities in the response to environmental pollutants and to inorganic mercury, respectively. Therefore, further investigations are required to validate these mechanisms to other pollutants.

8.1.2.3 Design of custom microarray and its use to study natural populations of zebra mussel

Chapter 7 describes the design of a custom microarray and its use in the measurement of seasonal variation of a well-establish zebra mussel population in Riba-Roja. The microarray was designed using published sequences in GeneBank from *Dreissena polymorpha* and similar species. Samples (half animals) were collected at 3 different stages: February (pre-spawning), June (spawning) and September (gonad re-absorption). Data analysis showed a clear distinction between two groups of samples, in which samples from February and June clustered together and distinguished neatly from September samples.

Functional analyses of transcripts showing significant variations among the three populations revealed that June mRNA samples showed an overrepresentation of transcripts related to reproduction process, like spermatogenesis, or a chordin like protein. On the other hand, transcripts related to protein degradation pathways, like ubiquitin, appeared overrepresented in September samples, together with some oxidative stress-related genes, like ferritin and heat shock proteins. These results are consistent with the known maturation stages of zebra mussel, as in February the gonads are in preparation, in June is the spawning season, and in September the gonads are re-absorbing (McMahon, R.F. 1996).

The results obtained with the custom microarray suggest that it could be a potential and useful tool to study the pathways affected by chemical substances or by natural variations as well as population changes. Together with the results from heavy metal and endocrine disruptor treatments (chapters 3 to 6), these data confirms the potential of zebra mussel as suitable sentinel specie in freshwater systems. However, further studies should be performed at molecular level like sequencing and characterizing new genes in order to get better information of the metabolic and regulatory pathways affected by xenobiotics.

8.2 Conclusions

1. Techniques based on the quantification of mRNA levels of specific genes allow the detection of early warning signals of environmental stress or the presence of xenobiotics in aquatic organisms.
2. The increase of metallothionein mRNA in kidney constitutes an excellent marker of exposure to subtoxic mercury levels in common carp, reflecting the accumulation of mercury in this organ
3. The use of scales as a target tissue is a fast, non-destructive, and relatively inexpensive test for toxic exposure that implies minimal suffering of the animal. This test also allows sequential testing in the same individual, facilitating time-course analyses and providing information on individual variability.
4. The analysis of MT and CYP1A gene expression in scales allow detecting an effect by pollutants exposure although the expression pattern detected in scales is different from liver.
5. The design of a battery of already established gene expression stress markers allowed us to explore different pathways of stress response in adults and larvae of zebra mussel, opening the possibility to analyze regulatory networks for stress-related genes in this species.
6. The study for the fist time of ABCB and ABCC membrane transport proteins in embryos and larvae stages of zebra mussel showed an early initiation of these mechanisms of defense, allowing embryos to survive in environments with moderate to high levels of pollution.
7. The design and use of a costume microarray for non-model specie showed a hybridization of the 90% of the probes with *Dreissena polymorpha mRNA*.
8. The study of transcriptomic seasonal variations in the invasive specie zebra mussel, using a custom microarray, revealed relevant information about the behavior and the response of this species to environmental changes in natural populations.

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ANNEX 1

Chapter 5: Development and Characterization of a new molecular marker for endocrine disruption

A.1.1. Analysis of TBT effect by costume Microarray.

The samples of digestive gland treated with TBT (TBT; 1ppb; SigmaeAldrich, St. Louis, MO) analyzed during the study of RxR characterization listed in chapter 5 were used in a new study using a microarray technique. Due to the lack of molecular information for *D. polymorpha* it was designed a costume microarray by Agilent® in order to obtain better view of pathways affected by contaminants. The microarray design is explained in chapter 7.

Data obtained from the hybridization of zebra mussel cDNA from control a treated tissue was analyzed by JMP® software. It revealed 262 probes significantly regulated, using a cutoff of $p< 0.05$ and a FDR= 5%, based on FDR determination experiment. With a fold change higher than 1.5, and after removing the duplicates probes, it was found 214 transcripts differentially expressed with more genes up-regulated (139; logFC>0) than down regulated (75; logFC<0).

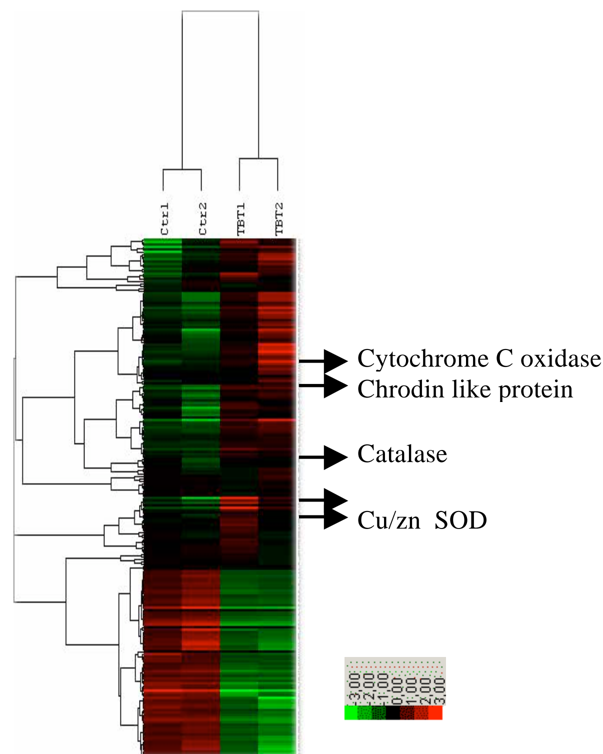

Fig A1.1: Hierarchical clustering for each control and TBT samples. In the figure is represented the probes that are significant regulated (*p*<0.05, FC±1.5) in digestive gland of zebra mussel. The color represents intensity of the single dye channel with red showing the probes with high intensity and with green the ones with low intensity.

A hierarchical cluster for unique significant probes (p<0.05; fc±1.5) showed a clear distinction between control and treated samples (fig A1.1). It was seen a higher number of up-regulated transcripts by TBT treatment (139 probes) than down-regulated (75 probes). Some of the most representative transcripts found statistically regulated are showed in table a.1.1 where the probes are listed in order of decreasing of fold change (expressed as logarithmic fold change; *p<0.05* and FC±1.5). When multiple probes were present for a single sequence like multixenobiotic resistance (MXR) that showed 11 transcripts significant regulated, the fold change reported was calculated from the average of all probes.

In the table A1.1 is listed the name of the gene for zebra mussel as it was submitted to the GeneBank (Gene ID). It was also added the putative sequence for this probes that did not have the sequence characterized, these probes were analyzed by Blast analysis (NCBI website) and cluster to similar sequences. After the Blast analysis, form the 214 transcripts 40 transcripts did not found a similar sequence.

Accession Num.	Gene ID	FC (log)	*p* values	Putative Sequence
Up regulated				
AJ506742	Multixenobiotic resistance protein	4.310	0.036	Multixenobiotic resistance protein [Dreissena polymorpha]
AF086634	Cyclin B	3.028	0.030	Cyclin B [Dreissena polymorpha]
EY436008	qm_NP8.F08	2.877	0.046	NADH dehydrogenase subunit 2 [Meretrix lusoria]
EY434718	qm_NP13.H06	2.793	0.013	Cytochrome P450 30 [Mercenaria mercenaria]
EY436637	qm_NP29R.H07	2.780	0.035	Heat shock protein 70 [Laternula elliptica]
EY436819	qm_NP28.H08	2.546	0.018	NAD-dependent deacetylase sirtuin-5 [Ictalurus punctatus]
EF520699	Venerupis CATALASE	2.111	0.037	Venerupis CATALASE
EY435315	qm_NP18.C09	1.899	0.031	NADH dehydrogenase subunit 4 [Meretrix petechialis]
AM230060	BG21_A03	1.804	0.013	Chordin-like 2 [Monodelphis domestica]
EY434041	qm_NP42.F08	1.541	0.039	Zinc finger protein 62-like [Acyrthosiphon pisum]
AY377969	Cu/Zn SOD	1.297	0.020	Cu/Zn SOD [Venerupis]
EY435882	qm_NP24R.H03	1.290	0.050	Nuclear receptor subfamily 1 ABC [Mytilus galloprovincialis]
EY436042	qm_NP8.C11	1.264	0.016	Cytochrome c oxidase subunit I [Dreissena polymorpha]
EF520698	Mn SOD	1.197	0.035	Mn SOD [Venerupis]
AF508218	Cyclin C	1.056	0.039	Cyclin C [Dreissena polymorpha]
AJ517645	atron12	0.953	0.031	U-box domain containing protein [Trichomonas vaginalis]
EY434099	qm_NP40.H04	0.916	0.023	NADH dehydrogenase subunit 3 [Cephalothrix sp.]
EY435719	qm_NP7.H02	0.798	0.044	NADH dehydrogenase subunit 1 [Paphia euglypta]
EY434111	qm_NP40.H05	0.790	0.005	cytochrome b [Meretrix lusoria]
EY436402	qm_NP31R.G06	0.675	0.049	Ubiquitin-protein ligase [Amblyomma variegatum]

Down regulated				
EY434542	qm_NP11.F02	-0.614	0.050	Glutathione S-transferase [Dreissena polymorpha]
EY436908	qm_NP32c.A06	-0.658	0.006	Ferritin [Sinonovacula constricta]
EY434440	qm_NP37.D12	-1.340	0.025	Metallothionein [Dreissena polymorpha]
AM229737	Byssal protein	-1.367	0.049	Byssal protein Dpfp1 precursor [Dreissena polymorpha]
EY435461	qm_NP5.E09	-1.379	0.028	Cytochrome b [Polyrhachis proxima]
EY435188	qm_NP16.D03	-1.707	0.019	NADH dehydrogenase subunit 6 [Cydistomyia duplonotata]

Table A1.1 List of some of the gene differentially expressed in digestive gland of adult zebra mussel after a treatment with TBT ($p > 0.05$, FDR 5%, FC≥1.5) separated by upregulated and downregulated List of some of the most representatine probes after TBT treatment.

After the microarray analysis it was seen a high induction of genes such as multixenobiotic resistance, in charge of transport chemical substances out of the cell in order to prevent damage, as well as an induction of oxidative stress. Keeping in mind that TBT is an antifouling known for its endocrine disruptor potential in molluscs (Castro, LFC. et al, 2007; Lima, D., et al 2011) it was expected to find indiction of transcripts related to reproduction like in the case of the chorine like protein. These results found in microarrays were similar with the ones found in chapter 5. However the few information about genes that is out for zebra mussel make difficult the identification of some pathways. It is seen the need to further studies and characterized some of the transcripts found in the array.

A.1.2 Validation of the Microarray results with qRT-PCR

Additional samples of digestive gland, 5 individuals for each treatment (Ctr, Act and TBT) from the same experiment, were used to compare the results obtained by microarray with the data measured by qRT-PCR and validate the effects of TBT in digestive gland of Zebra mussel. Gene related to oxidative stress (SOD and CAT) and the membrane transport p-glycoprotein (MXR) that were seen to be induced by TBT in the microarrays were utilized to validate the TBT effects using the primers listed in table A1.2.

Gene Name	Acc. num	Primer Name	Primer (5′-3′)	Length (bp)
p- glycoprotein	AJ506742	MxR	CACCTGGACGTTACCAAAGAAGATATA[a] TCACCAACCAGCGTCTCATATTT[b]	104
Glutathione peroxidase	DQ459994	GPx	GAACGGCGTGGAGTTGATG[a] GAGGAAAATTCGGCACGAAA[b]	88
Super Oxide Dismutase	AY377970	SOD	GACAGCATGGCTTCCATGTG[a] AGGAGCCCCGTGAGTTTTG[b]	100
Cytochrome Oxidase I	AM749000	COI	GACATTCAGGCCCTGCGATA[a] GATGTGCAGAACAAAGGGACC[b]	151
Catalase	EF 681763	CAT	ATCAGCCTGCGACCAGAGAC[a] GTGTGGCTTCCATAGCCGTT[b]	101
Ribosomal 3	AJ517687	S3	CAGTGTGAGTCCCTGAGATACAAG[a] AACTTCATGGACTTGGCTCTCTG[b]	158

Table A1.2. List of pair of primers with its access number in the GeneBank and size of amplicon used in our study to validate the Microarrays results. [a] Forward primer [b] reverse primer.

mRNA gene expression measured by qRT-PCR was in agreement with the ones obtained with microarray where multixenobiotic resistance gets induced as well as the oxidative stress genes, CAT and GPx). On the other hand, SOD did not show a variation in qRT-PCR analysis which it is possible that the oligonucleotide probes form zebra mussel microarray it is not specific to the amplicon that is quantified by qRT-PCR (fig A1.2). The discrepancy of results between microarray and qRT-PCR is also observed in other studies, depending on the probe designs (Martyniuk, C.J, et al 2010)

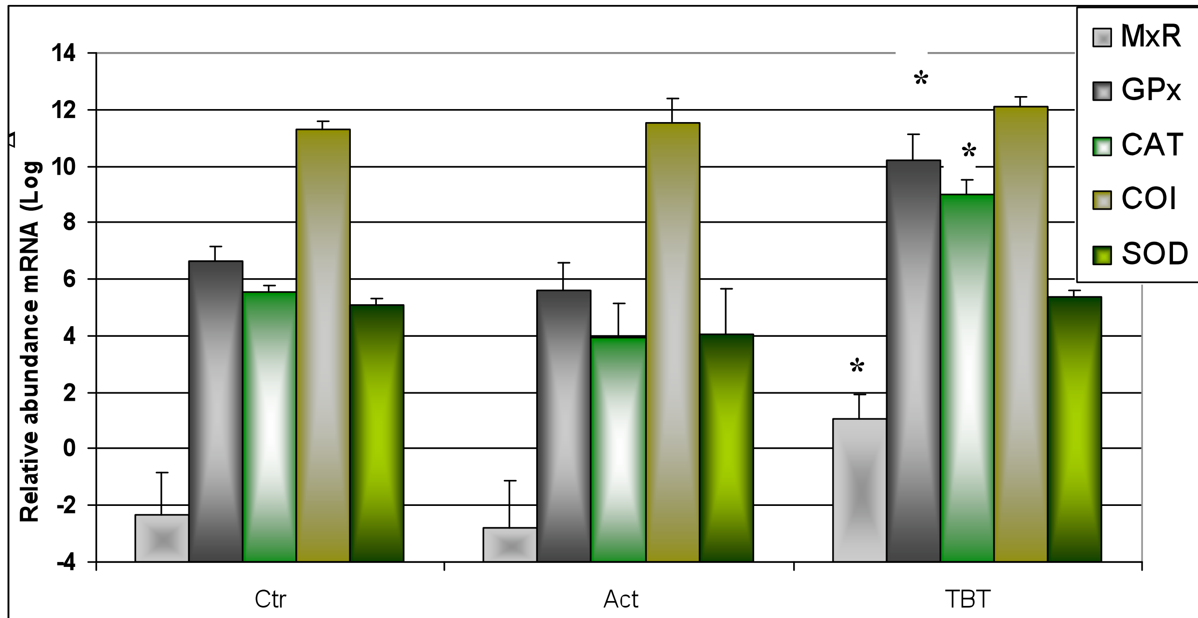

Fig A1.2: Graphic where it shows the relative abundance mRNA de genes of oxidative stress (GPx, CAT, COI and SOD) and the membrane protein transporter of xenobiotics (MxR). The asterisk shows the groups that are different from control treatments (ANOVA $p<0.05$ Tukey).

ANNEX 2

Chapter 6: Study of ABC membrane transportation proteins family in Larvae and adult Zebra mussel and its role in detoxification.

A2.1 mRNA expression of P-gycoprotein in different larvae stages

These results have been published:

Melissa Faria, Anna Navarro, Till Luckenbach, Benjamin Piña and Carlos Barata 2011 Characterization of the multixenobiotic resistance (MXR) mechanism in embryos and larvae of the zebra mussel (*Dreissena polymorpha*) and studies on its role in tolerance to single and mixture combinations of toxicants. *Aquatic Toxicology* 101: 78-87

- Methodology

Chemical

The five tested compounds, cyclosporine A (CsA), verapamil (VER), reversin 205 (REV205), MK571, and celestolide (Cel), and vinblastine, rhodamine B (RhB), calcein=AM (Ca=AM) and serotonin creatinine sulphate monohydrate were purchased from Sigma–Aldrich (Steinheim, Germany). Ethanol and DMSO (analytical grade) were obtained from Merck (Darmstadt, Germany).

Experimental Animal

Sexually mature zebra mussels (*D. polymorpha*) were collected in shallow water (0.5–1m deep) from the Ebro River in the Riba-Roja reservoir (NE, Spain) during the May–August period in 2009 and 2010. Within 3 h of collection, animals were transported in local water in aerated 10 L plastic containers to the lab and acclimated to 20°C and ASTM hard water (ASTM, 1995) for at least 24 h. Spawning of the zebra mussels was induced by exposure to 10^{-3}M serotonin creatinine sulphate monohydrate for 15 min. The mussels were then transferred to clean ASTM hard water. Spawning occurred within 15–30 min in males and within 1–3 h in females. A composite of about 10^{6} eggs from at least 5 females was pooled in 200mL ASTM hard water and fertilized with 1mL of sperm from at least 3 males.

Only gametes obtained from clean ASTM hard water (no serotonin) were used (Faria et al., 2010).

For studying the transcription levels of P-glycoprotein (P-gp1) gene and efflux MXR transporter activity in 1 d old trochophora and 2 d and 3 d D shape veliger larvae, about 10^5 of fertilized eggs were either collected (1 h of age) or cultured in a 10 L ASTM hard water under constant oxygenation (>90% air saturation), temperature (20°C) and photoperiod (14 h:10 h; light:dark) without food. Culture of zebra mussel larvae for more than 3 d requires addition of food and handling greater numbers of individuals to account for high mortality rates (Vanderploeg et al., 1996). Eggs (1 h after being fertilized) and larvae were collected and concentrated by filtration into 50 mL centrifuge tubes and then used in efflux MXR transporter assays. For determining gene transcription profiles eggs and larvae were further concentrated by gentle centrifugation (1000 rpm, 10 min). Pellets were re-suspended in 5mL and equally aliquoted in 1.5 mL eppendorf tubes. After a brief centrifugation the supernatant was discharged and the pellet, containing larvae, was re-suspended in RNAlater® (Sigma–Aldrich) and stored at −80°C.

RNA extraction and qRT-PCR

Total RNA was isolated from the tissues using Trizol reagent® (Invitrogen). The RNA concentration was measured by spectrophotometric absorption at 260nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, DE) and the RNA quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was treated with DNAseI to remove genomic DNA contamination. Quantities from 1µg to 100 ng of DNAseI-treated RNA were retrotranscribed to cDNA using First Strand cDNA Synthesis Kit (Roche®, Germany) and stored at −20 ◦C. Aliquots of 10 ng of total RNA were used to quantify specific transcripts in LightCycler® 480 Real-Time PCR System (Roche) using SYBR® Green Mix (Takara Bio Inc., Siga, Japan) and specific primers designed from reference genes AJ517687 (encoding *D. polymorpha* S3 ribosomal protein) and AJ506742 (encoding putative multixenobiotic resistance protein with high degree of identity with p-glycoprotein, P-gp1; Tutundjian and Minier, 2007), using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA). PCR efficiency values for both genes were calculated using at least five serial 1:3 dilutions for each gene (Pfaffl, 2001); primer sequences were refined until obtaining efficiency values better than 95%. Primer sequences: S3 forward: 5′ CAGTGTGAGTCCCTGA

GATACAAG-3′; S3 reverse: 5′-AACTTCATGGACTTGGCTCTCT-3′; P-gp1 forward: 5′ CACCTGGACGTTACCAAAGAAGATATA-3′; P-gp1 reverse: 5′- TCACCAACCAGCGTC TCATATTT-3′. The corresponding amplicons were sequenced in an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) and compared to the corresponding references in GenBank by the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Relative mRNA abundances of both reference (S3) and test (P-gp1) genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by triplicates). Ribosomal protein S3 was selected as reference gene after examination of its variability among all available samples (not shown). Variations on P-gp1 transcript abundance were calculated as ΔCp values (ΔCp=CpS3 −CpP-gp1). To facilitate the interpretation of results, these values were expressed as mRNA copies of target gene per 1000 copies of the reference gene mRNA (‰ of reference gene, $1000\times2^{\Delta Cp}$).

Data analysis

One way ANOVAs of relative mRNA expression were performed using ΔCp values, as this parameter followed normal distributions, as assessed by the Kolmogorov–Smirnov test.

- Results

mRNA expression of a P-gp1 like gene in mussels embryos and larvae

P-gp1 mRNA was barely detected in *D. polymorpha* eggs, but its abundance increased by a factor of 10 at the first day after fertilization (fig. A2.1). These levels decreased moderately in the two consecutive days, although maintaining values significantly higher (3–4 fold) than egg levels (fig. A2.1).

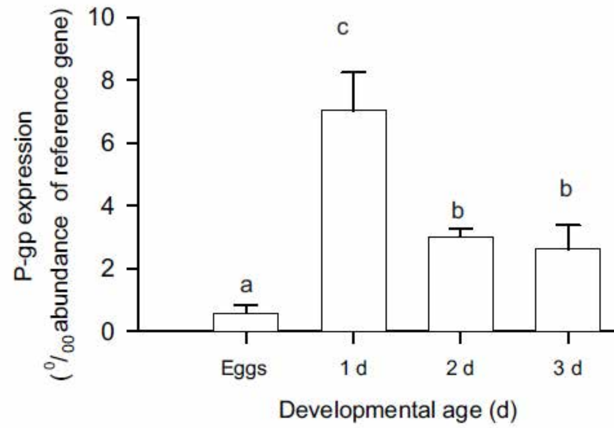

Fig A2.1 Relative expression of P-pgl levels depicted as ‰ of S3 ribosomal mRNA abundance (mean±SE, n=5) in *D. polymorpha* eggs, trochophora larvae of 1 d and D shape veliger larvae of 2 and 3 d old. Low case letters indicate significantly ($P < 0.05$) different sets of data after ANOVA and Tukey's post hoc tests.

A2.2 – Alignemnet of MRP sequences.

The sequenced obtained for ABCC membrane transport was aligned to closer sequences using BLASTx software. The figure A2.2 shows an aligment of NBD-1 aminoacid sequences from ABC transporters from various species.

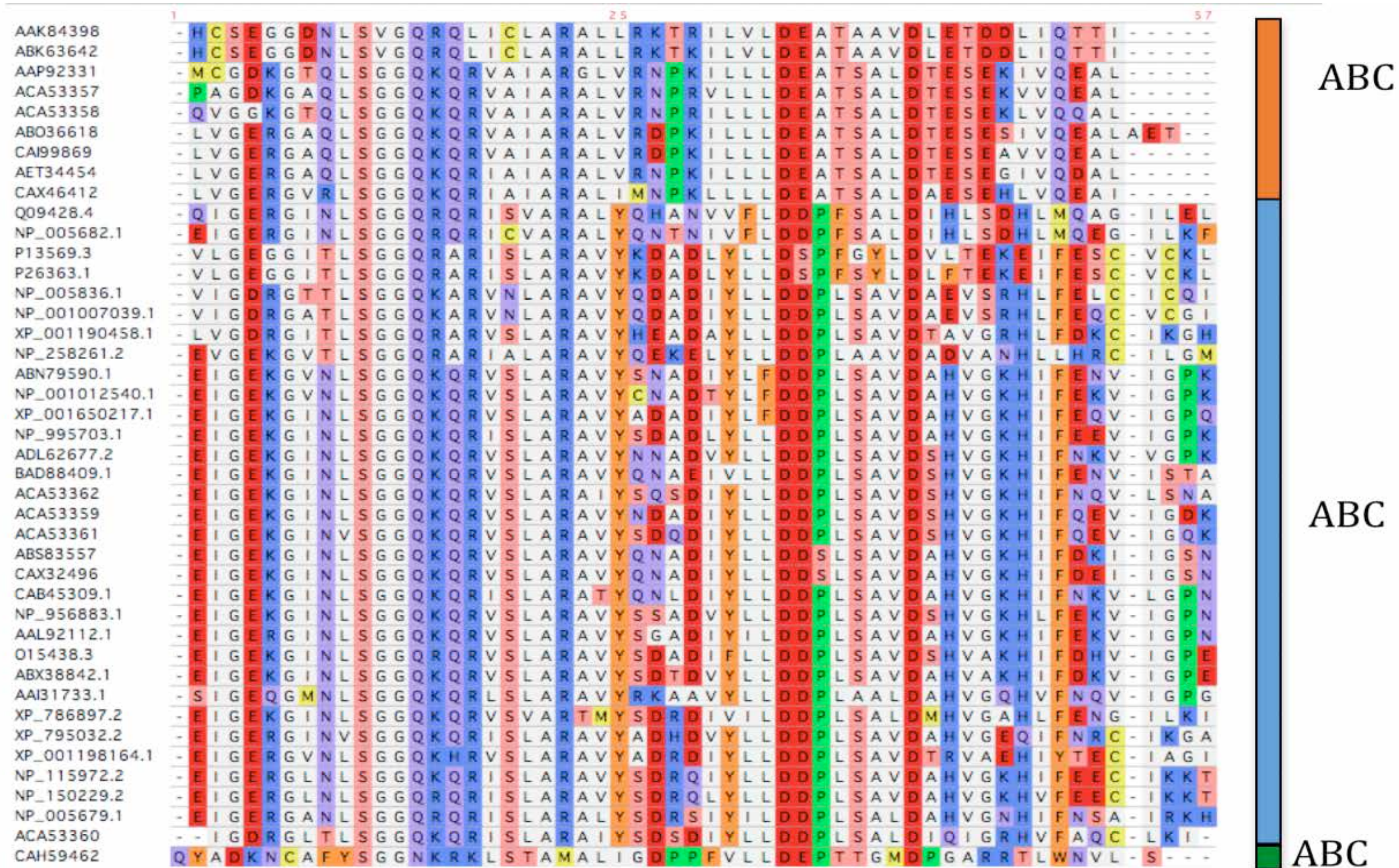

Fig. A2.2: Alignment of NBD-1 sequences from ABCA, B and C subfamilies from a range of species. Sequence names are provided in Figure 2. The *D. polymorpha* ABCC sequence is framed in red.

A2.3 Inhibitor assay

For determining MXR transporter activities in eggs/larvae and in gill tissue of zebra mussel, dye accumulation assays were performed. For disrupting ABCB1 and ABCC like efflux activities in mussel eggs / larvae and in gill tissue we used the model inhibitors of mammalian ABCB1 and ABCC transporters, reversin 205 and MK571, respectively. Preliminary Ca- AM assays with mussel gills showed that 10 µM approximately correspond to the IC50 for reversin 205 and IC25 for MK571

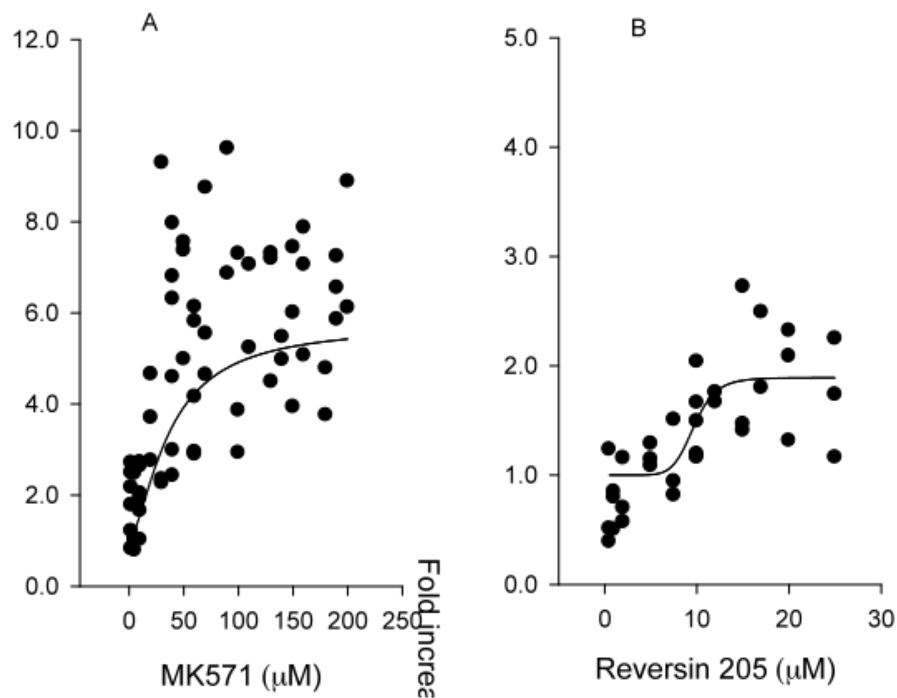

Fig A2.3. Fold increase changes of Ca-AM versus control of gills exposed to increasing concentrations of MK571 (A) and REV203 (B). Fitted lines to the Hill function are also dep

A2.4 Transport activity of MRP transport protein

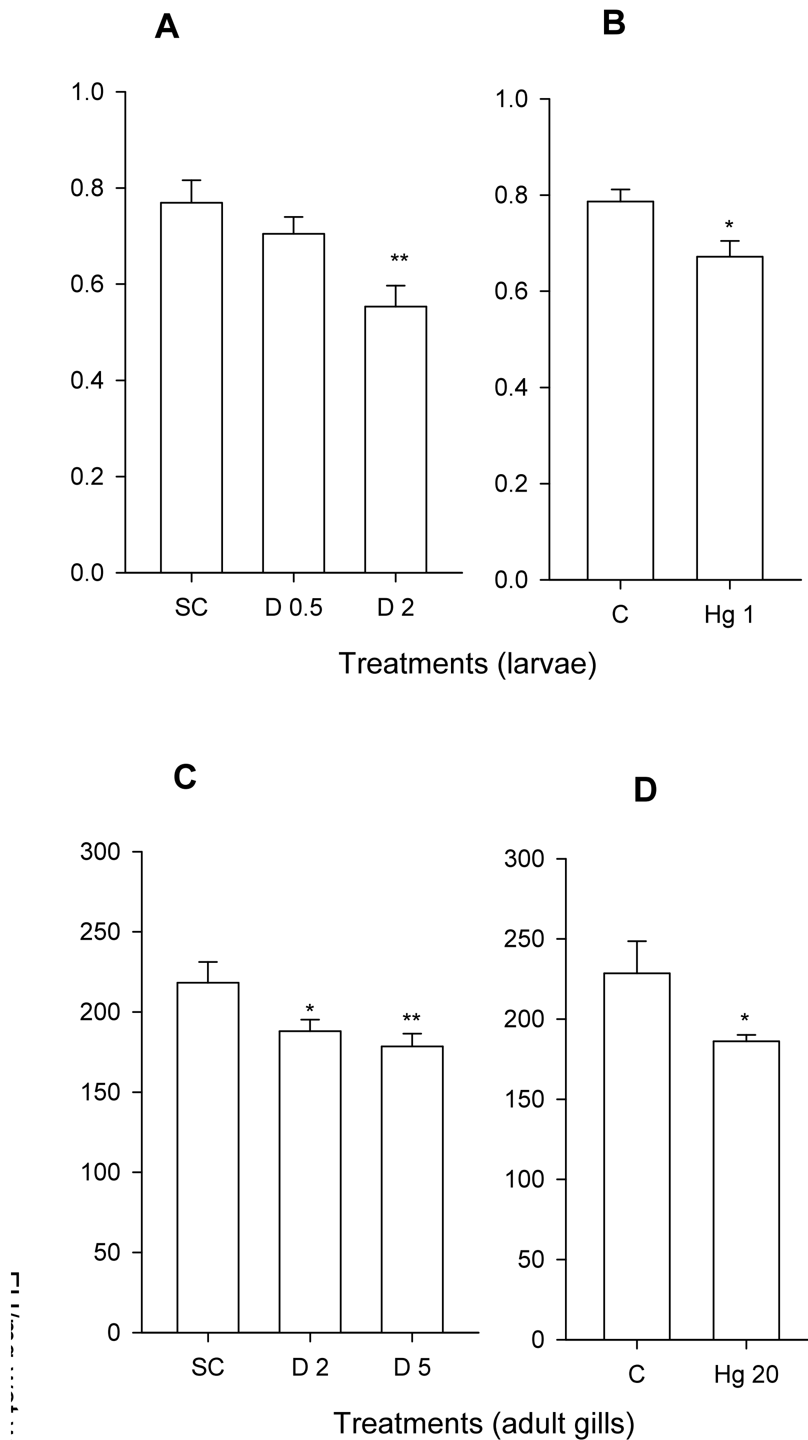

Fig A2.4. Basal transporter activity measured as fluorescence of calcein (mean ± SE, N=7) in whole larvae (A, B) and in gills of adult individuals (C, D) upon exposure to following ANOVA and Dunnet's post-hoc or Student's t tests. Dacthal and mercury treatments and their respective controls are depicted separately since they were from different experiments. dacthal and mercury. Single and double asterisks indicate significant (0.05<P<0.1; P<0.05, respectively) differences between pre-exposure treatments and controls

ANNEX 3

Chapter 7: Development of microarray for a non-model specie, Zebra mussel, to study the pathway of genes

A.3.1 Analysis Anova of microrrays values for the seasonal study of Dreissena polymorpha.

This table shows the complete list of significant probes that correspond to each cluster of genes from the figure 7,2 of Chapter 7. The grouped genes that were represented in the hierarchical cluster analysis created the clusters. Cluster 1 showed the transcripts over expressed during the months of February and June, Cluster 2 collect the transcripts over express during September and Cluster 3 the group of transcripts that were over expressed only in June.

	Gene	Acc. Num.	*p* Value
Cluster 1			
	N/A	EY436769	<0.001
	Hypothetical protein [Schistosoma mansoni]	EY435991	<0.001
	Hypothetical protein MELLADRAFT_115517 [Melampsora larici]	EY436738	<0.001
	WD repeat-containing protein 43 [Monodelphis domestica]	AJ517680	<0.001
	NADH dehydrogenase subunit 5 [Utterbackia peninsularis]	EY434995	0,0018
	N/A	AM230365	0,0026
	6a fibrillar collagen [Paracentrotus lividus]	EY435898	0,0069
	Putative period clock protein [Mytilus galloprovincialis]	AJ517673	<0.001
	pol-like protein [Ciona intestinalis]	EY435956	0,0083
	NADH dehydrogenase subunit 5 [Questa ersei]	EY435637	0,0041
	N/A	EY436688	<0.001
	Hypothetical protein OB0763 [Oceanobacillus iheyensis]	EY434582	0,0031
	Unnamed protein product [Oikopleura dioica]	EY434042	0,0017
	MAK16-like protein RBM13 [Esox lucius]	EY436793	0,0053
	Elongation factor 1-alpha [Pocillopora damicornis]	DN793469	<0.001
	N/A	EY434949	<0.001
	Aldehyde dehydrogenase family 9 member A1-A [Epinephelus coioides]	AJ517731	0,0051
	Unnamed protein product [Tetraodon nigroviridis]	EY436774	0,0031
	N/A	AM230313	0,0022
	splicing factor 3B subunit 2 [Danio rerio]	AJ517660	0,0042
	N/A	EY435457	0,0097
	Cu/Zn SOD [Venerupis sp]	AY377969	0,0022
	hypothetical protein GTHECHR2082 [Guillardia theta]	EY434915	0,0035
	threonyl-tRNA synthetase-like [Oryctolagus cuniculus]	EY434143	0,0016
	cytochrome c oxidase subunit I [Dreissena polymorpha]	EY435684	0,0032
	N/A	EY435900	0,0011
	39S ribosomal protein L37, mitochondrial precursor [Rattus norvegicus]	AJ517617	0,0035
	N/A	EY436474	0,0035

heat shock protein 70 [Meretrix meretrix]	EY436967	0,0081
Uncharacterized protein C1orf85-like protein [Camponotus floridanus]	AJ517711	0,0006
heat shock protein 70 [Laternula elliptica]	EY436108	0,0084
hypothetical protein [Plasmodium vivax]	AM230138	0,0044
endonuclease-reverse transcriptase [Schistosoma mansoni]	EY436020	0,0005
ribosomal protein [Mytilus galloprovincialis]	EY435029	0,0049
Elongation factor Tu GTP-binding domain [Xenopus tropicalis]	EY434712	0,0010
type II secretion system protein [Clostridium sp]	AJ517682	0,0055
ribosomal protein S8 [Haliotis diversicolor]	EY434224	0,0050
cytochrome c oxidase subunit I [Paphia euglypta]	EY435865	0,0058
heat shock protein 70 [Meretrix meretrix]	EY433824	0,0069
N/A	EY436635	0,0004
hypothetical protein PANDA_007074 [Ailuropoda melanoleuca]	EY436086	0,0049
unnamed protein product [Oncorhynchus mykiss]	EY434880	0,0055
Putative 40S ribosomal protein RPS2 [Novocrania anomala]	EY434559	0,0021
N/A	AJ517540	<0.001
N/A	EY434452	<0.001
hypothetical protein EAG_08928 [Camponotus floridanus]	EY435949	0,0081
hypothetical protein [Paramecium tetraurelia]	EY435796	0,0015
(Asp-Glu-Ala-Asp) box polypeptide 47-like [Saccoglossus kowalevskii]	EY433778	<0.001
putative ribosomal protein L35 [Sipunculus nudus]	EY434282	<0.001
Similar to nucleolar protein 10 isoform 1 [Strongylocentrotus purpuratus]	AJ517745	0,0017
ribosomal protein L3 [Chlamys farreri]	EY436930	0,0023
Dynein intermediate chain 2, ciliary [Harpegnathos saltator]	EY435669	<0.001
receptor of activated kinase C [Crassostrea angulata]	EY436190	0,0085
Hypothetical protein LOC100594049 [Nomascus leucogenys]	EY435741	0,0010
Ribosomal protein S2 [Homo sapiens]	EY436173	0,0046
MGC80147 protein [Xenopus laevis]	AJ517722	0,0023
cytochrome c oxidase subunit I [Dreissena polymorpha]	EY434023	0,0008
heat shock protein 70 [Meretrix meretrix]	EY433707	0,0080
Receptor of Activated Kinase C 1 [Mya arenaria]	EY434276	0,0035
Cyclin D mRNA [Dreissena polymorpha]	AF508219	0,0091
N/A	EY436069	0,0096
U3 small nucleolar ribonucleoprotein [Strongylocentrotus purpuratus]	EY433985	<0.001
39S ribosomal protein L38, mitochondrial [Apis mellifera]	EY436527	<0.001
hypothetical protein HS_0483 [Haemophilus somnus]	EY433894	0,0014
cytochrome b [Meretrix lusoria]	EY436639	<0.001
Eukaryotic translation initiation factor 2B[Saccoglossus kowalevskii]	EY435644	<0.001
NADH dehydrogenase subunit 5 [Nymphon gracile]	EY436722	0,0045
Methyltransferase 5 domain containing 1 [Mus musculus]	AJ517602	0,0023
component 1 Q subcomponent-binding protein [Callithrix jacchus]	EY436729	0,0010
putative accessory gland protein [Gryllus firmus]	EY433868	<0.001
CjCel9A mRNA for cellulase [Corbicula japonica]	AB264777	0,0024
16S ribosomal RNA gene, [Corbicula fluminea]	AF038999	0,0047
Alpha-tubulin mRNA [Dreissena polymorpha]	AF508224	0,0012

ribosomal protein l17 [Haliotis discus discus]	AJ517521	0,0051
hypothetical protein SORBIDRAFT_04g004730 [Sorghum bicolor]	AJ517529	0,0038
60S ribosomal protein L4 [Danio rerio]	AJ517564	0,0035
Sec61 alpha 1 subunit [Acyrthosiphon pisum]	AJ517578	0,0079
serine protease [Hyriopsis cumingii]	AJ517593	0,0021
similar to ribonuclease P/MRP 30kDa subunit [Ciona intestinalis]	AJ517594	0,0048
THO complex subunit 5 homolog [Meleagris gallopavo]	AJ517654	<0.001
Protein FAM32A [Harpegnathos saltator]	AJ517657	0,0024
similar to cytochrome c-1 isoform 3 [Canis familiaris]	AJ517658	0,0060
hypothetical protein [Thermobia domestica]	AJ517679	0,0021
N/A	AM229806	0,0095
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230045	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230047	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230048	<0.001
hypothetical protein DDB_G0288781 [Dictyostelium discoideum AX4]	AM230049	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230051	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230053	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230054	0,0017
Kielin/chordin-like protein-like [Xenopus tropicalis]	AM230055	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230056	<0.001
hypothetical protein BRAFLDRAFT_74063 [Branchiostoma floridae]	AM230057	<0.001
chordin-like 2 [Monodelphis domestica]	AM230059	<0.001
hypothetical protein [Plasmodium vivax]	AM230094	0,0040
Ubiquitin-protein ligase, putative [Candida dubliniensis]	AM230100	0,0011
cytochrome oxidase subunit 2 [Saissetia coffeae]	AM230126	0,0016
cytochrome oxidase subunit 3 [Nemertoderma westbladi]	AM230127	0,0016
dihydrofolate reductase-like [Anolis carolinensis]	AM230141	0,0020
60S ribosomal protein RPL26 [Phoronis muelleri]	AM230169	0,0019
Chordin-like 2 [Monodelphis domestica]	AM230184	<0.001
hypothetical protein [Oryza sativa Japonica]	AM230190	0,0010
hypothetical protein DDB_G0288781 [Dictyostelium discoideum]	AM230211	<0.001
chordin-like 2 [Monodelphis domestica]	AM230215	0,0017
hypothetical protein LOC734595 [Xenopus laevis]	AM230231	0,0035
hypothetical protein DDB_G0288781 [Dictyostelium discoideum]	AM230240	<0.001
hypothetical protein BRAFLDRAFT_122288 [Branchiostoma floridae]	AM230286	<0.001
N/A	AM230303	0,0017
hypothetical protein DDB_G0288781 [Dictyostelium discoideum]	AM230321	<0.001
type I restriction enzyme [Helicobacter pullorum	AM230334	0,0054
N/A	AM230375	0,0031
cytochrome oxidase III [Lasaea sp.]	AM230436	<0.001
hypothetical protein DDB_G0288781 [Dictyostelium discoideum]	AM230438	<0.001
predicted protein [Populus trichocarpa]	AM502279	<0.001

short-chain dehydrogenase/reductase SDR [Actinosynnema mirum]	AM502283	0,0021
Histone 3 H3[Corbicula sp]	AY070161	<0.001
histone 3 H3 [Dreissena p.]	AY070165	<0.001
histone H3 (H3) gene [Paphia vernicosa]	DQ184878	<0.001
histone H3 (H3) gene [Anomalocardia auberiana]	DQ184885	<0.001
histone H3 gene [Mercenaria mercenaria]	DQ280008	0,0015
histone H2B (H2B) gene [Venerupis decussatus]	EF670671	0,0055
conserved hypothetical protein, unlikely [Trypanosoma brucei]	EY433625	0,0031
hypothetical protein BRAFLDRAFT_124005 [Branchiostoma floridae]	EY433639	0,0066
hypothetical protein [Thermobia domestica]	EY433651	<0.001
beta tubulin [Aplysia californica]	EY433654	<0.001
ribosomal protein [Mytilus galloprovincialis]	EY433663	0,0037
NADH dehydrogenase subunit 1 [Paphia euglypta]	EY433696	<0.001
hypothetical protein DAPPUDRAFT_300560 [Daphnia pulex]	EY433708	0,0054
N/A	EY433724	0,0038
ribosomal protein rpl7a [Arenicola marina]	EY433733	0,0038
cytochrome c oxidase subunit II [Katharina tunicata]	EY433734	<0.001
Tetraspanin 39D-like protein [Daphnia pulex]	EY433757	0,0047
N/A	EY433771	0,0024
dihydrofolate reductase-like [Anolis carolinensis]	EY433849	0,0033
hypothetical protein [Thermobia domestica]	EY433852	0,0026
acetolactate synthase small subunit [Paracoccidioides brasiliensis]	EY433853	0,0070
hypothetical protein LOAG_03196 [Loa loa]	EY433908	<0.001
high mobility group 1 protein [Saccostrea kegaki]	EY433912	<0.001
beta-tubulin [Mesenchytraeus solifugus]	EY433919	0,0018
	U67347	0,0060
hypothetical protein [Cryptosporidium muris RN66]	EY433942	0,0034
peptidyl-prolyl cis-trans isomerase [Haliotis discus discus]	EY433997	0,0064
hypothetical protein LOC100443621, partial [Pongo abelii]	EY434008	0,0057
N/A	EY434009	0,0093
glutathione S-transferase [Danio rerio]	EY434057	0,0021
cytochrome oxidase subunit 1 and subunit 2 [Acanthamoeba castellanii]	EY434117	<0.001
hypothetical protein [Entamoeba dispar]	EY434147	0,0028
ACYPI007233 [Acyrthosiphon pisum]	EY434160	0,0012 0049
N/A	EY434183	0,0073
N/A	EY434185	<0.001
EF-hand calcium-binding protein [Pinctada fucata]	EY434190	0,0037
hypothetical protein [Saccoglossus kowalevskii]	EY434191	0,0037
ProFiliN family member (pfn-3)-like [Saccoglossus kowalevskii]	EY434215	<0.001
conserved hypothetical protein [Shewanella putrefaciens]	EY434220	<0.001
N/A	EY434226	0,0018
hypothetical protein [Thermobia domestica]	EY434230	0,0053
hypothetical protein BRAFLDRAFT_114927 [Branchiostoma floridae]	EY434240	0,0087
ribosomal protein rpl7a [Arenicola marina]	EY434279	0,0065
Zgc:55461 [Danio rerio]	EY434296	<0.001
hypothetical protein LOC100157567 [Sus scrofa]	EY434344	0,0036
hypothetical protein [Entamoeba dispar]	EY434349	0,0066

mitochondrial ATP synthase subunit 9 [Sinonovacula constricta]	EY434381	0,0065
hypothetical protein [Thermobia domestica]	EY434396	0,0005
NADH dehydrogenase subunit 5 [Toxolasma parvus]	EY434426	0,0024
THUMP domain containing 3 [Xenopus tropicalis]	EY434471	0,0032
N/A	EY434484	0,0010
cytochrome oxidase subunit 1 and subunit 2 [Acanthamoeba castellanii]	EY434554	0,0041
histone H2AV [Scylla paramamosain]	EY434565	<0.001
Ribosomal protein S2 [Chlamys farreri]	EY434579	0,0047
cytochrome c subunit II [Venerupis philippinarum]	EY434581	0,0048
expressed hypothetical protein-like [Saccoglossus kowalevskii]	EY434592	0,0022
cytochrome c oxidase subunit I [Meretrix petechialis]	EY434596	0,0039
hypothetical protein SNOG_04873 [Phaeosphaeria nodorum]	EY434600	0,0069
N/A	EY434639	0,0078
cytochrome c oxidase subunit I [Meretrix petechialis]	EY434650	0,0047
hypothetical protein [Thermobia domestica]	EY434682	0,0024
cytochrome oxidase subunit II [Nicrophorus guttula]	EY434684	0,0043
mitochondrial ATP synthase subunit 9 [Sinonovacula constricta]	EY434688	<0.001
hypothetical protein LOC100414382, partial [Callithrix jacchus]	EY434695	0,0021
N/A	EY434746	0,0071
N/A	EY434749	<0.001
N/A	EY434860	0,0084
hypothetical protein LOC100443621, partial [Pongo abelii]	EY434876	0,0026
cytochrome oxidase subunit 3 [Nemertoderma westbladi]	EY434904	0,0025
N/A	EY434936	0,0042
cytochrome c oxidase subunit I [Dreissena polymorpha]	EY434941	0,0044
putative 40S ribosomal protein RPS2 [Novocrania anomala]	EY434964	0,0083
hypothetical protein [Thermobia domestica]	EY434979	0,0075
predicted protein [Nematostella vectensis]	EY435065	<0.001
tubulin, beta 2B [Danio rerio]	EY435105	<0.001
WD40-like domain-containing protein [Dictyostelium discoideum AX4]	EY435123	0,0090
N/A	EY435177	0,0067
tubulin, beta 2B [Danio rerio]	EY435184	0,0012
growth arrest-specific 8-like [Saccoglossus kowalevskii]	EY435195	0,0013
SecY-independent transporter protein [Chrysodidymus synuroideus]	EY435200	<0.001
NADH dehydrogenase subunit 1 [Paphia euglypta]	EY435219	0,0012
N/A	EY435278	0,0039
NADH dehydrogenase subunit 4 [Meretrix petechialis]	EY435315	0,0096
cAMP-regulated phosphoprotein [Taeniopygia guttata]	EY435373	<0.001
Hypothetical protein SORBIDRAFT_01g011950 [Sorghum bicolor]	EY435407	0,0034
predicted protein-like [Saccoglossus kowalevskii]	EY435444	0,0077
hypothetical protein [Strongylocentrotus purpuratus]	EY435448	0,0019
N/A	EY435516	0,0060
NADH dehydrogenase subunit 4 [Meretrix lusoria]	EY435565	0,0022
N/A	EY435591	0,0082
N/A	EY435622	0,0099
hypothetical protein [Thermobia domestica]	EY435643	0,0006

Cytochrome c oxidase subunit I [Paphia euglypta]	EY435656	0,0064
N/A	EY435680	0,0044
hypothetical protein [Thermobia domestica]	EY435739	0,0026
DEAD/H box 56 RNA helicase/noh61 [Strongylocentrotus purpuratus]	EY435750	0,0039
flagelliform silk protein, putative [Ixodes scapularis]	EY435754	0,0092
tetratricopeptide repeat domain 33-like [Saccoglossus kowalevskii]	EY435798	0,0003
mitochondrial ATP synthase B subunit [Clonorchis sinensis]	EY435851	0,0037
N/A	EY435869	0,0048
epidermin response regulator [Staphylococcus epidermidis]	EY435886	0,0085
hypothetical protein [Strongylocentrotus purpuratus]	EY435929	0,0075
alpha tubulin [Schistosoma mansoni]	EY435947	0,0015
predicted protein [Nematostella vectensis]	EY435966	0,0039
hypothetical protein BRAFLDRAFT_206139 [Branchiostoma floridae]	EY435977	0,0038
ribosomal protein S3a [Crassostrea gigas]	EY436003	0,0048
hypothetical protein LOC100414382, partial [Callithrix jacchus]	EY436035	0,0005
ATP synthase a chain [Culex quinquefasciatus]	EY436079	0,0080
Transmembrane and TPR repeat-containing protein 4 [Harpegnathos saltator]	EY436080	0,0087
putative tubulin beta chain variant 1 [Taeniopygia guttata]	EY436097	0,0047
hypothetical protein [Thermobia domestica]	EY436102	<0.001
N/A	EY436114	0,0020
hypothetical protein DAPPUDRAFT_213372 [Daphnia pulex]	EY436116	0,0006
cytochrome c oxidase subunit II [Scoloplos cf. Armiger]	EY436119	0,0098
ATP synthase alpha chain, mitochondrial precursor [Brugia malayi]	EY436121	0,0047
hypothetical protein [Thermobia domestica]	EY436128	0,0041
Ribosomal protein S2 [Homo sapiens]	EY436147	0,0085
cytochrome c oxidase subunit Va [Nasonia vitripennis]	EY436250	0,0061
EF hand family protein [Trichomonas vaginalis G3]	EY436256	0,0029
ATP synthase alpha chain, mitochondrial precursor [Brugia malayi]	EY436263	0,0006
unnamed protein product [Vitis vinifera]	EY436294	0,0097
trafficking protein particle complex subunit 3 [Gallus gallus]	EY436304	0,0085
N/A	EY436313	0,0034
putative intron-encoded protein [Chaetosphaeridium globosum]	EY436315	0,0045
Ubiquitin-conjugating enzyme E2 L3 [Lepeophtheirus salmonis]	EY436322	0,0044
N/A	EY436326	0,0083
N/A	EY436393	0,0037
cytochrome c oxidase subunit I [Paphia euglypta]	EY436408	0,0016
hypothetical protein [Entamoeba dispar]	EY436426	0,0021
hypothetical protein, partial [Strongylocentrotus purpuratus]	EY436433	0,0008
mitochondrial ATP synthase F chain [Culex quinquefasciatus]	EY436441	0,0036
Conserved Plasmodium protein [Plasmodium falciparum]	EY436468	0,0077
hypothetical protein [Thermobia domestica]	EY436515	0,0065
cytochrome c oxidase subunit IV [Urechis caupo]	EY436535	0,0072
hypothetical protein [Thermobia domestica]	EY436540	0,0008
predicted protein [Nematostella vectensis]	EY436541	0,0035

putative intron-encoded protein [Chaetosphaeridium globosum]	EY436544	0,0041
hypothetical protein LOC100191710 [Zea mays]	EY436551	0,0069
ubiquitin-conjugating enzyme E2L	EY436552	0,0005
ADP,ATP carrier protein 3 [Lepeophtheirus salmonis]	EY436561	0,0083
complement factor B precursor [Nematostella vectensis]	EY436568	0,0035
unknown [Picea sitchensis]	EY436576	0,0050
hypothetical protein [Thermobia domestica]	EY436579	0,0038
N/A	EY436586	0,001004346
hypothetical protein TTHERM_00564180 [Tetrahymena thermophila]	EY436587	0,0047
N/A	EY436592	0,0004
NADH dehydrogenase subunit 2 [Sinonovacula constricta]	EY436596	0,0006
ribosomal protein L3 [Chlamys farreri]	EY436638	0,0078
Cytochrome oxidase III [Lasaea sp.]	EY436643	0,0012
Cytochrome oxidase subunit I [Paphia undulata]	EY436680	0,0035
N/A	EY436701	0,0089
ubiquitin-conjugating enzyme E2L [Bombyx mori]	EY436714	<0.001
LOC562370 protein [Danio rerio]	EY436740	0,0063
NADH dehydrogenase subunit 4 [Meretrix lusoria]	EY436771	0,0028
hypothetical protein [Thermobia domestica]	EY436856	0,0070
hypothetical protein [Thermobia domestica]	EY436910	0,0015
Ribosomal protein S2 [Homo sapiens]	EY436945	0,0084
conserved hypothetical protein [Prevotella buccae]	EY436960	0,0054
ribosomal protein S10 [Crassostrea gigas]	EY436961	0,0061
Predicted protein [Nematostella vectensis]	EY436977	0,0010
hypothetical protein LOC100414382, partial [Callithrix jacchus]	EY436980	0,0017
28S ribosomal protein S26, mitochondrial [Harpegnathos saltator]	EY436986	0,002475783
hypothetical protein GlnoM_p14 [Glaucocystis nostochinearum]	EY436993	<0.001
cytochrome c oxidase subunit II	EY437032	0,0062
cubilin-like	EY437038	0,0052
hypothetical protein LOC100414382, partial	EY437040	0,0013
cytochrome c oxidase subunit 2	EY437043	0,0064
cytoplasmic actin mRNA [Dreissena polymorpha]	AF082863	<0.001
DMC1 mRNA [Dreissena polymorpha]	AF508222	<0.001
alpha tubulin [Schistosoma mansoni]	EY435297	0,0049
predicted protein [Nematostella vectensis]	EY434011	0,0021
hypothetical protein DFA_07668 [Dictyostelium fasciculatum]	AJ517676	0,0020
N/A	AM230373	<0.001
ADP-ribosylation factor-like protein 3-like [Nomascus leucogenys]	AM230117	0,0085
chordin-like 2 [Monodelphis domestica]	AM230311	<0.001
Hypothetical protein HPF16_1436 [Helicobacter pylori]	EY436311	<0.001
Hypothetical protein lmo0070 [Listeria monocytogenes]	EY434439	<0.001
Cluster 2		
N/A	AM230362	0,0035
SH3 domain protein [Klebsiella pneumoniae]	AJ517619	0,0031
Phosphoenolpyruvate carboxykinase [Crassostrea gigas]	EY434568	<0.001
Phosphoenolpyruvate carboxykinase [Crassostrea gigas]	EY435953	<0.001
Probable E3 ubiquitin-protein ligase MYCBP2 [Xenopus tropicalis]	EY436329	0,0093

argininosuccinate synthase [Danio rerio]	EY435891	0,0097
C2H2 type zinc finger containing protein [Coccidioides posadasii]	EY436699	0,0020
N/A	EY434128	0,0061
Unnamed protein product [Homo sapiens]	AJ517581	0,0045
NADH dehydrogenase subunit 2 [Meretrix lusoria]	EY436008	0,0095
Cytochrome b-245 heavy chain-like [Monodelphis domestica]	EY436817	<0.001
Hypothetical protein TcasGA2_TC008668 [Tribolium castaneum]	EY43477	0,0031
N/A	EY436659	0,0007
AraC family transcriptional regulator [Paenibacillus sp.]	EY435190	0,0055
Ymf77 [Tetrahymena pigmentosa	EY436213	0,0081
N/A	EY435772	0,0037
predicted protein [Mycobacterium tuberculosis]	EY435968	0,0035
hypothetical protein Bfra3_04746 [Bacteroides fragilis]	AM230102	<0.001
phage terminase-like protein [Candidatus Hamiltonella]	EY434882	0,0066
60S ribosomal protein L18a-like [Monodelphis domestica]	EY435356	0,0090
N/A	EY433829	0,0034
ribosomal protein L3 [Chlamys farreri]	EY434034	0,0084
hypothetical protein ELI_3856 [Eubacterium limosum]	EY436611	<0.001
N/A	EY436602	<0.001
N/A	EY434210	0,0042
N/A	EY434193	<0.001
GJ22804 [Drosophila virilis]	AM230333	0,0044
unnamed protein product [Macaca fascicularis]	EY436413	0,0027
pol-like protein [Ciona intestinalis]	EY436742	0,0017
Translation elongation factor 1-alpha [Dreissena polymorpha]	AJ250733	0,0018
E6 protein [Phocoena spinipinnis papillomavirus]	AJ517518	0,0018
similar to ficolin 2 [Ciona intestinalis]	AJ517534	0,0043
Cathepsin L2 cysteine protease [Pinctada fucata]	AJ517568	0,0001
outer membrane adhesin like protein [Pseudomonas mendocina	AJ517576	0,0083
Excision repair protein Rad15, putative [Toxoplasma gondii]	AJ517583	0,0003
N/A	AJ517585	0,0011
N/A	AJ517591	<0.001
hypothetical protein BRAFLDRAFT_87227 [Branchiostoma floridae]	AJ517609	0,0052
Nose Resistant to Fluoxetine family member (nrf-6)-like [Saccoglossus kowalevskii]	AJ517613	0,0008
GF14635 [Drosophila ananassae]	AJ517614	0,0033
CG4523 [Drosophila yakuba]	AJ517621	0,0036
Proactivator polypeptide [Acromyrmex echinatior]	AJ517622	<0.001
hypothetical protein EAI_09853 [Harpegnathos saltator]	AJ517631	<0.001
cytoplasmic actin [Dreissena polymorpha]	AJ517647	0,0029
chloramphenicol acetyltransferase [Promoter probe vector pEvoGlowRed]	AJ517667	0,0084
ferric-chelate reductase, putative [Ricinus communis]	AJ517685	<0.001
hypothetical protein [Podospora anserina S mat+]	AJ517699	0,0011
hypothetical protein [Tuber melanosporum Mel28]	AJ517705	0,0095
neural precursor cell expressed, developmentally down-regulated [Ectocarpus siliculosus]	AJ517714	0,0098
N/A	AJ517716	0,0062
CRE-LNTL-1 protein [Caenorhabditis remanei	AJ517723	0,0018
N/A	AM229771	0,0037

Myc homolog [Crassostrea virginica]	AM230116	0,0041
hypothetical protein Krac_11050 [Ktedonobacter racemifer]	AM230155	0,0038
N/A	AM230200	0,0028
MHC class II alpha chain [Oncorhynchus mykiss]	AM230238	0,0051
hypothetical protein MCJ_006000 [Mycoplasma conjunctivae]	AM230245	0,0088
glucosyl transferase [Staphylococcus saprophyticus	AM230275	0,0042
hypothetical protein EAG_03672 [Camponotus floridanus]	AM230290	0,0041
N/A	AM230305	0,0018
unknown [Picea sitchensis]	AM230307	0,0066
similar to mKIAA0708 protein [Taeniopygia guttata]	AM230316	<0.001
hypothetical protein EAI_02435 [Harpegnathos saltator]	AM230371	<0.001
hypothetical protein BRAFLDRAFT_67985 [Branchiostoma floridae]	AM230394	0,0096
hypothetical protein CLONEX_03036 [Clostridium nexile]	AM230409	0,0014
carbohydrate binding protein [Agrobacterium vitis	AM230418	0,0026
hypothetical protein PGTG_07232 [Puccinia graminis]	AM230425	0,0028
ubiquitin/actin fusion protein 3 [Lotharella globosa]	AM503944	0,0049
collagen type XXI-like protein [Haliotis diversicolor]	AM503947	0,0049
retinoid X receptor mRNA [Mytilus galloprovincialis]	EF644351.1	<0.001
ferritin subunit [Meretrix meretrix]	EY433633	<0.001
ribosomal protein l [Haliotis discus discus]	EY433645	0,0047
	EY435752	0,0023
hypothetical protein RUMHYD_03590 [Blautia hydrogenotrophica]	EY433652	0,0031
Complement factor B-like protein [Ruditapes decussatus]	EY433655	0,0013
N/A	EY433657	0,0018
predicted protein [Nematostella vectensis]	EY433677	0,0068
cytoplasmic actin [Dreissena polymorpha]	EY433678	<0.001
N/A	EY433694	0,0017
N/A	EY433703	<0.001
hypothetical protein [Thermobia domestica]	EY433706	<0.001
N/A	EY433714	0,0024
cathepsin L, putative [Ixodes scapularis]	EY433754	<0.001
N/A	EY433784	0,0094
rCG27216, isoform CRA_a [Rattus norvegicus]	EY433808	0,0096
N/A	EY433812	0,0038
N/A	EY433820	0,0076
cyclohexa-1,5-dienecarbonyl-CoA hydratase [Rhodomicrobium vannielii]	EY433825	0,0062
predicted protein [Nematostella vectensis]	EY433838	0,0087
39S ribosomal protein L53, mitochondrial [Danio rerio]	EY433848	0,0075
translation elongation factor 1-alpha [Dreissena polymorpha]	EY433850	0,0051
unnamed protein product [Vitis vinifera]	EY433854	0,0065
N/A	EY433859	0,0017
hypothetical protein Mbar_A1908 [Methanosarcina barkeri]	EY433863	0,0081
predicted protein [Nematostella vectensis]	EY433886	0,0071
N/A	EY433911	0,0085
unnamed protein product [Tetraodon nigroviridis]	EY433950	0,0024
hypothetical protein LOC100612796 [Pan troglodytes]	EY433957	0,0011
ribosomal protein rpl8 [Eurythoe complanata]	EY434010	0,0041
Amidohydrolase family [Thermomicrobium roseum	EY434015	0,0053
ferritin subunit [Ruditapes philippinarum]	EY434067	0,0078

N/A	EY434068	<0.001
N/A	EY434081	0,0013
translation elongation factor 1-alpha [Dreissena polymorpha]	EY434089	0,0057
hypothetical protein ARALYDRAFT_493940 [Arabidopsis lyrata subsp. lyrata]	EY434092	<0.001
N/A	EY434097	<0.001
N/A	EY434102	0,0030
N/A	EY434108	0,0064
lysozyme murein hydrolase [Enterobacteria phage]	EY434125	0,0017
N/A	EY434133	0,0073
beta actin [Hippoglossus hippoglossus]	EY434149	0,0051
hypothetical protein LOC100497909 [Xenopus tropicalis]	EY434162	<0.001
N/A	EY434165	0,0018
ferritin subunit [Meretrix meretrix]	EY434167	0,0034
	EY434169	0,0013
conserved hypothetical protein [Culex quinquefasciatus]	EY434209	<0.001
N/A	EY434212	0,0035
Actin [Ciona intestinalis]	EY434236	0,0071
N/A	EY434251	0,0039
ferritin [Sinonovacula constricta]	EY434261	0,0022
plakophilin-2 [Monodelphis domestica]	EY434287	<0.001
N/A	EY434288	0,0041
hypothetical protein CLOHIR_00220 [Clostridium hiranonis]	EY434289	<0.001
Tumor necrosis factor-like protein [Dreissena rostriformis bugensis]	EY434297	<0.001
hypothetical protein LOC100608502, partial [Pan troglodytes	EY434308	0,0012
N/A	EY434321	0,0021
predicted protein [Naegleria gruberi]	EY434325	0,0026
N/A	EY434329	0,0075
2OG-Fe(II) oxygenase [Cyanothece sp.]	EY434350	0,0011
GL14865 [Drosophila persimilis]	EY434357	<0.001
kelch-like protein 35-like [Anolis carolinensis]	EY434379	0,0032
SJCHGC00527 protein [Schistosoma japonicum]	EY434397	<0.001
cytoplasmic actin [Dreissena polymorpha]	EY434419	0,0052
N/A	EY434425	0,0097
Metallothionein [Dreissena polymorpha]	EY434440	0,0066
N/A	EY434460	0,0016
N/A	EY434468	0,0018
N/A	EY434469	0,0100
hypothetical protein BRAFLDRAFT_247809 [Branchiostoma floridae]	EY434472	0,0044
N/A	EY434513	0,0084
similar to BEL12_AG transposon polyprotein [Nasonia vitripennis]	EY434515	0,0059
novel protein similar to vertebrate furin [Danio rerio]	EY434519	0,0038
hypothetical protein LOC100493167 [Xenopus tropicalis]	EY434521	0,0045
cytoplasmic actin [Dreissena polymorpha]	EY434522	0,0014
hypothetical protein DAPPUDRAFT_230342 [Daphnia pulex]	EY434545	0,0003
predicted protein [Nematostella vectensis]	EY434546	0,0029
gustatory receptor candidate 19 [Tribolium castaneum]	EY434550	0,0078
hypothetical protein [Plasmodium berghei]	EY434552	0,0006
major facilitator superfamily MFS_1 [Clostridium	EY434566	0,0097

lentocellum]		
DEAD/DEAH box helicase domain protein [Treponema brennaborense]	EY434569	<0.001
hypothetical protein [Lepeophtheirus salmonis]	EY434587	0,0056
RecQ helicase [Heliconius erato]	EY434597	<0.001
regulator of polyketide synthase expression [Thermoanaerobacter tengcongensis]	EY434598	0,0037
ferritin subunit [Meretrix meretrix]	EY434599	0,0098
collagen alpha-1(XIV) chain-like [Danio rerio]	EY434608	0,0055
N/A	EY434615	0,0085
outer dense fiber protein 3 [Xenopus laevis]	EY434617	0,0006
hypothetical protein [Plasmodium yoelii yoelii]	EY434618	0,0041
N/A	EY434619	0,0070
N/A	EY434627	0,0020
predicted protein [Nematostella vectensis]	EY434654	0,0059
hypothetical protein [Monosiga brevicollis]	EY434662	0,0090
unnamed protein product [Mus musculus]	EY434666	0,0036
N/A	EY434673	0,0040
zinc finger protein 484 isoform 1 [Xenopus tropicalis]	EY434679	0,0053
hypothetical protein ACP_1496 [Acidobacterium capsulatum]	EY434709	0,0015
cytochrome P450 30 [Mercenaria mercenaria]	EY434718	0,0019
similar to Activated in blocked unfolded protein response protein 2 [Gallus gallus]	EY434780	<0.001
conserved hypothetical protein [Fusobacterium sp.]	EY434782	0,0012
similar to Phosphoenolpyruvate carboxykinase 2 [Strongylocentrotus purpuratus]	EY434797	<0.001
ORF65a [Sulfolobus islandicus]	EY434809	0,0016
predicted protein [Physcomitrella patens]	EY434830	0,0098
N/A	EY434836	0,0045
N/A	EY434839	0,0016
similar to translational activator [Arabidopsis thaliana]	EY434849	0,0014
N/A	EY434869	0,0058
BUD13 homolog [Sus scrofa]	EY434870	<0.001
oxidoreductase, short chain dehydrogenase/reductase family [Paenibacillus polymyxa]	EY434889	<0.001
similar to tubulin, alpha 1 isoform 2 [Canis familiaris]	EY434896	0,0013
phage terminase-like protein [Candidatus Hamiltonella]	EY434899	0,0049
N/A	EY434905	<0.001
hypothetical protein [Paramecium tetraurelia]	EY434917	0,0036
predicted protein [Nematostella vectensis]	EY434956	0,0035
phosphoglycerate mutase 1 family protein [Bacillus cereus]	EY434959	<0.001
N/A	EY434989	<0.001
Fibropellin-1 [Camponotus floridanus]	EY434991	0,0079
translation elongation factor 1-alpha [Dreissena polymorpha]	EY434992	0,0028
predicted protein-like [Saccoglossus kowalevskii]	EY435003	<0.001
unnamed protein product [Vitis vinifera]	EY434759	0,0020
N/A	EY435036	<0.001
ferritin [Branchiostoma belcheri tsingtauense]	EY435056	0,0020
hypothetical protein, conserved in Plasmodium species [Plasmodium knowlesi]	EY435060	0,0028
hypothetical protein CPR_0130 [Clostridium perfringens]	EY435063	<0.001
hypothetical protein BRAFLDRAFT_69334 [Branchiostoma floridae]	EY435071	0,0029

hypothetical protein NEMVEDRAFT_v1g176499 [Nematostella vectensis]	EY435073	0,0014
similar to gelsolin [Strongylocentrotus purpuratus]	EY435075	0,0077
Calmodulin [Pinctada fucata]	EY435085	<0.001
Tubulin alpha chain [Ascaris suum]	EY435090	<0.001
N/A	EY435103	0,0055
conserved hypothetical protein [Ajellomyces capsulatus H88]	EY435106	0,0024
similar to tubulin, alpha 1 isoform 3 [Canis familiaris]	EY435115	0,0014
TPR Domain containing protein [Trichomonas vaginalis G3]	EY435121	0,0031
ATPase subunit 6 [Trypanosoma cruzi]	EY435126	0,0076
NLRC3-like [Danio rerio]	EY435133	0,0053
	EY435134	0,0049
heat shock protein 90 [Laternula elliptica]	EY435137	0,0093
hypothetical protein NEMVEDRAFT_v1g155648 [Nematostella vectensis]	EY435164	0,0060
N/A	EY435174	0,0068
N/A	EY435178	0,0013
ribosomal protein L5 [Argopecten irradians]	EY435180	0,0068
N/A	EY435191	<0.001
hypothetical protein, partial [Ornithorhynchus anatinus]	EY435192	0,0020
N/A	EY435209	0,0008
cAMP-dependent protein kinase catalytic subunit, putative [Pediculus humanus corporis]	EY435220	0,0029
hypothetical protein LOC100454926, partial [Pongo abelii]	EY435222	0,0057
tribbles homolog 2 [Danio rerio]	EY435226	0,0025
cytoplasmic actin [Dreissena polymorpha]	EY435231	0,0027
Branched chain amino acid ABC transporter, permease[Bacillus cereus]	EY435271	0,0050
unnamed protein product [Tetraodon nigroviridis]	EY435303	0,0029
N/A	EY435338	0,0018
similar to predicted protein [Hydra magnipapillata]	EY435355	0,0032
hypothetical protein DAPPUDRAFT_48614 [Daphnia pulex]	EY435358	0,0095
membrane protein [Comamonas testosteroni]	EY435361	0,0047
hypothetical protein [Entamoeba dispar]	EY435362	<0.001
N/A	EY435375	0,0056
cysteine/glutathione ABC transporter membrane/ATP-binding component [Salmonella enterica]	EY435385	0,0058
predicted protein [Nematostella vectensis]	EY435387	0,0077
hypothetical protein [Plasmodium chabaudi chabaudi]	EY435404	0,0005
predicted protein [Nematostella vectensis]	EY435428	0,0030
ferritin subunit [Meretrix meretrix	EY435437	0,0014
hypothetical protein DICPUDRAFT_83909 [Dictyostelium purpureum]	EY435438	0,0033
similar to RAS-like family 11 member A [Gallus gallus]	EY435450	<0.001
ribosomal protein L5 [Crassostrea gigas]	EY435454	0,0086
hypothetical protein [Thermobia domestica]	EY435455	0,0060
cytochrome b [Polyrhachis proxima]	EY435461	0,0072
predicted protein [Nematostella vectensis]	EY435469	0,0015
conserved hypothetical protein [Fusobacterium sp.]	EY435476	0,0041
tubulin A [Hyriopsis schlegelii]	EY435488	0,0055
conserved unknown protein [Ectocarpus siliculosus]	EY435492	<0.001
hypothetical protein PSYMP_21861 [Pseudomonas syringae]	EY435497	0,0017
hypothetical protein TcasGA2_TC006367 [Tribolium	EY435524	0,0015

castaneum]		
phosphoenolpyruvate carboxykinase [Acanthopagrus schlegelii]	EY435531	0,0034
inhibitor of apoptosis 2 [Bombyx mori]	EY435543	0,0053
hypothetical protein BRAFLDRAFT_84801 [Branchiostoma floridae]	EY435556	0,0027
N/A	EY435562	0,0020
hypothetical protein BRAFLDRAFT_67520 [Branchiostoma floridae]	EY435567	<0.001
conserved hypothetical protein [Pediculus humanus corporis]	EY435573	0,0029
predicted protein [Nematostella vectensis]	EY435594	0,0010
similar to Tubulin polymerization-promoting protein [Ciona intestinalis]	EY435617	0,0011
alpha tubulin [Schistosoma mansoni]	EY435623	<0.001
hypothetical protein [Thermobia domestica]	EY435626	<0.001
hypothetical protein BRAFLDRAFT_123768 [Branchiostoma floridae]	EY435628	0,0044
N/A	EY435630	<0.001
N/A	EY435645	<0.001
similar to carotene-9,10-monooxygenase [Taeniopygia guttata]	EY435653	0,0083
N/A	EY435674	0,0014
N/A	EY435705	0,0013
Hypothetical protein [Ornithorhynchus anatinus]	EY435706	<0.001
DEHA2G13640p [Debaryomyces hansenii]	EY435718	0,0090
N/A	EY435722	<0.001
N/A	EY435724	<0.001
translation elongation factor 1-alpha [Dreissena polymorpha]	EY435766	0,0075
cytosolic malate dehydrogenase [Mytilus galloprovincialis]	EY435792	0,0036
N/A	EY435799	0,0028
putative polysaccharide polymerase [beta proteobacterium]	EY435826	0,0059
ficolin-1-like [Xenopus tropicalis]	EY435827	<0.001
similar to Cathepsin L [Hydra magnipapillata]	EY435831	<0.001
beta-actin [Canis lupus familiaris]	EY435835	0,0036
similar to tetraspanin [Nasonia vitripennis]	EY435836	0,0012
Tribbles homolog 2-like [Saccoglossus kowalevskii]	EY435861	0,0020
predicted protein-like [Saccoglossus kowalevskii]	EY435863	0,0084
putative threonine synthase [Treponema vincentii]	EY435879	0,0021
hypothetical protein CRE_19542 [Caenorhabditis remanei]	EY435880	0,0029
cathepsin C [Homo sapiens]	EY435894	0,0028
N/A	EY435932	0,0015
CG8568-like [Saccoglossus kowalevskii]	EY435933	<0.001
N/A	EY435934	0,0071
translation elongation factor 1-alpha [Dreissena polymorpha	EY435937	0,0046
heat shock protein 90 [Laternula elliptica]	EY435944	<0.001
similar to reverse transcriptase-like [Strongylocentrotus purpuratus]	EY435950	0,0036
Chromobox protein homolog 7 [Dicentrarchus labrax]	EY435962	<0.001
translation elongation factor 1-alpha [Dreissena polymorpha]	EY435964	0,0096
Predicted protein [Nematostella vectensis]	EY435973	0,0014
alpha tubulin [Schistosoma mansoni]	EY435983	<0.001
similar to notch homolog [Strongylocentrotus purpuratus]	EY435993	0,0056

hypothetical protein Mbar_A1908 [Methanosarcina barkeri]	EY435998	0,0015
serine/threonine-protein kinase DCLK1 isoform 3 [Mus musculus]	EY436012	0,0074
GD18419 [Drosophila simulans]	EY436014	<0.001
glycyl-tRNA synthetase [Bacillus sp.	EY436022	0,0083
S protein [Hepatitis B virus]	EY436038	0,0026
ABC transporter permease component [Clostridium botulinum]	EY436047	<0.001
N/A	EY436052	<0.001
Predicted protein [Nematostella vectensis]	EY436070	0,0019
conserved Plasmodium membrane protein [Plasmodium falciparum]	EY436081	0,0043
Hypothetical protein BRAFLDRAFT_120859 [Branchiostoma floridae]	EY436084	0,0170
N/A	EY436089	0,0050
cytoplasmic actin [Dreissena polymorpha]	EY436166	0,0020
TPR repeat-containing protein [Acaryochloris marina MBIC11017]	EY436186	0,0044
N/A	EY436189	0,0019
N/A	EY436198	0,0045
hypothetical protein BRAFLDRAFT_97108 [Branchiostoma floridae]	EY436210	<0.001
N/A	EY436211	<0.001
N/A	EY436221	0,0035
hypothetical protein BRAFLDRAFT_234817 [Branchiostoma floridae]	EY436225	0,0012
hemolysin-type calcium-binding protein [Rhodobacteraceae bacterium]	EY436230	<0.001
hypothetical protein HMPREF0659_A5501 [Prevotella melaninogenica	EY436238	<0.001
N/A	EY436273	0,0028
beta-thymosin [Sycon raphanus]	EY436288	<0.001
hypothetical protein stu0550 [Streptococcus thermophilus]	EY436296	0,0031
hypothetical protein BRAFLDRAFT_71485 [Branchiostoma floridae]	EY436298	0,0014
hypothetical protein [Thermobia domestica]	EY436325	0,0084
hypothetical protein PANDA_021802 [Ailuropoda melanoleuca]	EY436328	0,0019
attachment glycoprotein [Human respiratory syncytial virus]	EY436361	<0.001
beta-galactosidase/beta-glucuronidase [Xanthomonas vesicatoria]	EY436386	0,0082
hypothetical protein [Monosiga brevicollis]	EY436398	0,0025
predicted Bcl-2 protein-like [Saccoglossus kowalevskii]	EY436399	0,0020
Fe2+/Zn2+ uptake regulation proteins [Pelotomaculum thermopropionicum]	EY436414	<0.001
similar to tubulin, alpha 1 isoform 3 [Canis familiaris]	EY436418	0,0071
ribonuclease BN [Nautilia profundicola]	EY436423	0,0035
hypothetical protein [Thermobia domestica]	EY436472	0,0061
GJ16369 [Drosophila virilis]	EY436486	<0.001
N/A	EY436492	0,0026
beta-thymosin [Sycon raphanus]	EY436502	<0.001
N/A	EY436507	0,0047
hypothetical protein LOC100573992 [Acyrthosiphon pisum]	EY436512	0,0064
predicted protein-like [Saccoglossus kowalevskii]	EY436516	0,0022
Hypothetical protein [Theileria annulata]	EY436530	<0.001
hypothetical protein HFELIS_16070 [Helicobacter felis]	EY436556	0,0048

hypothetical protein PANDA_021802 [Ailuropoda melanoleuca]	EY436557	<0.001
probable E3 ubiquitin-protein ligase MYCBP2 [Rattus norvegicus]	EY436558	0,0051
calmodulin [Pinctada fucata]	EY436595	<0.001
ferritin [Mytilus chilensis]	EY436606	0,0057
cytoplasmic actin [Dreissena polymorpha]	EY436617	0,0020
selenoprotein K [Ciona intestinalis]	EY436627	0,0091
N/A	EY436628	0,0071
ABC transporter, ATP-binding protein [Bacillus thuringiensis	EY436647	<0.001
conserved Plasmodium protein [Plasmodium falciparum]	EY436653	<0.001
60S ribosomal protein L13A [Mytilus edulis]	EY436661	0,0044
N/A	EY436691	0,0033
N/A	EY436692	0,0032
cytoplasmic actin [Dreissena polymorpha]	EY436696	0,0025
similar to alpha kinase [Hydra magnipapillata]	EY436698	0,0045
ferritin subunit [Meretrix meretrix]	EY436700	<0.001
hypothetical protein CHLNCDRAFT_56640 [Chlorella variabilis]	EY436713	0,0061
Chromobox protein homolog 7 [Dicentrarchus labrax]	EY436747	0,0070
NADH dehydrogenase subunit 4 [Tetrahymena pigmentosa]	EY436756	0,0011
inositol-3-phosphate synthase A [Capsaspora owczarzaki]	EY436759	0,0185
hypothetical protein [Brugia malayi]	EY436763	<0.001
N/A	EY436775	0,0018
tubulin alpha-3C/D chain-like [Nomascus leucogenys]	EY436778	0,0062
ADP,ATP carrier protein 3 [Lepeophtheirus salmonis]	EY436790	0,0067
Ferritin subunit [Meretrix meretrix]	EY436791	<0.001
cyclin-dependent kinase-like [Xenopus tropicalis]	EY436795	0,0071
conserved hypothetical protein [Ixodes scapularis]	EY436801	<0.001
similar to GA10554-PA [Strongylocentrotus purpuratus]	EY436805	0,0017
hypothetical protein CLOHIR_00220 [Clostridium hiranonis]	EY436806	<0.001
N/A	EY436812	0,0016
Ferritin subunit [Meretrix meretrix]	EY436834	0,0010
ribosomal protein [Mytilus galloprovincialis]	EY436836	0,0074
cytoplasmic actin [Dreissena polymorpha]	EY436880	0,0041
N/A	EY436882	<0.001
hypothetical protein [Brugia malayi]	EY436888	0,0068
N/A	EY436890	<0.001
hypothetical protein ATP_00032 [Candidatus Phytoplasma]	EY436896	0,0054
translation elongation factor 1-alpha [Dreissena polymorpha]	EY436899	0,0034
ferritin [Sinonovacula constricta]	EY436908	<0.001
unknown [Schistosoma japonicum]	EY436917	0,0015
putative kinase [Desulfurococcus kamchatkensis]	EY436944	0,0100
similar to GA21569-PA [Nasonia vitripennis]	EY436947	0,0031
VSP [Giardia lamblia]	EY436956	0,0063
N/A	EY436962	<0.001
hypothetical protein [Saccoglossus kowalevskii]	EY436973	0,0050
triphosphoribosyl-dephospho-CoA synthase [Lactobacillus reuteri]	EY436979	0,0052
hypothetical protein [Meretrix lusoria]	FK829247	0,0041
Metallothionein mRNA [Dreissena polymorpha]	U67347	0,0057
ADP,ATP carrier protein 3 [Lepeophtheirus salmonis]	EY434503	0,0041

putative olfactory receptor 4A8-like [Macaca mulatta]	EY434275	<0.001
ADP,ATP carrier protein 3 [Lepeophtheirus salmonis]	EY436333	<0.001
B-cell translocation gene 1 [Crassostrea gigas]	EY435605	0,0053
ribosomal protein l17 [Haliotis discus discus]	EY434929	0,0059
N/A	EY434953	0,0031
Cytoplasmic actin [Dreissena polymorpha]	AJ517553	0,0091
Hypothetical protein [Cryptosporidium muris]	EY436876	<0.001
Hypothetical protein DAPPUDRAFT_230342 [Daphnia pulex]	EY435095	0,0037
Heat shock protein 70 mRNA [Dreissena polymorpha]	EF526096	0,0020
predicted protein [Nematostella vectensis	EY436234	0,0017
DNA-binding protein inhibitor id-1 [Ictalurus furcatus]	EY434058	0,0021
Serine/threonine-protein kinase pim-3-like [Xenopus tropicalis]	EY436685	0,0012
N/A	AJ517719	0,0032
Tyrosine protein kinase-like protein [Lumpy skin	EY434237	0,0059
N/A	AJ517544	0,0060
N/A	EY434159	<0.001
bZIP transcription factor family protein [Tetrahymena thermophila]	EY435000	0,0099
cytochrome P450 30 [Mercenaria mercenaria]	EY435627	0,0082
Ribosomal protein L22 [Lepidochitona cinerea]	AJ517690	0,0049
similar to Activated in blocked unfolded protein response protein 2 [Gallus gallus]	EY435596	0,0019
transcription factor Tal [Crassostrea gigas]	EY435539	0,0027
Similar to ABC transmembrane transporter [Ciona intestinalis]	EY436141	0,0021
N/A	EY433693	0,0087
Ribosomal protein l17 [Haliotis discus discus]	EY434394	0,0068
Collagen alpha-1(XXVIII) chain-like [Meleagris gallopavo]	AJ517744	0,0019
B-cell translocation gene 1 [Crassostrea gigas]	EY435876	<0.001
Phosphatidylinositide phosphatase SAC2 [Acromyrmex echinatior]	EY436799	0,0068
Lipid phosphate phosphohydrolase 1 [Danio rerio]	EY435697	0,0053
Hypothetical protein BRAFLDRAFT_220425 [Branchiostoma floridae]	EY433914	0,0064
Predicted protein [Nematostella vectensis]	EY434967	<0.001
Cluster 3		
N/A	EY434339	<0.001
N/A	EY434751	0,0019
Hypothetical protein [Saccoglossus kowalevskii]	EY435914	0,0069
Transcription elongation factor B polypeptide 1 [Acromyrmex echinatior]	EY434562	0,0700
ribosomal protein L9 [Argopecten irradians]	EY433958	0,0043
breast cancer susceptibility protein [Caluromys philander]	EY436603	0,0098
Dynein intermediate chain 2, ciliary-like [Saccoglossus kowalevskii]	EY436136	0,0100
histone H4 (H4) gene [Venerupis decussatus]	EF670673	<0.001
glycoprotein [rabies virus]	EY434547	0,0089
hypothetical protein BRAFLDRAFT_117811 [Branchiostoma floridae]	EY433866	0,0061
beta-tubulin [Halocynthia roretzi]	EY433887	0,0100
putative SERF-like protein [Amblyomma americanum]	EY434166	0,0012
Similar to sperm associated antigen 17 [Ciona intestinalis]	EY433929	0,0023
cytochrome c oxidase subunit I [Dreissena polymorpha]	EY434603	0,0011
hypothetical protein TcasGA2_TC030624 [Tribolium castaneum]	EY434976	0,0076

CAAX amino terminal protease family protein [Streptococcus mitis]	EY435821	0,0022
selenocysteine lyase [Candidatus Carsonella ruddii]	EY435007	0,0096
Hypothetical protein DFA_02248 [Dictyostelium fasciculatum]	AJ517642	0,0038
Hypothetical protein PrwipMp21 [Prototheca wickerhamii]	EY434351	0,0045
Hypothetical protein [Entamoeba dispar]	EY434776	<0.001
Metal dependent phosphohydrolase [Petrotoga mobilis]	EY436573	0,0077
hypothetical protein LOC779590 [Xenopus tropicalis]	AJ517671	0,0039
B-cell leukemia/lymphoma 2-like [Oryctolagus cuniculus]	AM230364	0,0066
chordin-like 2 [Monodelphis domestica]	AM230412	<0.001
N/A	EY435666	<0.001
Conserved Plasmodium protein [Plasmodium falciparum]	EY436467	0,0076
N/A	EY434858	0,0051
TlyC family hemolysin [Campylobacter coli]	AM230144	0,0024
similar to EF-hand calcium binding domain 5 [Strongylocentrotus purpuratus]	EY436494	0,0067
Hypothetical protein BRAFLDRAFT_275083 [Branchiostoma floridae]	EY436939	0,0051
N/A	EY435615	<0.001
Caspase short class (AGAP011951-PA) [Anopheles gambiae]	EY434382	0,0036
CBR-SRE-1 protein [Caenorhabditis briggsae]	EY434502	0,0020
Enkurin [Danio rerio]	EY434137	0,0018
transcription factor IIB [Haliotis discus discus]	EY435946	<0.001
Hypothetical protein LOC100163607 [Acyrthosiphon pisum]	EY434186	<0.001
chromodomain-helicase-DNA-binding protein 9 isoform [Pan troglodytes]	EY433990	<0.001
tektin 3-like [Saccoglossus kowalevskii]	EY435552	<0.001
Hypothetical protein MG_525 [Mycoplasma genitalium G37]	EY435160	0,0049
Hypothetical protein Tb11.1350 [Trypanosoma brucei	EY434594	<0.001
Similar to spermatogenesis associated 6 [Canis familiaris]	EY434681	<0.001
Hypothetical protein P700755_04193 [Psychroflexus torquis]	EY434495	<0.001
outer dense fiber protein 3 [Xenopus laevis]	EY434025	<0.001
cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase [Anolis carolinensis]	EY434173	0,0050
Hypothetical protein [Saccoglossus kowalevskii]	EY434180	<0.001
Hypothetical protein BRAFLDRAFT_263840 [Branchiostoma floridae]	EY433996	<0.001

A3.1 Complete list of significant transcripts for each cluster of probes from the hierarchical cluster analysis (ANOVA, $p<0.01$ and fold change of 1.5). In the table is shown the putative gene name of the transcript after a Blast analysis at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the accession number foe each transcript and the *p value*.